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Neuroprotection in non-transgenic and transgenic mouse models of Alzheimer's disease by positive modulation of σ_1 receptors

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Abstract

The sigma-1 (σ_1) receptor is an endoplasmic reticulum (ER) chaperone protein, enriched in mitochondria-associated membranes. Its activation triggers physiological responses to ER stress and modulate Ca^{2+} mobilization in mitochondria. Small σ_1 agonist molecules activate the protein and act behaviorally as antidepressant, anti-amnesic and neuroprotective agents. Recently, several chemically unrelated molecules were shown to be σ_1 receptor positive modulators (PMs), with some of them a clear demonstration of their allostericity. We here examined whether a σ_1 PM also shows neuroprotective potentials in pharmacological and genetic models of Alzheimer's disease (AD). For this aim, we describe (±)-2-(3-chlorophenyl)-3,3,5,5-tetramethyl-2-oxo-[1,4,2]-oxazaphosphinane (OZP002) as a novel σ_1 PM. OZP002 does not bind σ_1 sites but induces σ_1 effects in vivo and boosts σ_1 agonist activity. OZP002 was antidepressant in the forced swim test and its effect was blocked by the σ_1 antagonist NE-100 or in σ_1 receptor knockout mice. It potentiated the antidepressant effect of the σ_1 agonist igmesine. In mice tested for Y-maze alternation or passive avoidance, OZP002 prevented scopolamine-induced learning deficits, in a NE-100 sensitive manner. Pre-administered IP before an ICV injection of amyloid $A\beta_{25-35}$ peptide, a pharmacological model of Alzheimer's disease, OZP002 prevented the learning deficits induced by the peptide after one week in the Y-maze, passive avoidance and novel object tests. Biochemical analyses of the mouse hippocampi showed that OZP002 significantly decreased $A\beta_{25-35}$-induced increases in reactive oxygen species, lipid peroxidation, and increases in Bax, TNFα and IL-6 levels. Immunohistochemically, OZP002 prevented $A\beta_{25-35}$-induced reactive astrogliosis and microgliosis in the hippocampus. It also alleviated $A\beta_{25-35}$-induced decreases in synaptophysin level and choline acetyltransferase activity. Moreover, chronically administered in APPswe mice during 2 months, OZP002 prevented learning deficits (in all tests plus place learning in the water-maze) and increased biochemical markers. This study shows that σ_1 PM with high neuropotective potential can be identified, combining pharmacological efficacy, selectivity and therapeutic safety, and identifies a novel promising compound, OZP002.

keywords: σ_1 Receptor; OZP002; Antidepressant; Anti-amnesic; Neuroprotection; Alzheimer's disease

1. Introduction

The sigma-1 (σ_1) receptor is a chaperone protein, particularly expressed in mitochondria-associated endoplasmic reticulum (ER) membranes (MAM) [1]. In the brain, the protein is expressed in neurons, astrocytes, oligodendrocytes and microglia [2-4]. On ER membranes, the σ_1 receptor is associated with several partner proteins including the glucose-related protein 78/binding immunoglobulin protein (BiP) [1]. σ_1 Receptor activation is physiologically induced by cellular stress such as oxidative stress [5]. It triggers or amplifies several cellular responses including the transcriptional regulation of Bcl-2 expression via the NF-κB pathway [5], dissociation from BiP and activation of ER stress responses [1], or modulation of inositol-1,4,5 trisphosphate (IP_3) receptor-gated Ca^{2+} mobilization into mitochondria [6]. In addition, σ_1 receptors may translocate after activation in the vicinity of other cellular compartments, or plasma membrane, and interact with different membrane proteins such as ion channels, kinases, G-protein coupled receptors, or trophic factor receptors [7-9]. σ_1 Receptor-mediated neuromodulation plays an important role in brain plasticity, mood regulation, learning and memory processes and neuroprotection [10-12].

The σ_1 receptor is proposed to be a molecular chaperone [1] and it can be activated by physiopathological signals [5]. It may therefore not necessitate an endogenous ligand to be activated and none has yet been unequivocally identified. However, the σ_1 receptor can be activated or inactivated by small molecules, so acting as σ_1 agonists or antagonists. Binding assays, using cells or brain membrane preparations, are based on selective agonist or antagonist radiotracers [12]. Several activity tests have been proposed, leading to a coherent pharmacology. These include the agonist-induced potentiation of bradykinin-induced Ca^{2+} mobilization [6], the agonist-induced dissociation from BiP [1], the agonist-induced attenuation of dizocilpine-induced learning impairments [13,14], or the antagonist-induced inhibition of mechanical hypersensitivity induced by capsaicin [15]. Finally, chemical and structural biology 2D and 3D models of the pharmacophore exist [16-18]. They are not fully coherent with the recent first description of a crystallized structure of the σ_1 protein [19],

but they allowed detailed structure/activity relationship studies and pertinent drug development programs [20,21].

Recently, the notion of putative positive modulators for σ_1 receptors has re-emerged. Historically, in the early 1990's, Musacchio's group described that the anticonvulsants ropizine and phenytoin induced allosteric modulation of the high affinity binding of [^{3}H]dextromethorphan or [^{3}H](+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine [(+)-3-PPP] on the (+)-pentazocine-sensitive σ_1 binding site [22,23]. Very recently, the D1 dopamine receptor ligand 6-chloro-2,3,4,5-tetrahydro-3-methyl-1-(3-methylphenyl)-1H-3-benzazepine-7,8-diol (SKF83959) was found to allosterically potentiate [^{3}H](+)-pentazocine binding to σ_1 receptor in brain and liver tissues [24]. The drug inhibited inflammation in lipopolysaccharide-stimulated microglia, in a σ_1 antagonist sensitive manner and potentiated the σ_1 agonist effect [4]. The novel drug (4R,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (E1R) failed to affect [^{3}H](+)-pentazocine binding to σ_1 receptor but markedly potentiated bradykinin-induced Ca^{2+} increase in NG-108 cells and potentiated σ_1 agonists-induced potentiation of stimulated contractions of rat vas deferens [25]. E1R was pro-mnesic by itself and attenuated scopolamine-induced amnesia. The SKF83959 derivative 3-methyl-phenyl-2,3,4,5-tetrahydro-1H-benzo[d]azepin-7-ol (SOMCL-668) also behaved as a σ_1 receptor positive modulator and showed antidepressant effects in the forced swimming test (FST), tail suspension test and in sucrose preference after chronic mild stress [26].

σ_1 Receptor agonists are currently developed in several neurodegenerative diseases related indications and since the initial description that σ_1 agonists are neuroprotective in pharmacological and genetic mouse models of Alzheimer's disease (AD) [27-29], evidences have been accumulated that σ_1 drugs are effective in other pathologies including Parkinson, Huntington, amyotrophic lateral sclerosis. σ_1 PMs have not yet been tested as neuroprotectants. We previously described a novel molecule, (±)-2-(3-chlorophenyl)-3,3,5,5-tetramethyl-2-oxo-[1,4,2]-oxazaphosphinane (OZP002) conceived as a hydroxybupropion analogue, where a phosphinolactone group has replaced the lactol group (Fig. 1) [30,31]. The compound showed a significant antidepressant activity in the forced swimming test (FST) [31] and we report here that the drug indeed acts as a σ_1 PM, since it did bind to σ_1

receptor but showed antidepressant and anti-amnesic effects that could be blocked by a σ_1 antagonist or were absent in σ_1 receptor knockout (KO) mice, and potentiated the σ_1 agonist-induced antidepressant and anti-amnesic effects. We therefore investigated its neuroprotective potential in a pharmacological and a transgenic mouse models of AD and report that σ_1 PMs with high neuropotective potential can be identified, which would lead to innovative drugs combining pharmacological efficacy, selectivity and therapeutic safety.

2. Materials and methods

2.1. Animals

Five hundred and fifty male Swiss OF-1 mice, aged 7-9 weeks and weighing 32 ± 2 g were purchased from Janvier (St Berthevin, France). The σ_1 receptor KO line (+/-)Oprs1$^{Gt(IRESBetageo)33Lex}$ was purchased from the Texas Institute for Genomic Medicine (Houston, Texas). Generated by gene trapping, the animals were backcrossed and maintained by heterozygous breeding in a C57BL/6J (wildtype, WT) background over > 10 generations [32]. Forty-four male C57BL/6 WT and σ_1 receptor KO mice, aged 7-9 weeks, were used in this study. Fifty-four female Tg2576 mice overexpressing APP_{Swe}, and C57BL/6xSJL WT controls were purchased from Taconic (Lelystad, Netherlands). Mice were housed between 2 to 12 months of age in the animal facility of the University of Montpellier (registration number D34-172-23). Animals were housed in groups with access to food and water *ad libitum*. They were kept in a temperature and humidity controlled facility on a 12-h/12-h light/dark cycle (lights on at 7:00 h). All animal procedures were conducted in strict adherence to the European Union Directive 2010/63 and the ARRIVE guidelines [33] and authorized by the National Ethic Committee (Paris) (APAFIS #1241-2552).

2.2. Drugs and injections

2-(3-chlorophenyl)-3,3,5,5-tetramethyl-2-oxo-[1,4,2]-oxazaphosphinane (OZP002) was synthesized in our laboratory (ICGM-AM2N) [30,31]. (R)-(+)-N-cyclopropylmethyl-α-ethyl-N-methyl-α-[(2E)-3-phenyl-2-propenyl)benzenemethanamine hydrochloride (igmesine) was a gift from Dr F.J. Roman (Pfizer, Fresnes, France). (-)-scopolamine hydrobromide (scopolamine), (±)-1-(3-chlorophenyl)-2-[(1,1-dimethylethyl)amino]-1-propanone hydrochloride (bupropion), (+)-(2S,3S)-2-(3-chlorophenyl)-3,5,5-trimethyl-2-morpholinol hydrochloride (hydroxybupropion), and 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone (haloperidol) were from Sigma-Aldrich (Saint-Quentin-Fallavier,

France). [1α,4(S),6β,14α,16β]-20-ethyl-1,6,14,16-tetramethoxy-4-[[[2-(3-methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl]oxy]methyl] aconitane-7,8-diol citrate salt (methyllycaconitine, MLA), (2S,13bS)-2-methoxy-2,3,5,6,8,9,10,13-octahydro-1H,12H-benzo[i]pyrano[3,4-g]indolizin-12-one hydrobromide (dihydro-β-erythroidine, DHβE) and 4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzene-ethanamine hydrochloride (NE-100) were from Tocris Bioscience (Bristol, UK). Drugs were solubilized in physiological saline (vehicle) and injected intraperitoneally (IP) or subcutaneously (SC), in a volume of 5 µl/g body weight. The amyloid β_{25-35} peptide ($A\beta_{25-35}$) and scrambled $A\beta_{25-35}$ peptide were from Polypeptides (Strasbourg, France), solubilized in sterile distilled water (3 mg/ml) and stored at -20°C. Before injection, peptides were aggregated by incubation at 37°C for 4 days. They were administered under isoflurane anesthesia, intracerebroventricularly (ICV) according to the method of Haley and McCormick [34], in a volume of 3 µl per mouse [27,35,36].

2.3. In vitro binding of [^{3}H](+)-pentazocine to σ_1 receptor

The assay was performed by CEREP (Celle l'Evescault, France) according to Ganapathy et al. [37] for OZP002 and in the laboratory for the comparison between OZP002, bupropion and hydroxybupropion at 1 µM. Increasing concentrations of OZP002 (10 nM to 10 µM) were incubated with 15 nM of [^{3}H](+)-pentazocine in membranes preparations from Jurkat human leukemic T cells (CEREP) or mouse brain membranes. Samples were incubated during 120 min at 37°C and non-specific binding level was determined using 10 µM of haloperidol. Assays were performed in duplicates and results were expressed as % of specific binding level.

2.4. σ_1 Protein/BiP dissociation assay

The assay was performed as described by Hayashi and Su [1] but in Jurkat human leukemic T cells. Cells were grown in complete RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics. Cells were treated with test compounds dissolved in culture medium for 30 min at 37°C. Cells were prepared in Tris/HCl 50 mM pH

7.4 containing 0.2% CHAPS and protease inhibitors (Roche). Crosslinking was performed by incubation with 150 µg/ml of dithiobis(succinimidyl propionate) (Thermofischer Scientific) at 4°C for 60 min. The reaction was stopped by adding Tris/HCl 50 mM pH 8.8. After 15 min incubation on ice, cells were lysed with Tris 50 mM pH 7.4, NaCl 150 mM, 1% Triton X/100 and protease inhibitors. After centrifugation at 12,000 *g* for 5 min, the supernatant was incubated with σ_1 protein antibody (Abcam) overnight at 4°C. Cell lysates were incubated with Sepharose Protein-A (Invitrogen) for 90 min. After centrifugation at 12,000 x g for 5 min, supernatants were discarded and pellets were suspended in 0.5 ml RIPA buffer, rinsed twice and the supernatants were assayed for BiP level by ELISA (USCN life sciences, SEC343Mu).

2.5. Forced swim test (FST)

Mice were placed in 2 l glass becher (diameter 12.5 cm), filled with 1.4 l water (22 ± 1°C), and forced to swim for 15 min on day 1 and 6 min on day 2 [38,39]. Mouse behavior was videotracked (Viewpoint, Lissieu, France). Duration of immobility was analyzed during the last 5 min of the second session. Drugs were injected IP 20 min (OZP002, Igmesine) or 30 min (NE-100) before the session on the second day.

2.6. Spontaneous alternation in the Y-maze (YMT)

Spontaneous alternation performance in the Y-maze, an index of spatial working memory was measured as described [27,35,36]. Mice were allowed to explore the maze for 8 min. The series of arm entries, including possible returns into the same arm, was checked and the percentage of alternation was calculated as (actual alternations / number of arm entries - 2) x 100. Animals that performed less than 8 arm entries or showed alternation percentage <20% or >90% were discarded from the calculations. Attrition was 11.3% in this study. In scopolamine experiments, drugs were injected IP 30 min (OZP002, Igmesine, NE-

100, MLA, DHβE) or SC 20 min (scopolamine) before the session. $A\beta_{25-35}$–treated and APP_{Swe} mice were tested without drug treatment before the test.

2.7. Step-through passive avoidance procedure

Long-term non-spatial memory was tested using a passive avoidance test [27]. The apparatus consisted of a white and a dark compartment with a grid floor separated by a guillotine door. A 60 W lamp lit the white compartment during the experimental period. Scrambled foot shocks (0.3 mA for 3 s) were delivered to the grid floor using a shock generator scrambler (Lafayette Instruments, Lafayette, MA, USA). The guillotine door was initially closed. Each mouse was placed into the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment, the door was closed and foot shock was delivered for 3 s. The step-through latency and the level of sensitivity to the shock was recorded during training. None of the treatments affected these parameters during training (data not shown). Retention was checked after 24 h. Each mouse was placed again into the white compartment. After 5 s, the door was raised and step-through latency recorded up to 300 s. In scopolamine experiments, drugs were injected IP 30 min (OZP002, Igmesine, NE-100, MLA, DHβE) or SC 20 min (scopolamine) before the training session. $A\beta_{25-35}$–treated and APP_{Swe} mice were tested without drug treatment before the test.

2.8. Place learning in the water-maze

Spatial reference memory was assessed using place learning in the water-maze, as previously described [40]. The water-maze was a circular pool (diameter 140 cm) and the platform (diameter 10 cm) was immersed under the water surface during acquisition. Swimming could be recorded using Videotrack® software (Viewpoint), with trajectories being analyzed as latencies and distances. Training consisted in 3 swims per day for 5 days. Start positions, set at each limit between quadrants, were randomly selected and each animal was allowed a 90 s swim to find the platform. Animals were left on the platform during 20 s.

Animals that did not find the platform after 90 s had elapsed were placed on it manually and left for 20 s. Median latency was calculated for each training day and expressed as mean ± SEM. A retention probe test was performed 48 h after the last training without platform. The platform was removed and each animal was allowed a free 60 s swim. The 60-s duration swimming was videotracked (Viewpoint) and time spent in the training (T) quadrant analyzed *vs*. the averaged time spent in the 3 others (o). Animals did not receive drug treatment before the training sessions or probe test.

2.9. Novel object test

Recognition memory was analyzed using a novel object test [40]. Mice were placed individually in a squared open-field. In session 1, animals were allowed to acclimate during 10 min. In session 2, after 24 h, two identical objects were placed at ¼ and ¾ of one diagonal of the 50 x 50 cm^2 arena. The mouse activity and nose position was recorded during 10 min (Nosetrack® software, Viewpoint). The number of contact with the objects and duration of contacts were measured. In session 3, after 24 h, the object in position 2 was replaced by a novel one differing in color, shape and texture. Each mouse activity was recorded during 10 min and analyzed. A preferential exploration index was calculated as the ratio of the number (or duration) of contacts with the object in position 2 over the total number (or duration) of contacts with the two objects. Animals showing no contact with one object or less than 10 contacts with objects, during the session 2 or 3, were discarded from the study. Attrition was 8.5% in this study. Animals did not receive drug treatment before the sessions.

2.10. Measure of reactive oxygen species (ROS) level using DCF fluorescence

After completion of behavioral experiments, mice were sacrificed by decapitation, their brains rapidly removed and hippocampus dissected out, weighed, frozen in liquid nitrogen and stored at -80°C. ROS accumulation was determined using 2',7'-

dichlorofluorescein (DCF) fluorescence in the mouse hippocampus *ex vivo*. DCF diacetate (0.5 µM) (Sigma-Aldrich) was applied to the SDS-soluble fraction of hippocampus extracts. After 30 min at 37°C, DCF fluorescence was quantified (excitation at 485 nm, emission at 530 nm) using a Fluoroskan Ascent spectrofluorimeter (Thermo Scientific, Waltham, USA), normalized for protein concentration.

2.11. Lipid peroxidation measures

Hippocampus was homogenized in cold methanol (1/10 w/v), centrifuged at 1,000 *g* during 5 min and supernatant collected. Homogenate was added to a solution containing $FeSO_4$ 1mM, H_2SO_4 0.25 M, xylenol orange 1 mM and incubated for 30 min at room temperature. Absorbance was measured at 580 nm ($A_{580}1$), and 10 µl of cumene hydroperoxide (CHP) 1 mM was added and incubated for 30 min at room temperature, to determine the maximal oxidation level. Absorbance was measured at 580 nm ($A_{580}2$). Lipid peroxidation level was determined according to: CHPeq. = $A_{580}1/A_{580}2$ x [CHP (nmol)] x dilution, calculated per wet tissue weight and as percentage of control group value.

2.12. Bax, Bcl-2, TNFα, IL-1β, IL-6, synaptophysin Elisa assays

Hippocampus was homogenized in 50 mM Tris-150 mM NaCl buffer, pH 7.5, and sonicated for 20 s. After centrifugation at 16,000 *g* for 15 min at 4°C, supernatants were used for Elisa assays (ref. SEB343Mu for Bax, ref. SEA778Mu for Bcl-2, ref. SEA133Mu for TNFα, ref. SEA425Mu for synaptophysin; ref. E90563Mu for IL-1β; Cloud-Clone/Uscn, Wuhan, PR China). For each assay, absorbance was read at 450 nm and sample concentration calculated using standard curve. Results are expressed as ng or pg of marker per mg of wet tissue and as percentage of the control group.

2.13. Immunohistology

Each mouse was anesthetized using ketamine, 80 mg/kg, and xylazine, 10 mg/kg, and transcardially perfused with saline solution (50 ml) and paraformaldehyde 4% (50 ml). Brains were post-fixed for 48 h in paraformaldehyde 4%, sucrose 30%. Sections were cut using a microtome, blocked with 0.01 M PBS, 0.1% Triton X-100 and 10% normal goat serum solution for 30 min and incubated at 4°C overnight with primary rabbit anti-Iba1 antibody (dilution 1:500, 019-19741, Wako) and mouse anti-glial fibrillary acidic protein (GFAP) antibody (dilution 1:400, ref. G3893, Sigma-Aldrich), diluted in PBS. After several washes, sections were incubated with appropriate biotinylated goat anti-rabbitsecondary antibody (dilution 1:200, ref. BA-1000, Vector laboratories) and goat anti-mouse secondary antibody (dilution 1:500, ref. BA-9200, Vector laboratories). Finally, sections were incubated for 1 h in avidin–biotin–peroxidase complex (PK-4000, Eurobio) and staining detected with diaminobenzidine. Successive baths of alcohol allow to dehydrate all sections, they were stained with Cresyl violet and cover slipped with mounting medium (Mountex, Histolab). Examination of the hippocampus structure was performed on slices located between antero-posterior coordinates -1.80 and -3.08 from Bregma [41], using an optical microscope (DM 2500, Leica) under white light at a magnification of x100. Astroglial and microglial cells were counted in three zones of the hippocampus: the polymorph layer (PoDG), molecular layer (Mol) and stratum radiatum (Rad). Data from GFAP immunolabeling were calculated as average of 5 slices per animal, with 5-6 animals per group, and expressed as number of immunoreactive cells per 100 μm^2. Internal variability of the counting was 2.7 ± 0.2% in PoDG, 3.0 ± 0.3% in Mol and 3.4 ± 0.2% in Rad. Data from Iba1 immunolabeling were calculated as average of 4 slices per animal, with 4-6 animals per group, and expressed as number of immunoreactive cells per 100 μm^2. Internal variability of the counting was 9.1 ± 1.0% in PoDG, 7.1 ± 0.8% in Mol and 4.9 ± 0.5% in Rad.

2.14. Measure of the choline acetyltransferase (ChAT) activity

Hippocampus was homogenized in 10 mM EDTA, 10 mM phosphate buffer (pH 7.4) using a Teflon-glass homogenizer. Triton X-100 was added in homogenates (1%, w/v) and

stored at −80°C. Incubation solution contained 50 µL of 10 mM choline bromide, 50 µl of 1 mM physostigmine, 50 µl of 10 mM phosphate buffer (pH 7.4), 150 µl of 0.67 mM [^{3}H]acetyl-CoA (about 220,000 dpm), and 200 µl of homogenate. Final concentrations in the reaction mixture were 1 mM in choline bromide, 0.1 mM in physostigmine and 0.2 mM in [^{3}H]acetyl-CoA. After incubation at 37 °C for 30 min, 2 ml of acetonitrile containing 10 mg of sodium tetraphenylborate (Kalibor) were added, with 10 ml of liquid scintillator (Microscint 0). The solution was vigorously shaken. Radioactivity was measured in 4 ml of the organic phase using by liquid scintillation (TriCarb 2300TR, Packard). ChAT activity was expressed as pmol of ACh formed per h and per mg of tissue.

2.15. Statistical analyses

Analyses were done using GraphPad Prism (GraphPad Software Inc.). Data were analyzed using one-way of two-way analyses of variance (ANOVA, *F* value), followed by the Dunnett's test. Passive avoidance latency, expressed as median and interquartile range, were analyzed using a nonparametric Kruskal-Wallis ANOVA and *post-hoc* comparisons done using the Dunn's test. Acquisition profiles in the water-maze were analyzed using the nonparametric repeated-measure Friedman's ANOVA (*Fr* value), followed by the Dunn's or Mann-Whitney's test. Probe test data were presented as time spent in the T and o quadrants and analyzed using the one-sample *t*-test *vs*. the chance level (15 s). The object preference, calculated from the number of contacts with the two objects, was analyzed using the one-sample *t*-test *vs*. the chance level (50%). The level of statistical significance were $p < 0.05$, $p < 0.01$ and $p < 0.001$. Statistical data are indicated in the figure legends.

2.16. Combination index calculations

Isobologram analyses, evaluating the nature of interaction of two drugs at a given effect level were performed according to the Fraser's concept [42] and previous reports [43,44]. The concentrations required to produce the given effect (e.g., IC_{50}) are determined

for drug A ($IC_{x,A}$) and drug B ($IC_{x,B}$) and indicated on the x and y axes of a two-coordinate plot, forming the two points ($IC_{x,A}$, 0) and (0, $IC_{x,B}$), which define the line of additivity. Concentrations of A and B in the combination that provides the same effect, denoted as ($C_{A,x}$, $C_{B,x}$), are placed in the same plot. Synergy, additivity, or antagonism is indicated when ($C_{A,x}$, $C_{B,x}$) is located below, on, or above the line, respectively. Operationally, a combination index (CI) is calculated as: $CI = C_{A,x}/IC_{x,A} + C_{B,x}/IC_{x,B}$, where $C_{A/B,x}$ are the concentrations of drug A/B used in the combination that generates x% of the maximal combination effect; CI is the combination index; and $IC_{x,A/B}$ is the concentration of drug A/B needed to produce x% of the maximal effect. A CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively.

3. Results

3.1. OZP002 is a σ_1 receptor positive modulator

The Cerep profile of OZP002, performed at 1 µM, revealed no significant inhibition in a panel of 76 receptor binding assays, but only moderate potentiation of [^{3}H]neurotensin binding to NTS1 receptors, [^{3}H]α-bungarotoxin binding to α_7 nAChR, and [^{3}H](±)-DOI binding to 5-HT_{2B} sites (Table 1). The drug did not affect [^{3}H](+)-pentazocine binding to σ_1 receptor, in the 10 nM-100 µM concentration range, with a non-significant +25% increase at sub-micromolar doses (Fig. 2a). The molecule therefore did not behave as its parent compounds, bupropion and hydroxybupropion, that show high σ_1 binding affinity [45]. A direct comparison of their inhibitory potency at 1 µM in mouse brain preparations showed that OZP002 failed to affect [^{3}H](+)-pentazocine binding while bupropion decreased it by ~45% and hydroxybupropion by ~63% (Fig. 2b). The activity of OZP was analyzed in a cellular response, the σ_1 protein/BiP dissociation assay. Increasing concentrations of the σ_1 agonist igmesine significantly decreased the level of BiP associated with σ_1 protein in Jurkat cells (Fig. 2c). OZP002, in the same concentration range, failed to show any effect. However, a mix of igmesine and OZP002, at the low, non-active concentration of 0.3 µM each significantly dissociated BiP from the σ_1 protein. The effect was completely prevented by NE-100 (Fig. 2c). The antidepressant-like activity of OZP002 was then analyzed in mice with pharmacological or genetic inhibition of σ_1 receptor. OZP002 effect was prevented by the σ_1 antagonist NE-100, used at a dose known to prevent neurosteroid antidepressant effect or to reduce alcohol preference in rats [46,47] (Fig. 2c) or absent in σ_1 receptor KO mice (Fig. 2d). Igmesine decreased immobility in the FST at 30 but not 10 mg/kg (Fig. 2e). A combination of igmesine (10 mg/kg) and OZP002 at a sub-active dose (5 mg/kg) led to a significant effect (Fig. 2e). Calculation of the combination index (CI), detailed in Table 2a, showed a CI = 0.84 suggestive of a synergy between the two drugs. These observations in the FST indicated that OZP002 acts as a σ_1 receptor positive modulator *in vivo*.

3.2. Anti-amnesic effect of OZP002 against scopolamine-induced learning impairments

σ_1 Agonists [48-50] or the σ_1 receptor positive modulator E1R [25] attenuated scopolamine-induced amnesia in mice. We tested OZP002, in the 0.03-1.5 mg/kg dose-range. The drug attenuated significantly scopolamine-induced alternation deficits (Fig. 3a) and, interestingly, biphasically potentiated at 0.1 mg/kg but attenuated at 1.5 mg/kg the scopolamine-induced exploratory activity (Fig. 3b). OZP002 attenuated scopolamine-induced passive avoidance deficits, by increasing step-through latency at 0.1-0.3 mg/kg (Fig. 3c) and decreasing escape latency at doses > 0.1 mg/kg (Fig. 3d). The combination study was performed with igmesine. Igmesine attenuated scopolamine-induced alternation deficit at 1 but not 0.3 mg/kg (Fig. 3e). Combination with the maximal inactive dose of OZP002 (0.03 mg/kg) led to a significant effect. Calculation showed a CI = 0.64, again suggestive of a synergy between the two drugs (Table 2b).

Antagonism studies were performed with OZP002 (1 mg/kg) and the σ_1 antagonist NE-100, the α_7 nAChR antagonist MLA or the $\alpha_4\beta_2$ nAChR antagonist DHβE (Fig. 4). NE-100 significantly prevented OZP002 anti-amnesic effect in the YMT (Fig. 4a) and attenuated it in the passive avoidance test (Fig. 4b). MLA pre-treatment significantly prevented OZP002 anti-amnesic effect at 3 mg/kg in the YMT (Fig. 4c) and attenuated it in the passive avoidance test at both doses tested (Fig. 4d). DHβE failed to significantly prevent OZP002 effect in both tests (Figs. 4e,f). These data showed that the behavioral effect of OZP002 not only involves a σ_1 component but also an interaction with α_7 nAChR.

3.3. Protective effects of OZP002 in the $A\beta_{25-35}$ mouse model of AD

σ_1 Agonists are neuroprotective drugs in the $A\beta_{25-35}$ mouse model of AD [27,28]. We tested the effects of OZP002 administered once before $A\beta_{25-35}$ (9 nmol ICV). Animals were tested after one week (Fig. 5a). OZP002, at doses higher than 0.7 mg/kg, prevented $A\beta_{25-35}$-induced alternation deficits (Fig. 5b). All doses tested alleviated the $A\beta_{25-35}$-induced impairment of passive avoidance (Fig. 5c). In the novel object test, the dose of 0.7 mg/kg

significantly prevented the $A\beta_{25-35}$-induced deficits in preferential exploration of the novel object during session 3, both in terms of number of contacts (Fig. 5d) and duration of contacts (Fig. 5e).

Several biochemical parameters measuring the extent of $A\beta_{25-35}$ toxicity were then assessed in the hippocampus or cortex of the animals. Oxidative stress was addressed by measuring ROS levels in the hippocampus (Fig. 6a) and peroxidation of membrane lipids the cortex (Fig. 6b). $A\beta_{25-35}$ induced a +27% increase in DCF fluorescence that was significantly attenuated in OZP002-treated animals at 0.7 mg/kg (Fig. 6a). The effect was U-shaped and limited to this active dose. $A\beta_{25-35}$ induced a +29% increase in lipid peroxidation that was significantly attenuated in OZP002-treated animals, at doses > 0.7 mg/kg (Fig. 6b). The effect also appeared U-shaped and 0.7 mg/kg was the most active dose. In the hippocampus, the levels of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were analyzed (Figs. 6c,d). $A\beta_{25-35}$ induced a +21% increase in Bax and failed to affect Bcl-2. OZP002 significantly attenuated Bax increase and failed to affect Bcl-2 at all doses tested. As a consequence, $A\beta_{25-35}$ increased Bax/Bcl-2 ratio (Fig. 6e). OZP002 prevented this increase (Figs. 6c,e).

Neuroinflammation was first analyzed by visualizing reactive astrocytes in the hippocampus using glial fibrillary acidic protein (GFAP) immunolabeling (Fig. 7) and reactive microglia using allograft inflammatory factor 1 (Iba1) immunolabeling (Fig. 8). $A\beta_{25-35}$ increased the number of labeled astrocytes and in their branching, resulting in densification of astrocytic clusters (Fig. 7a). This was observed throughout the cortex and hippocampal formation. Astrocytes were regularly disseminated throughout the polymorph, molecular, and stratum radiatum layers (PoDG, Mol, and Rad, respectively, as shown in Fig. 7a) surrounding the pyramidal cell layers (indicated by asterisks) and lacunosa molecular layer (LMol). The OZP002 (0.7 mg/kg) treatment did not affect the GFAP labeling pattern in Sc.Aβ-treated mice, but attenuated significantly the $A\beta_{25-35}$-induced densification of astrocytic labeling (Fig. 7b). Indeed, the two-way ANOVA showed a peptide and interaction effect if almost all areas analyzed. The level of Iba1 positive cells was very low in Sc.Aβ-treated mice throughout the hippocampal formation (Fig. 8a). $A\beta_{25-35}$ injection provoked after 7 days a marked increase in

the number of labeled microglial cells only in the Rad layer (Figs. 8a,b), an effect that was prevented by the OZP002 treatment (Fig. 8b).

We then analyzed the release of the cytokines TNFα and IL-6 in the hippocampus. $A\beta_{25-35}$ induced a +30% increase in TNFα (Fig. 9a). OZP002 significantly attenuated the increase, at 0.7 mg/kg (Fig. 9a). $A\beta_{25-35}$ induced a +15% increase in IL-6 (Fig. 9b). OZP002 attenuated the increase at 0.7 mg/kg (Fig. 9b). The level of synaptophysin was analyzed as a marker of synapse alteration. $A\beta_{25-35}$ induced a 22% decrease in synaptophysin (Fig. 9c). OZP002 attenuated the decrease, at 0.7 mg/kg (Fig. 9c). Choline acetyltransferase activity, the enzyme responsible of ACh synthesis, was analyzed as a marker of cholinergic activity. $A\beta_{25-35}$ induced a -28% decrease in ChAT activity (Fig. 9d). OZP002, at 0.7 mg/kg, prevented the decrease (Fig. 9d). OZP002 decreased synaptophysin at the highest dose tested in Sc.Aβ mice (Fig. 9c), without affecting other markers.

3.4. Protective effects of OZP002 in the APP_{Swe} mouse model of AD

Since we previously observed that σ_1 receptor agonists (PRE-084, ANAVEX2-73) tested in the $A\beta_{25-35}$ model, were active after a two month treatment when translated to a chronic regimen in APP_{Swe} mice [29], we treated APP_{Swe} mice 3 times a week with OZP002 at 0.7 or 2 mg/kg, at 10-12 months of age (Fig. 10a). Alternation performances of the animals were checked after 1 and 2 months of treatment (Figs. 10b,c). Vehicle solution (V)-treated APP_{Swe} mice showed significant impairment of alternation at 11 months of age (Fig. 10b). The chronic OZP002 treatment at 2 mg/kg alleviated the deficit. Results obtained at the end of the chronic treatment were less clear: mice at 12 month of age showed a decrease in alternation that was only attenuated (no significant difference with V-treated APP_{Swe} mice) by OZP002 at both doses tested (Fig. 10c). Measures of the number of arm entries showed, in parallel, that no variation in locomotor response was observed among groups (Fig. 10d after 1 month of treatment, Fig. 10e after 2 months).

Animals were then tested for place learning in the water-maze (Figs. 11a-c). V- and OZP002 (2 mg/kg)-treated WT animals learned the platform location, since acquisition

profiles significantly decreased over trials (Fig. 11a). V-treated or OZP002-treated APP_{Swe} mice showed a tendency to decreased swimming duration during training (Fig. 11b), but Friedman ANOVA failed to reach significance. Retention was analyzed and it appeared that V-treated WT but not V-treated APP_{Swe} explored preferentially the T quadrant. OZP002 treatment restored learning in APP_{Swe} mice, since a preferential exploration of the T quadrant was measured. It must be noted however that, although a significant difference was observed between the T and o quadrants data, OZP002-treated WT mice performed poorly in the probe test (Fig. 11c).

Animals were finally tested for object recognition. Total numbers of contacts, analyzed for sessions 2 and 3 (Fig. 11d) as an index of general motivation to perform the test, showed a 30-40% decrease for V-treated APP_{Swe} mice that was recovered by the OZP002 treatment (Fig. 11d). The novel object preference showed mild differences in terms of contacts (Fig. 11e). But, in terms of duration, both V- and OZP002-treated WT showed a significant preference for the novel object (Fig. 11f). V-treated APP_{Swe} mice did not. OZP002 treatment tended to increase the novel object preference up to 59%, but the value was not significantly different from random level (50%) (Fig. 11f).

Animals were then sacrificed and their brain collected for biochemical analyses. Among the biochemical analyses performed in the hippocampus of OZP002-treated APP_{Swe} mice (Fig. 12), lipid peroxidation and Bax levels were significantly increased in APP_{Swe} mice as compared to WT mice, and levels were normalized by the OZP treatment. Tissue levels in cytokines (TNFα and IL1β) were not different from controls in APP_{Swe} mice, but the OZP002 treatment significantly decreased TNFα level. Synaptophysin level was significantly decreased in both V- and OZP-002-treated APP_{Swe} mice.

4. Discussion

Chemical synthesis and initial pharmacological characterization of OZP002 have been previously reported (compound **4c** in [31]). No PK/PD data are yet available for this compound. However, the initial screening in the FST, and the dose-response analysis performed in the scopolamine study (presented in Fig. 3), suggested that the drug presents a good bioavailability and a poor susceptibility to phosphatase hydrolysis on the internal phosphate ester. It was indeed active in the sub-mg/kg dose range *in vivo*.

Although structurally unrelated, several σ_1 receptor positive modulators have been described so far. They showed different pharmacological or behavioral outcomes. First, SKF83959 and its derivative SOMCL-668, markedly potentiated [^{3}H](+)-pentazocine binding to σ_1 receptor in brain and periphery [24,51], while E1R and OZP002 failed to affect [^{3}H](+)-pentazocine binding ([25]; this study). Second, SOMCL-668 and OZP002 showed antidepressant effects ([26]; this study), while E1R did not [52]. Third, E1R and OZP002 showed anti-amnesic effects in the scopolamine model of learning impairment ([25]; this study). Demonstrations that σ_1 receptor positive modulators increase σ_1 agonist effects and that their effects, alone or in combination of a σ_1 agonist, are blocked by σ_1 antagonists have been provided for all drugs and on several physiological responses. SKF83959 induced anti-inflammatory activity in BV2 microglia and potentiated the anti-inflammatory activity induced by the σ_1 agonist PRE-084 or dehydroepiandrosterone (DHEA), a σ_1 acting steroid. These effects were blocked by the σ_1 antagonist BD1047 [4]. SKF83959 and SOMCL-668 exhibited anti-seizure activity in different status epilepticus mouse models and potentiated the effect of the σ_1 agonist (+)-SKF-10,047. These effects were blocked by BD1047 [51]. E1R increased bradykinin-induced Ca^{2+} mobilization in NG-108 cells alone and potentiated the PRE-084 effect, in a NE-100-sensitive manner [25]. We here report that OZP002 did not bind to σ_1 receptor at least on the [^{3}H](+)-pentazocine binding site. Some differences have been reported in the past regarding the pharmacological properties of the different σ_1 radioligands used to analyze σ_1 binding [53]. It is however unlikely that OZP002 would show a high affinity for other σ_1 radioligand binding without affecting [^{3}H](+)-pentazocine binding, at concentration

up to 100 µM. So, two possible mechanisms could be proposed to the σ_1 receptor-related effect of the compound: either OZP002 boosts a signalling pathway triggered by the σ_1 receptor or the drug acts as a positive modulator. OZP002 shows antidepressant activity by itself and potentiated the igmesine effect, in a NE-100 sensitive manner (Fig. 2). The drug also shows anti-amnesic effect in the scopolamine model alone and potentiated the PRE-084 effect, in a NE-100 sensitive manner (Fig. 3). Precise calculations of the CI were performed and indicated that the two-drug combinations are synergic confirming that the two drugs do not activate σ_1 receptor through a strictly similar mechanism, leading to pure additivity. Indeed, a moderate synergic effect is expected when combining a modulator and an orthosteric ligand [51]. Whether all these σ_1 positive modulators act similarly on σ_1 receptor at the structural level has still to be determined. Highly pertinent 3D models of the σ_1 receptor have been established for ligands inhibiting σ_1 binding [17,18]. These models must now be used to characterize the mode of interaction of each modulator with the σ_1 receptor.

OZP002 appears to be as effective as igmesine in the FST, in terms of maximum decrease in immobility, and to prevent the scopolamine-induced learning deficits. The observation that a positive modulator induces σ_1 agonist effects *in vivo*, fully prevented in σ_1 receptor KO mice or using a selective σ_1 antagonist, suggests that the drug may potentiate the endogenous activation of the σ_1 receptor. An endogenous σ_1 ligand has not yet been unambiguously identified. The only endogenous modulators shown to physiologically interact with σ_1 receptor are a trace amine, N,N-dimethyltryptamine [54] and neurosteroids. Indeed, pregnenolone, DHEA, and their sulfate esters are high-to-moderate affinity σ_1 agonists while progesterone is a potent antagonist [36,55]. As steroid release is a physiological response to brain stimulation and as σ_1 receptor modulator affects DHEA responses [4], a classical mechanism can be proposed to explain the effect OZP002. However, σ_1 receptors can also be activated by physiological signals. Whether positive modulators can also potentiate such alternative mechanism of σ_1 receptor activation remains to be determined.

We observed that the OZP002-induced attenuation of scopolamine-induced amnesia is not only blocked by NE-100 but also by MLA, a selective α_7 nAChR antagonist. Several observations relate the effects of σ_1 receptor ligands to a rapid activation of α_7 nAChRs. The

effect of pregnenolone sulfate against $A\beta_{25-35}$-induced hippocampus pyramidal cells loss and PI3K-Akt activation or ERK inhibition were blocked by a σ_1 antagonist or an α_7 nAChR antagonist [56]. A synergistic protective effect was observed by combining donepezil and the σ_1 agonists PRE-084 or ANAVEX2-73 against $A\beta_{25-35}$-induced learning deficits [44]. Similarly as observed here, NE-100 or MLA, but not DHβE blocked the effect of the (donepezil + PRE-084) combination. An interplay between σ_1 receptors and α_7 nAChRs is conceivable. The α_7 nAChR is a Ca^{2+} permeable ion channel that present several Ca^{2+} binding sites [57]. It is therefore regulated by modulation in Ca^{2+} levels. , as is the σ_1 receptor [6]. Agonists of the σ_1 receptor could affect biodynamics or bioavailability of α_7 nAChRs by interacting with several proteins and receptors of the plasma membrane [58]. Moreover, the σ_1 receptor and α_7 nAChR are localized at the vicinity or in the mitochondrial membrane, which could suggest a close interplay of the two receptors.

Finally, we report here that OZP002 also prevents amyloid toxicity and memory impairment in two mouse models of AD. First, OZP002 administered once before $A\beta_{25-35}$ prevented the onset of learning deficits after one week. Second, OZP002 administered chronically during 2 months in APP_{Swe} mice prevented learning impairments. In both models, the drug treatment alleviated the markers of the toxicity assessed: oxidative stress, apoptosis, inflammation (astrocyte and microglial reactions, release of cytokines), synaptic alterations and cholinergic tonus. Indeed, the σ_1 receptor is not only expressed in neuron, but has been described to play major roles in brain plasticity and neuroinflammation through its activation following insults or neurodegeneration in reactive astroglia [59,60] and microglia [61,62]. OZP002 therefore appears as a potent neuroprotectant *in vivo* in preclinical AD mouse models. Interestingly, the drug has active dose (0.7 mg/kg in the acute model and 2 mg/kg chronically), very close to what has been described for PRE-084, the mixed mAChR/σ_1 receptor ligand ANAVEX2-73 or donepezil [27,28]. However, for most of the parameters examined and in the two AD mouse models, the drug showed a U-shaped dose-response with a limited active dose-range. This has already been observed for several pharmacological drugs and particularly the σ_1 agonists [27,28]. Whether it may limit the translational potential of the drug in clinical use will have to be addressed. However, these

data not only suggest that σ_1 receptor positive modulators share all the behavioral effects of σ_1 agonists, but also that, besides σ_1 agonists, σ_1 receptor modulators could also be considered as potential neuroprotectants in neurodegenerative diseases. The use of modulators is usually considered as safer than agonists for a variety of receptors, since they are expected to present less side-effects. Moreover σ_1 agonists are usually considered as presenting a low level of side effects due to the intrinsic modulatory nature of the σ_1 chaperone. Indeed, clinically tested σ_1 agonists appeared devoid of major undesirable side effects [63,64].

In conclusion, OZP002, initially synthesized as a potential antidepressant acting through, putatively, monoaminergic systems appeared *de facto* to be an antidepressant drug acting rather as a σ_1 receptor positive modulator. The drug presents a promising efficacy *in vivo* in depression, but also against learning and memory deficits and in neuroprotection. It appeared to be preclinically models as effective as potent drugs acting partly as σ_1 agonists such as donepezil or ANAVEX2-73, to prevent toxicity and memory impairment in pharmacologic and genetic mouse models of Alzheimer's disease. Its translational potential into clinic must however be further investigated.

Conflict of interest statement

T. Maurice, J.-N. Volle, D. Virieux, and J.-L. Pirat are co-inventors and CNRS, INSERM, University of Montpellier, and ENSCM owners of the European patent PCT/EP2017/060129, describing OZP002.

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FIGURE LEGENDS

Fig. 1. Chemical structure of (±)-2-(3-chlorophenyl)-3,3,5,5-tetramethyl-2-oxo-[1,4,2]-oxazaphosphinane (OZP002).

Fig. 2. OZP002 acts as a positive modulator of σ_1 receptor. (a) Lack of displacement of [^{3}](+)-pentazocine binding to σ_1 receptors *in vitro* in Jurkat cells membranes. (b) Comparison of the ability of OZP002, bupropion and hydroxybupropion at 1 µM to displace [^{3}](+)-pentazocine binding to σ_1 receptors *in vitro* in mouse brain membranes. Data are average of 2 determinations. (c) σ_1 Protein/BiP dissociation assay: Cell lysates were collected, the σ_1 protein immunoprecipitated and σ_1 protein coupled to BiP assayed by Elisa. Igmesine or OZP002 were applied at 0.3-3 µM and NE-100 at 10 µM. Data are expressed as percentage of control. (d) Antagonism by the selective σ_1 antagonist NE-100 of the antidepressant-like effect of OZP002 in mice submitted to the forced swimming test. (e) lack of antidepressant effect of OZP002 in σ_1 receptor KO mice. (f) Potentiation effect of OZP002 on igmesine antidepressant response. Mice were forced to swim during 15 min on day 1 and 6 min on day 2. Drugs, OZP002 (5, 10 mg/kg) or igmesine (10, 30 mg/kg), were injected IP 20 min before the session on day 2. NE-100 (10 mg/kg) was injected IP 10 min before the drugs. Vehicle solution (V) was distilled water. Immobility was measured during the 5 last min of the session. In (c-f), the number of animals per group is indicated within the columns. $F_{(9,63)}$ = 4.03, $p < 0.001$ in (c); $F_{(3,42)}$ = 4.45, $p < 0.01$ in (d); $F_{(3,49)}$ = 6.64, $p < 0.001$ in (e); $F_{(6,96)}$ = 5.59, $p < 0.0001$ in (f). ** $p < 0.01$, *** $p < 0.001$ *vs.* V-treated group, # $p < 0.05$, ## $p < 0.01$ *vs.* OZP002-treated group, Dunnett's test.

Fig. 3. Anti-amnesic effects of OZP002 against scopolamine-induced learning deficits in mice. (a) Alternation performance and (b) total number of arm entries in the Y-maze test; (c) step-through latency and (d) escape latency in the passive avoidance test; and (e) combination study with igmesine in the Y-maze test. OZP (0.03-1.5 mg/kg IP) or igmesine (0.3, 1 mg/kg IP) was injected 10 min before scopolamine (0.5 mg/kg SC) administered 20

min before the Y-maze test session or passive avoidance training session. Passive avoidance retention was measured 24 h after training. In (e), igmesine and OZP002 were injected alone or simultaneously, 10 min before scopolamine. Vehicle solution (V) was physiological saline. Data in (a, b) are presented as mean ± SEM and in (c, d) as median and interquartile range. The number of animals per group is indicated in (a, c, e). ANOVA: $F_{(6,94)}$ = 5.32, $p < 0.0001$ in (a), $F_{(6,94)}$ = 10.6, $p < 0.0001$ in (b), H = 31.4, $p < 0.0001$ in (c), H = 24.3, $p < 0.001$ in (d), $F_{(6,88)}$ = 8.10, $p < 0.0001$ in (e). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* (V+V)-treated group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* (Scop+V)-treated group; Dunnett's test in (a, b, e), Dunn's test in (c, d).

Fig. 4. Involvement of σ_1 receptors or nAChRs in the anti-amnesic effect of OZP002. Effects of: (a, b) the σ_1 antagonist NE-100, (c, d) the α_7 nAChR antagonist methyllycaconitine (MLA), or (e, f) the $\alpha_4\beta_2$ nAChR antagonist dihydro-β-erythroidine (DHβE) on the anti-amnesic effect of OZP002 (O) against scopolamine (S)-induced learning deficits, in the: (a, c, e) spontaneous alternation test and (b, d, f) passive avoidance test. NE-100 (1 mg/kg IP), MLA (1-3 mg/kg IP) or DhβE (1-3 mg/kg IP) were administered simultaneously with OZP002 (0.1 mg/kg IP), which was injected 10 min before scopolamine (0.5 mg/kg SC) administered 20 min before the Y-maze test session or passive avoidance training session. Passive avoidance retention was measured 24 h after training. Vehicle solution (V) was physiological saline. Data in (a, c, e) are presented as mean ± SEM and in (b, d, f) as median and interquartile range, with the number of mice indicated below the columns. ANOVA: $F_{(4,77)}$ = 10.0, $p < 0.0001$ in (a); H = 30.5, $p < 0.0001$ in (b); $F_{(5,87)}$ = 7.19, $p < 0.0001$ in (c); H = 31.3, $p < 0.0001$ in (d); $F_{(5,82)}$ = 5.82, $p = 0.0001$ in (e); H = 28.8, $p < 0.0001$ in (f). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* (V/V+V)-treated group; # $p < 0.05$, ## $p < 0.01$ *vs.* (Scop/V+V)-treated group; ° $p < 0.05$, °° $p < 0.01$ *vs.* (Scop/OZP002+V)-treated group;Dunnett's test in (a, c, e), Dunn's test in (b, d, f).

Fig. 5. Protection by OZP002 of the $A\beta_{25-35}$-induced memory deficits in mice. (a) Experimental procedure: On day 1, OZP002 was injected IP once 20 min before the $A\beta_{25-35}$

peptide (9 nmol ICV). On day 8, animals were tested for learning and memory responses in different tests and then sacrificed for biochemical analyses. Note that different batches of animals went through YMT/ST-PA and NOT. Abbreviations: YMT, Y-maze test; ST-PA, step-through passive avoidance; NOT, novel object test; Sacrif, sacrifice and brain dissection; bioch, biochemical analyses. (b) Spontaneous alternation in the Y-maze, (c) passive avoidance response, and (d, e) novel object test. Animals were treated with OZP002 (0.3-3 mg/kg IP) 20 min before $A\beta_{25-35}$ or Sc.Aβ control peptide (9 nmol ICV). mice were tested in the Y-maze on day 8, then trained in the passive avoidance test on day 9 and retention analyzed on day 10. For the NOR, only the most active dose of OZP002, 0.7 mg/kg IP, was tested. The number of animals per group is indicated in (b, c, d). ANOVA: $F_{(6,167)} = 4.58$, $p < 0.001$ in (a); $F_{(6,167)} = 2.65$, $p < 0.05$ in (b); $H = 20.5$, $p < 0.01$ in (c). ** $p < 0.01$ *vs.* (Sc.Aβ+V)-treated group; # $p < 0.05$, ## $p < 0.01$ *vs.* ($A\beta_{25-35}$+V)-treated group; Dunnett's test in (a) and Dunn's test in (c). ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$ *vs.* chance level (50 s); one-sample *t*-test in (d, e).

Fig. 6. Neuroprotective effect of OZP002 in $A\beta_{25-35}$-treated mice. (a) ROS level in the hippocampus, (b) lipid peroxydation level in the frontal cortex, (c) Bax level in the hippocampus, (d) Bcl-2 level and (e) Bax/Bcl-2 ratio. Animals were treated with OZP002 (0.3-3 mg/kg IP) 20 min before $A\beta_{25-35}$ or Sc.Aβ control peptide (9 nmol ICV) and sacrificed after 9 days. All data were expressed as percentage of control (Sc.Aβ+V) group data. The number of animals per group is indicated in (a-c). One-way ANOVA: $F_{(6,47)} = 2.60$, $p < 0.05$ in (a); $F_{(6,62)} = 5.03$, $p < 0.001$ in (b); $F_{(5,36)} = 3.36$, $p < 0.05$ in (c); $F < 1$ in (d); $F_{(5,36)} = 3.72$, $p < 0.01$ in (e). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* the (Sc.Aβ+V)-treated group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* the ($A\beta_{25-35}$+V)-treated group; Dunnett's test.

Fig. 7. Morphological analysis of the effect of OZP002 on the astrocytic reaction in the hippocampus of $A\beta_{25-35}$-treated mice using GFAP immunolabeling: (a) Typical micrographs and (b) quantifications. Coronal 25 µm thick sections were stained with anti-GFAP antibody and three 100 μm^2 areas of the hippocampus analyzed as shown in (a). Five slices were

counted per animals and the number of mice per group is indicated in (b). Abbreviations: DG, dendate gyrus; LMol, lacunosum molecular layer; Mol, molecular layer; PoDG, polymorph layer; Rad, stratum radiatum. Scale in (a): 20 µm. Two-way ANOVA in (b): $F_{(1,19)} = 13.3$, $p < 0.01$ for the $A\beta_{25-35}$ treatment, $F_{(1,19)} = 0.454$, $p > 0.05$ for the OZP002 treatment and $F_{(1,19)} = 5.31$, $p < 0.05$ for the interaction in PoDG; $F_{(1,19)} = 6.20$, $p < 0.05$ for the $A\beta_{25-35}$ treatment, $F_{(1,19)} = 0.172$, $p > 0.05$ for the OZP002 treatment and $F_{(1,19)} = 2.22$, $p > 0.05$ for the interaction in Mol; $F_{(1,19)} = 4.41$, $p < 0.05$ for the $A\beta_{25-35}$ treatment, $F_{(1,19)} = 0.350$, $p > 0.05$ for the OZP002 treatment and $F_{(1,19)} = 7.93$, $p < 0.05$ for the interaction in Rad. * $p < 0.05$, ** $p < 0.01$ *vs*. the (Sc.Aβ+V)-treated group; # $p < 0.05$ *vs*. the ($A\beta_{25-35}$+V)-treated group; Dunnett's test.

Fig. 8. Morphological analysis of the effect of OZP002 on the microglial reaction in the hippocampus of $A\beta_{25-35}$-treated mice using Iba1 immunolabeling: (a) Typical micrographs and (b) quantifications. Coronal 25 µm thick sections were stained with anti-Iba1 antibody and three 100 μm^2 areas of the hippocampus analyzed as shown in (a). Four slices were counted per animals and the number of mice per group is indicated in (b). Abbreviations: DG, dendate gyrus; LMol, lacunosum molecular layer; Mol, molecular layer; PoDG, polymorph layer; Rad, stratum radiatum. Scale in (a): 20 µm. Two-way ANOVA in (b): $F_{(1,17)} = 0.305$, $p > 0.05$ for the $A\beta_{25-35}$ treatment, $F_{(1,17)} = 0.251$, $p > 0.05$ for the OZP002 treatment and $F_{(1,17)} = 2.18$, $p > 0.05$ for the interaction in PoDG; $F_{(1,17)} = 2.20$, $p > 0.05$ for the $A\beta_{25-35}$ treatment, $F_{(1,17)} = 1.28$, $p > 0.05$ for the OZP002 treatment and $F_{(1,17)} = 0.700$, $p > 0.05$ for the interaction in Mol; $F_{(1,17)} = 8.24$, $p < 0.05$ for the $A\beta_{25-35}$ treatment, $F_{(1,17)} = 3.28$, $p > 0.05$ for the OZP002 treatment and $F_{(1,17)} = 3.67$, $p > 0.05$ for the interaction in Rad. * $p < 0.05$ *vs*. the (Sc.Aβ+V)-treated group; Dunnett's test.

Fig. 9. Neuroprotective effect of OZP002 of $A\beta_{25-35}$-treated mice. TNFα level (a), IL6 level (b), synaptophysin level (c) and ChAT activity (d) in the mouse hippocampus. Animals were treated with OZP002 (0.3-3 mg/kg IP) 20 min before $A\beta_{25-35}$ or Sc.Aβ control peptide (9 nmol ICV) and sacrificed after 9 days. Data in (a-c) were expressed as percentage of control

(Sc.Aβ+V) group data. The number of animals per group is indicated in the columns. One-way ANOVA: $F_{(5,56)} = 1.09$, $p > 0.05$ in (a); $F_{(5,67)} = 3.82$, $p < 0.01$ in (b); $F_{(5,30)} = 4.55$, $p < 0.01$ in (c); $F_{(3,23)} = 4.97$, $p < 0.01$ in (d). * $p < 0.05$, ** $p < 0.01$ *vs.* the (Sc.Aβ+V)-treated group; # $p < 0.05$, ## $p < 0.01$ *vs.* the ($A\beta_{25-35}$+V)-treated group; Dunnett's test.

Fig. 10. Chronic treatment of APP_{Swe} mice with OZP002. (a) Experimental procedure: OZP002 was administered IP 3 times-a-week in APP_{Swe} mice, at 10 to 12 month of age. Animals were tested at the indicated timepoints. Abbreviations: YMT, Y-maze test; WMT, water-maze test; NOT, novel object test; Sacrif, sacrifice and brain dissection; bioch, biochemical analyses. (b, c) Alternation performances and (d, e) number of arm entries of OZP002-treated APP_{Swe} mice, in the Y-maze, after 1 month (b, d) and 2 months (c, e) of treatment. Animals were treated with OZP002 (0.7 or 2 mg/kg IP) 3 times per week during 1 or 2 months. The number of animals per group is indicated in (d, e). One-way ANOVA: $F_{(5,49)} = 3.84$, $p < 0.01$ in (b); $F_{(5,54)} = 1.54$, $p > 0.05$ in (c); $F < 1$ in (d) and (e). * $p < 0.05$, ** $p < 0.01$ *vs.* V-treated WT group; # $p < 0.05$ *vs.* V-treated APP_{Swe} group; Dunnett's test in (b, c).

Fig. 11. A 2-month chronic treatment with OZP002 prevented learning deficits in APP_{Swe} mice. (a-c) Place learning in the water-maze and (d-f) novel object test performances of OZP002-treated APP_{Swe} mice after 2 months of treatment. Water-maze: acquisition profiles for V- and OZP002 (2 mg/kg)-treated WT mice (a) or APP_{Swe} mice (b). (c) Presence in the training (T) and others (o) quadrants during the probe test, performed 48 h after the last training session. The number of animals per group is indicated in (a-b). Repeated-measure non-parametric Friedman ANOVA: $Fr = 21.7$, $p < 0.001$ for V-treated WT group, $Fr = 21.8$, $p < 0.001$ for OZP002-treated WT group in (a); $Fr = 13.0$, $p < 0.05$ for V-treated APP_{Swe} group, $Fr = 12.2$, $p < 0.05$ for OZP002-treated APP_{Swe} mice in (b). Novel object test: total number of contacts with the objects during sessions 2 and 3 of the novel object test (d) and preference for the position #2/novel object in terms of contacts (e) or duration of contacts (f). The number of animals per group is indicated in (d). ° $p < 0.05$, °° $p < 0.01$ *vs.* chance level: 15 s in (c), 50% in (e,f), one-sample *t*-test. $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ *vs.* other (o)

quadrants in (c). * $p < 0.05$ *vs*. V-treated WT group in (d).

Fig. 12. Biochemical analyses of the effect of a chronic treatment with OZP002 in the hippocampus of APP_{Swe} mice. Lipid peroxidation was analyzed by colorimetric methods. Expression levels of Bax, IL1β, TNFα and Synaptophysin, level were analyzed using commercial Elisa kits. Data were expressed as percentage of control vehicle (V)-treated WT group. The number of animals per group is indicated in the columns. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. the V-treated WT group (100%); # $p < 0.05$ *vs*. the V-treated APP_{Swe} group; Student's *t*-test.

Table 1. Pharmacological profiling of OZP002. OZP002 was tested at one concentration in 76 *in vitro* binding assays designed with appropriate reference compounds in duplicates at CEREP facility.

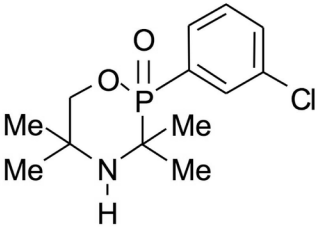
Pharmacological targets	*Inhibition at 1 µM (%)*
Sigma σ_1 receptor	-20.4 ± 0.6%
Neurotensin NTS1 receptor	-22.9 ± 1.5%
Nicotinic neuronal α_7 acetylcholine receptor	-23.2 ± 9.0%
Serotonin 5-HT_{2B} receptor	-25.5 ± 2.7%

Tested but not active *(inhibition < 20%):*
Purinergic receptors: A_1, A_{2A}, A_3; P2X, P2Y
Peptide receptors: Angiotensin AT1, AT2 receptors; AVP V_{1a} receptor; Bradykinin B2 receptor; non-selective Bombesin BB receptors; CGRP receptor; Cholescystokinin CCK1, CCK2 receptors; Endothelin ETA, ETB receptors; Galanin GAL1, GAL2 receptors; Melatonin MT1 (ML1A) receptor; Neurokinin NK1, NK2, NK3 receptors; Neuropeptide Y Y1, Y2 receptors; Opiate δ2 (DOP), κ (KOP), μ (MOP) receptors; nociceptin NOP (ORL1) receptor; PAC1 (PACAP) receptor; Somatostatine sst receptor; VIP VPAC1 (VIP1) receptor;
Neurotransmitter receptors: non-selective GABA receptors; central and peripheral Benzodiazepine receptors; Phencyclidine (PCP) receptor; Muscarinic M1, M2, M3, M4, M5 acetylcholine receptors; Nicotinic neuronal $\alpha_4\beta_2$ and muscle-type acetylcholine receptors; Cannabinoid CB1 receptor; Histamine H1, H2 receptors
Monoamine receptors: Dopamine D1, D2S, D3, D4.4, D5 receptors; Adrenergic α_1 (non-selective), α_2 (non-selective), β_1, β_2 receptors; Serotonin 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6, 5-HT_7 receptors
Monoamine transporters: norepinephrine transporter, dopamine transporter, 5-HT transporter
Cellular chaperone: Sigma (non-selective), sigma 2 receptors
Trophic factor receptor: PDGF receptor
Cytokine and hormones receptors: CXCR2 receptor; CCR1 receptor; TNF-α receptor; Melanocortin MC4 receptors; Prostaglandin EP2, EP4, IP (PGI2) receptors
Nuclear receptors: Glucocorticoid GR receptor; PPARγ receptor
Ion channels: L-type Ca^{2+} channel, KV channel, SKCa channel, Na^+ channel site 2 , GABA-gated Cl^- channel

Table 2. Calculation of combination index (CI) for the igmesine/OZP002 mix.

Treatment (mg/kg IP)	*PP (%)*	$C_{x,Igmesine}$	$C_{x,OZP002}$	***CI***
***(a)** FST (Fig. 2d)*				
Igmesine (10)	84.6 ± 7.4			
Igmesine (30)	63.8 ± 7.6 [1]			
OZP002 (5)	100.1 ± 5.4			
OZP002 (10)	67.5 ± 6.3 [2]			
Igmesine (10) + OZP002 (5)	68.6 ± 4.1	25.3 ± 1.9	11.3 ± 0.7	**0.84 ± 0.06**
***(b)** YMT (Fig. 3e)*				
Igmesine (0.3)	-5.4 ± 15.9			
Igmesine (1)	52.7± 15.7 [3]			
OZP002 (0.03)	17.4 ± 11.7			
OZP002 (0.1)	48.9 ± 11.5 [4]			
Igmesine (0.3) + OZP002 (0.03)	46.0 ± 17.0	0.95 ± 0.13	0.09 ± 0.1	**0.64 ± 0.08**

PP was calculated using 100% for V-treated animals and 0% for 0 s immobility in the FST (a) and using 100% for V-treated animals and 0% for scopolamine (0.5 mg/kg SC)-treated animals in the YMT (b). $C_{x,Igmesine/OZP002}$ was calculated using the linear regression from responses with the drug alone: [1] $y = -1.181x + 98.551$; [2] $y = -3.2461x + 105.44$; [3] $y = 58.092x - 9.4208$; [4] $y = 482.41x + 1.2115$.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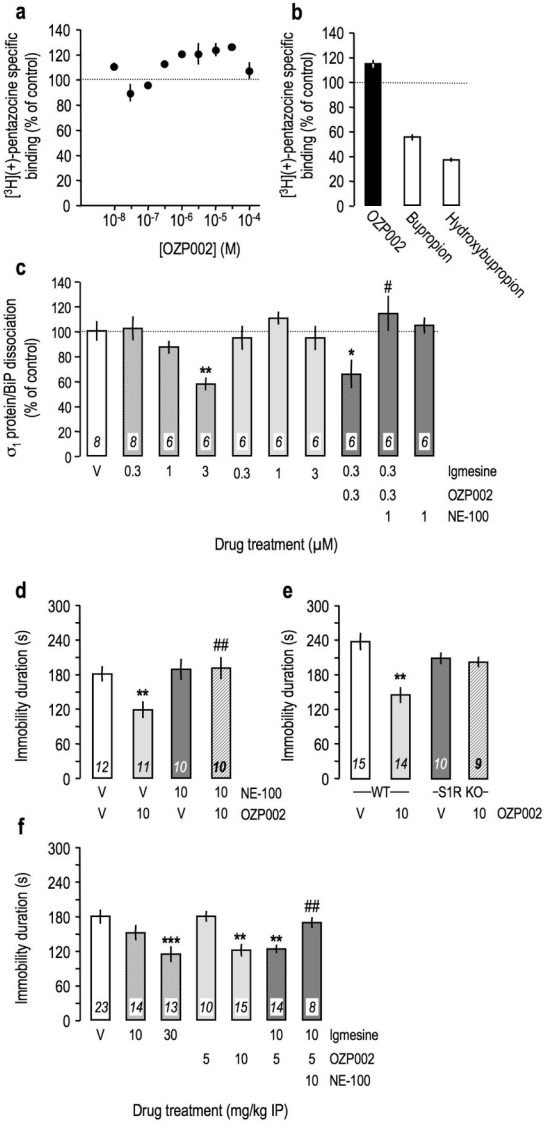
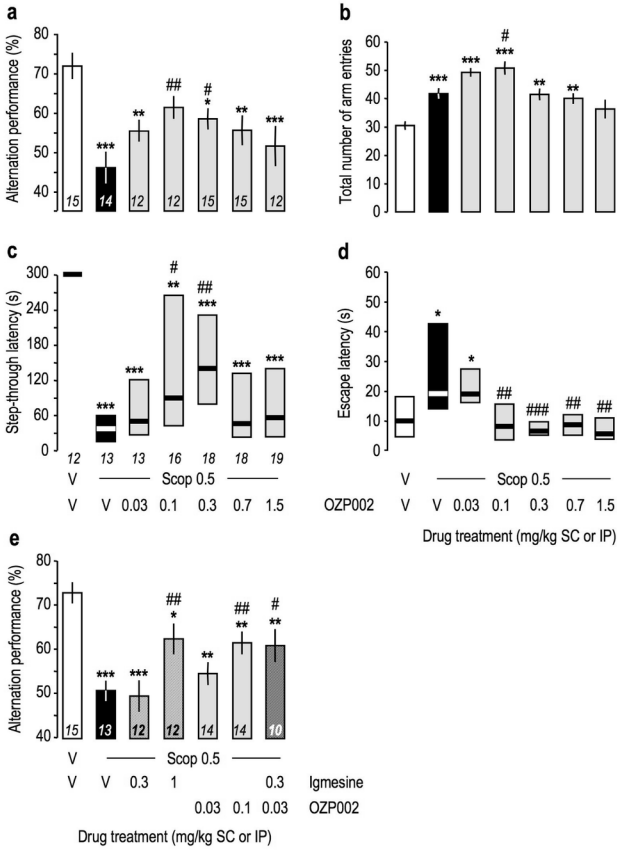
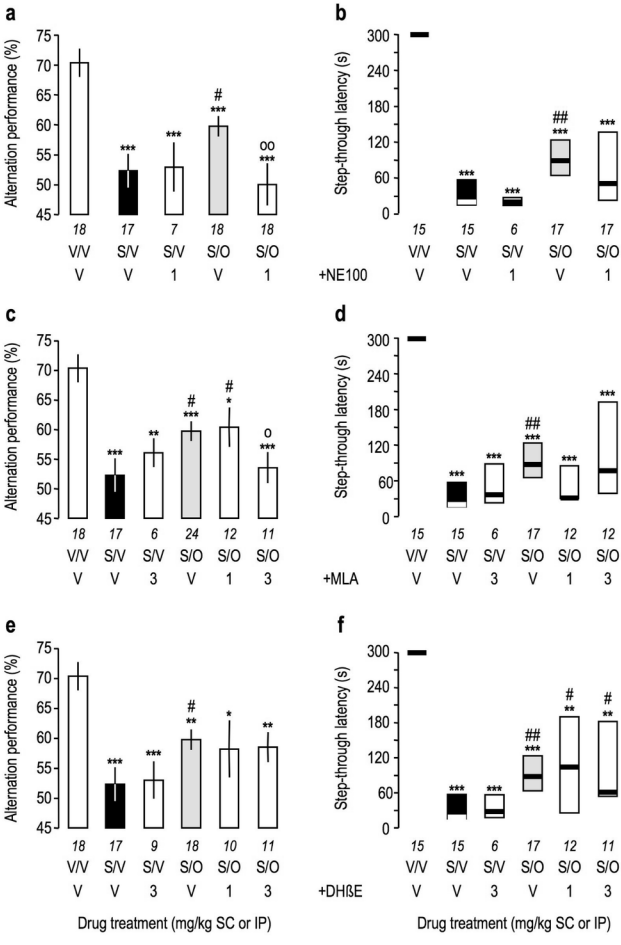
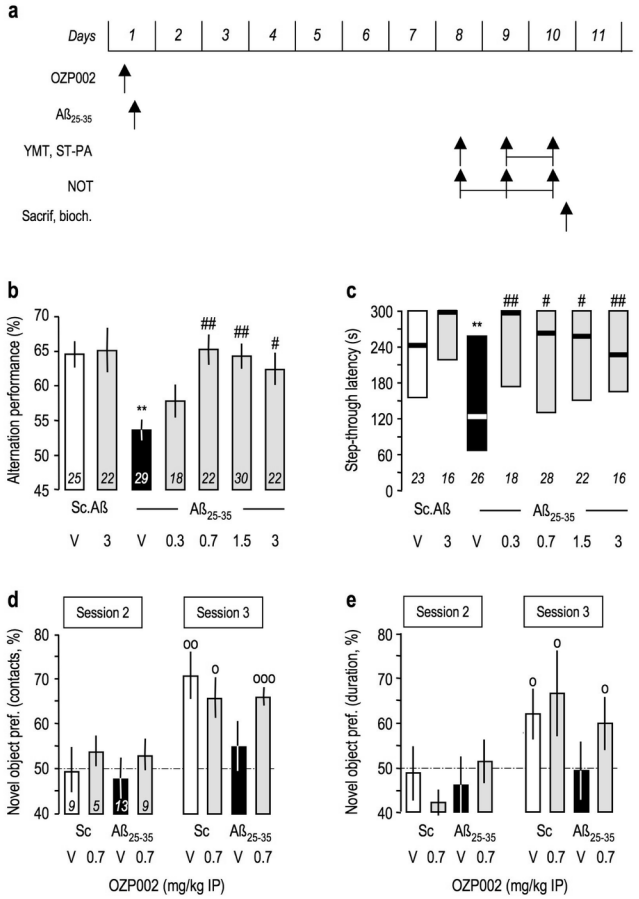
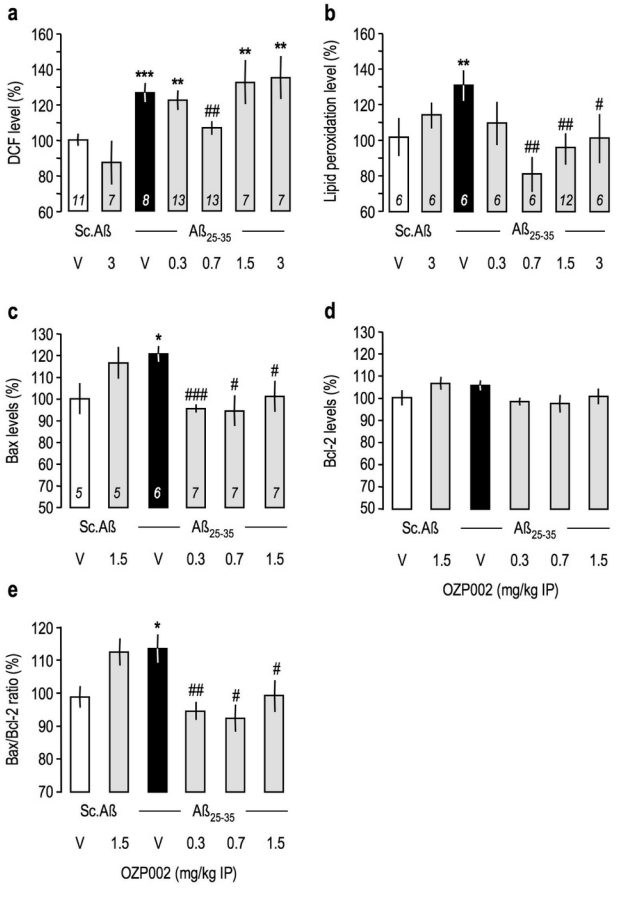
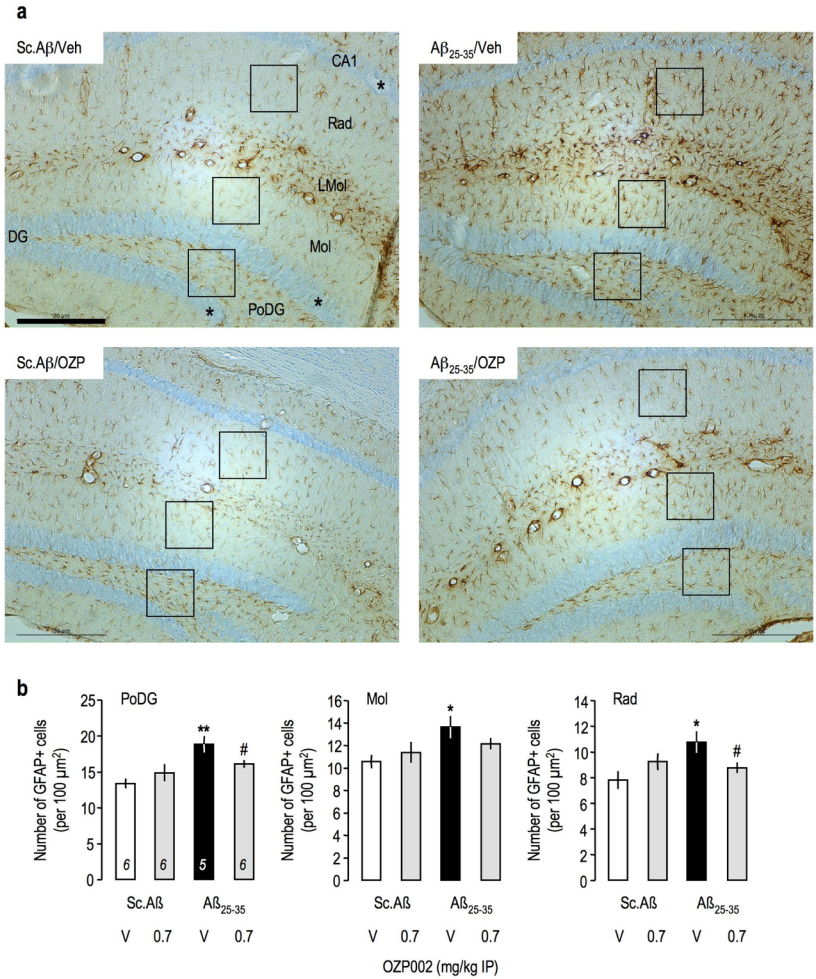

a
[3H](+)-pentazocine specific binding (% of control)
[OZP002] (M)
b
[3H](+)-pentazocine specific binding (% of control)
OZP002
Bupropion
Hydroxybupropion
c
σ1 protein/BiP dissociation (% of control)
Igmesine
OZP002
NE-100
Drug treatment (μM)
d
Immobility duration (s)
NE-100
OZP002
e
Immobility duration (s)
—WT—
–S1R KO–
OZP002
f
Immobility duration (s)
Igmesine
OZP002
NE-100
Drug treatment (mg/kg IP)

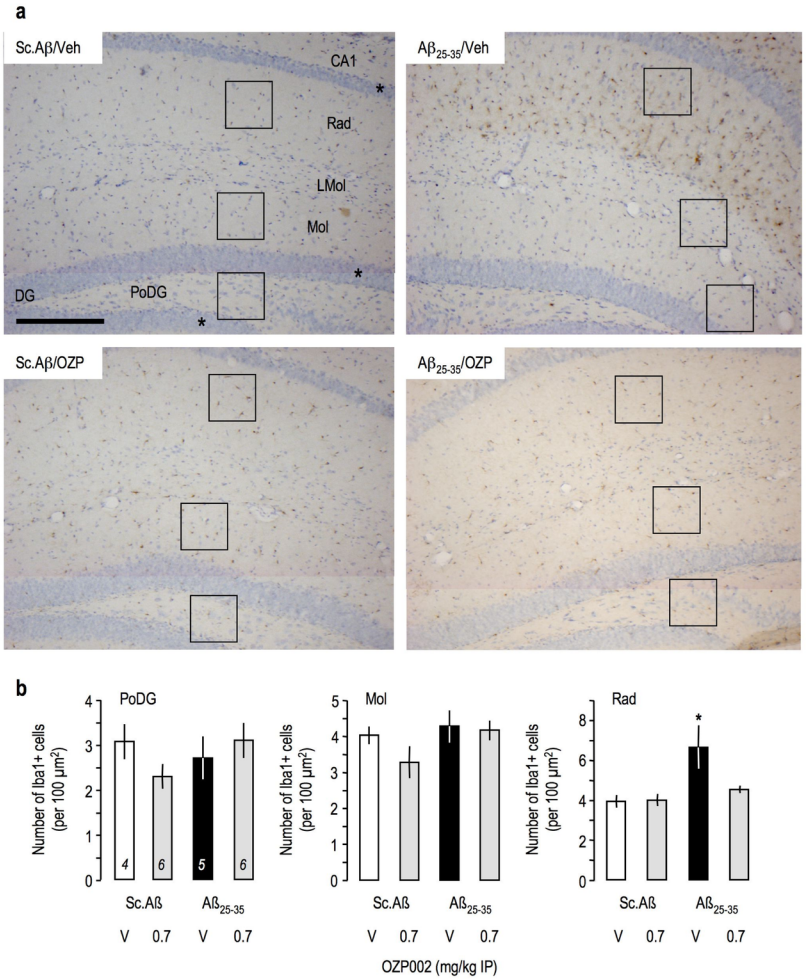

a
Alternation performance (%)
80
70
60
50
40
15
14
12
12
15
15
12
b
Total number of arm entries
60
50
40
30
20
10
0
c
Step-through latency (s)
300
240
180
120
60
0
12
13
13
16
18
18
19
V
Scop 0.5
V V 0.03 0.1 0.3 0.7 1.5
d
Escape latency (s)
60
50
40
30
20
10
0
V
Scop 0.5
OZP002 V V 0.03 0.1 0.3 0.7 1.5
Drug treatment (mg/kg SC or IP)
e
Alternation performance (%)
80
70
60
50
40
15
13
12
12
14
14
10
V
Scop 0.5
V V 0.3 1 0.3 Igmesine
0.03 0.1 0.03 OZP002
Drug treatment (mg/kg SC or IP)

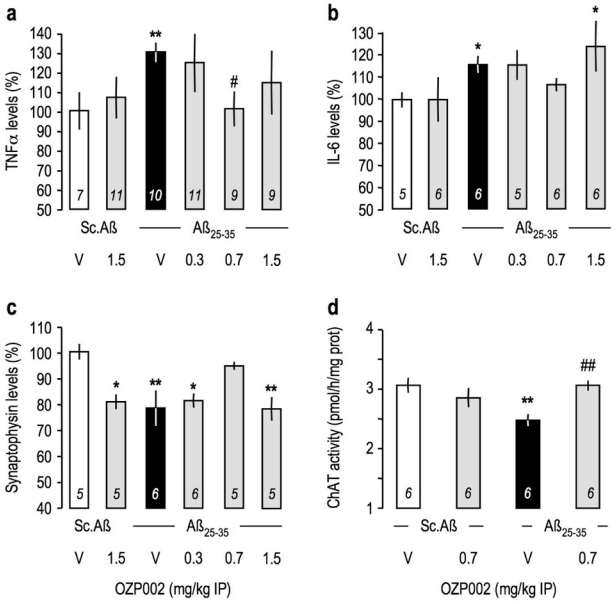

a
b
c
d
e
f
Alternation performance (%)
Step-through latency (s)
18 17 7 18 18
15 15 6 17 17
V/V S/V S/V S/O S/O
V V 1 V 1
+NE100
18 17 6 24 12 11
15 15 6 17 12 12
V/V S/V S/V S/O S/O S/O
V V 3 V 1 3
+MLA
18 17 9 18 10 11
15 15 6 17 12 11
V/V S/V S/V S/O S/O S/O
V V 3 V 1 3
+DHßE
Drug treatment (mg/kg SC or IP)
Drug treatment (mg/kg SC or IP)

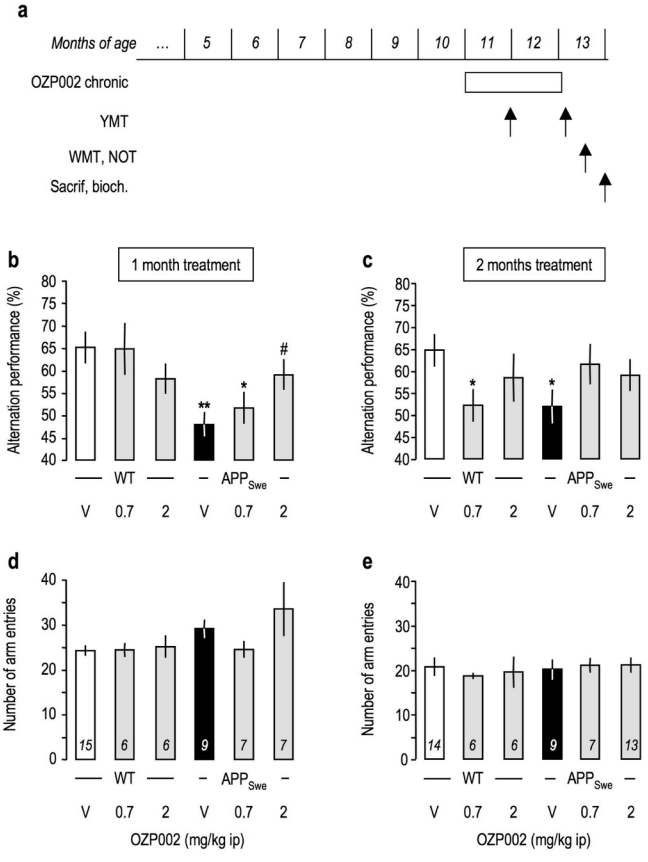

a
Days
1
2
3
4
5
6
7
8
9
10
11
OZP002
Aß25-35
YMT, ST-PA
NOT
Sacrif, bioch.
b
Alternation performance (%)
70
65
60
55
50
45
25
22
29
18
22
30
22
##
##
#
**
Sc.Aß
Aß25-35
V
3
V
0.3
0.7
1.5
3
c
Step-through latency (s)
300
240
180
120
60
0
23
16
26
18
28
22
16
##
#
#
##
**
Sc.Aß
Aß25-35
V
3
V
0.3
0.7
1.5
3
d
Session 2
Session 3
Novel object pref. (contacts, %)
80
70
60
50
40
9
5
13
9
oo
o
ooo
Sc
Aß25-35
Sc
Aß25-35
V
0.7
V
0.7
V
0.7
V
0.7
OZP002 (mg/kg IP)
e
Session 2
Session 3
Novel object pref. (duration, %)
80
70
60
50
40
o
o
o
Sc
Aß25-35
Sc
Aß25-35
V
0.7
V
0.7
V
0.7
V
0.7
OZP002 (mg/kg IP)

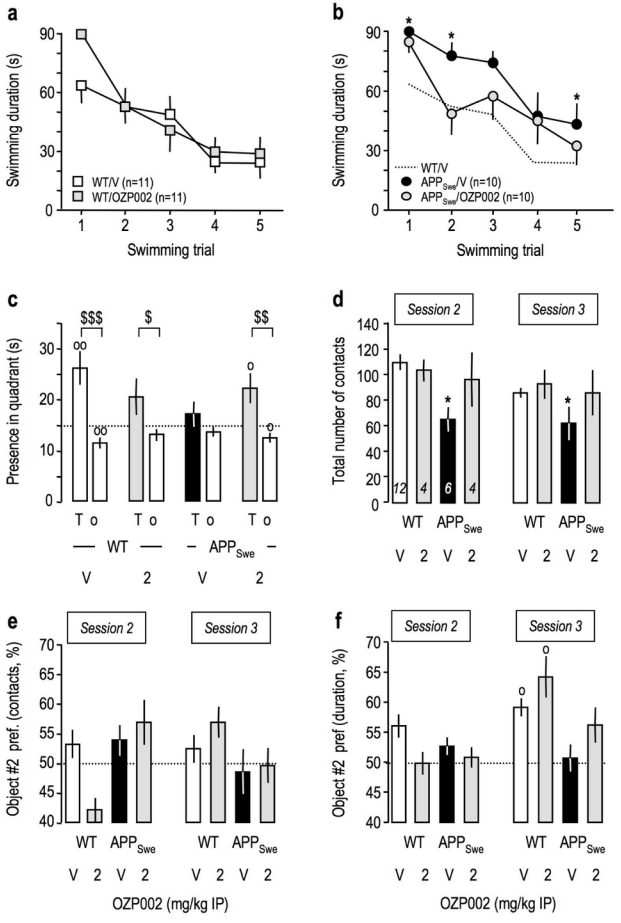

a
DCF level (%)
160
140
120
100
80
60
***
**
##
**
**
11
7
8
13
13
7
7
Sc.Aß
Aß25-35
V
3
V
0.3
0.7
1.5
3
b
Lipid peroxidation level (%)
160
140
120
100
80
60
**
##
##
#
6
6
6
6
6
12
6
Sc.Aß
Aß25-35
V
3
V
0.3
0.7
1.5
3
c
Bax levels (%)
130
120
110
100
90
80
70
60
50
*
###
#
#
5
5
6
7
7
7
Sc.Aß
Aß25-35
V
1.5
V
0.3
0.7
1.5
d
Bcl-2 levels (%)
130
120
110
100
90
80
70
60
50
Sc.Aß
Aß25-35
V
1.5
V
0.3
0.7
1.5
OZP002 (mg/kg IP)
e
Bax/Bcl-2 ratio (%)
120
110
100
90
80
70
*
##
#
#
Sc.Aß
Aß25-35
V
1.5
V
0.3
0.7
1.5
OZP002 (mg/kg IP)

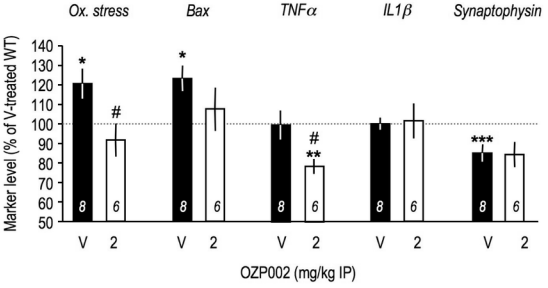

a
Sc.Aβ/Veh
CA1
Rad
LMol
DG
Mol
PoDG
Aβ25-35/Veh
Sc.Aβ/OZP
Aβ25-35/OZP
b
PoDG
Mol
Rad
Number of GFAP+ cells (per 100 µm²)
Sc.Aß
Aß25-35
V
0.7
OZP002 (mg/kg IP)


a
Sc.Aβ/Veh
CA1
Rad
LMol
Mol
DG
PoDG
Aβ25-35/Veh
Sc.Aβ/OZP
Aβ25-35/OZP
b
PoDG
Mol
Rad
Number of Iba1+ cells (per 100 µm²)
Sc.Aß
Aß25-35
V
0.7
OZP002 (mg/kg IP)


a
TNFα levels (%)
Sc.Aß
Aß25-35
V 1.5 V 0.3 0.7 1.5
b
IL-6 levels (%)
Sc.Aß
Aß25-35
V 1.5 V 0.3 0.7 1.5
c
Synaptophysin levels (%)
Sc.Aß
Aß25-35
V 1.5 V 0.3 0.7 1.5
OZP002 (mg/kg IP)
d
ChAT activity (pmol/h/mg prot)
Sc.Aß
Aß25-35
V 0.7 V 0.7
OZP002 (mg/kg IP)


a
Months of age
...
5
6
7
8
9
10
11
12
13
OZP002 chronic
YMT
WMT, NOT
Sacrif, bioch.
b
1 month treatment
Alternation performance (%)
80
75
70
65
60
55
50
45
40
**
*
#
WT
APPSwe
V
0.7
2
V
0.7
2
c
2 months treatment
Alternation performance (%)
*
*
WT
APPSwe
V
0.7
2
V
0.7
2
d
Number of arm entries
40
30
20
10
0
15
6
6
9
7
7
WT
APPSwe
V
0.7
2
V
0.7
2
OZP002 (mg/kg ip)
e
Number of arm entries
40
30
20
10
0
14
6
6
9
7
13
WT
APPSwe
V
0.7
2
V
0.7
2
OZP002 (mg/kg ip)

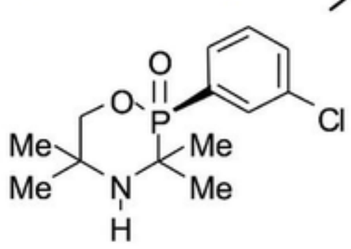

a
Swimming duration (s)
WT/V (n=11)
WT/OZP002 (n=11)
Swimming trial
b
Swimming duration (s)
WT/V
APPSwe/V (n=10)
APPSwe/OZP002 (n=10)
Swimming trial
c
Presence in quadrant (s)
$$$
$
$$
T o T o T o T o
WT
APPSwe
V 2 V 2
d
Session 2
Session 3
Total number of contacts
12 4 6 4
WT APPSwe WT APPSwe
V 2 V 2 V 2 V 2
e
Session 2
Session 3
Object #2 pref. (contacts, %)
WT APPSwe WT APPSwe
V 2 V 2 V 2 V 2
OZP002 (mg/kg IP)
f
Session 2
Session 3
Object #2 pref (duration, %)
WT APPSwe WT APPSwe
V 2 V 2 V 2 V 2
OZP002 (mg/kg IP)


Ox. stress
Bax
TNFα
IL1β
Synaptophysin
Marker level (% of V-treated WT)
140
130
120
110
100
90
80
70
60
50
*
#
*
#
**
***
8
6
V
2
OZP002 (mg/kg IP)

Sigma-1 Antagonists
(e.g., NE-100)

Sigma-1 Agonists
(e.g., Igmesine)

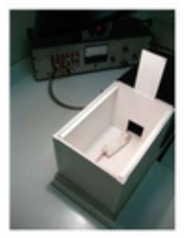
Sigma-1 PAM
(OZP-002)



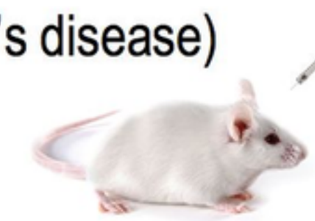
Sigma-1
receptor

Antidepressant

Anti-amnesiant



Neuroprotectant
(Alzheimer's disease)



$A\beta_{25-35}$ ICV injection

$hAPP_{Swe}$ line