Amyloid toxicity induced by oligomeric $A\beta_{25-35}$ injection is enhanced after pharmacologic or genetic invalidation of the σ_1 chaperone protein

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INTRODUCTION

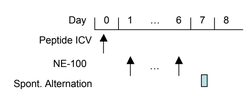
● Selective or non-selective ligands that specifically activate the σ_1 chaperone protein —so-called σ_1 receptor— have been repeatedly shown to mediate a potent neuroprotective activity. The σ_1 protein is a sensor/regulator of local calcium exchange among cellular organelles and thus appears to play a key role in cellular, and notably neuronal, homeostasis. By using in vitro or in vivo models of toxicity, σ_1 agonists were reported to be anti-oxidant, anti-ischemic and anti-inflammatory agents. In animal models of neurodegenerative pathologies, reference σ_1 agonists, including PRE-084, protected against apoptosis and neuronal death and maintained cognitive functions, particularly in Alzheimer's disease models induced by direct application of amyloid peptide oligomeric preparations in neuronal cell cultures or *in vivo*, by direct injection into the cerebral ventricles.

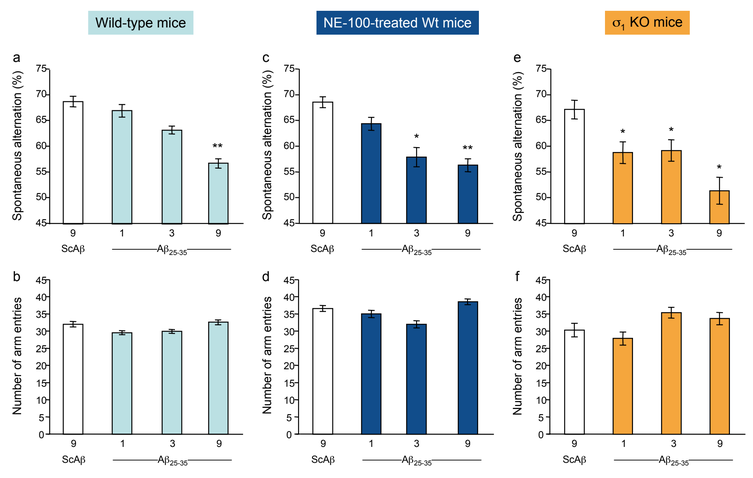
● We here analyzed whether activation of the σ_1 protein may play a role as endogenous protection system mobilized during the cellular insult by examining the consequences of its invalidation in mice submitted to a central injection of $A\beta_{25-35}$ oligomeric preparations. Wild-type (WT, C57BL/6j) or σ_1 KO mice received $A\beta_{25-35}$ at 1, 3 or 9 nmol icv 7 days before analyses of spatial working memory deficits (using spontaneous alternation in the Y maze) or long-term contextual memory deficits (using a step-down passive avoidance test). Animals were then sacrificed and the levels of lipid peroxidation in hippocampus extracts was analyzed as an index of $A\beta_{25-35}$-induced oxidative stress. The level of Bax protein expression was analyzed as an inde of apoptosis. The level of cholinesterase activity (ChAT) was measured as an index of the alteration of cholinergic systems. A group of mice received ip injection of the selective σ_1 antagonist NE-100 (3 mg/kg) once daily from day -1 to 4 after the peptide.

● In WT animals, $A\beta_{25-35}$ provoked learning deficits in both tests and oxidative stress at the highest dose, 9 nmol. The NE-100 treatement resulted in a facilitation of the toxicity, significant at the 3 nmol dose. In σ_1 KO mice, all three doses led to behavioral deficits, of higher intensity, and the two highest doses to increased lipid peroxidation levels. It therefore appeared clearly that the pharmacologic or genetic invalidation of the σ_1 protein directly augmented the impact of amyloid peptide toxicity.

FIGURE 1

Memory impairments after $A\beta_{25-35}$ injection in wild-type, NE-100-treated or σ_1 KO mice: Spontaneous alternation in the Y-maze

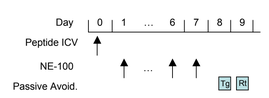


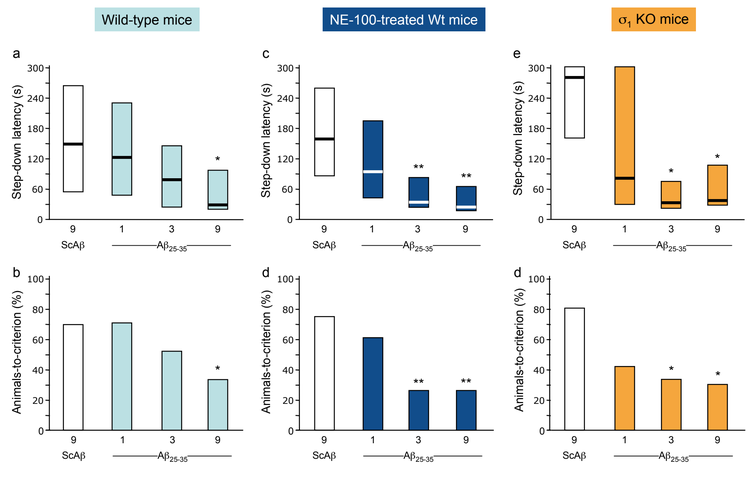


(a, c, e) Spontaneous alternation performance in the Y-maze. $F_{(3,85)} = 7.13$, $p < 0.01$ in (a), $F_{(3,74)} = 4.19$, $p < 0.01$ in (c), $F_{(3,47)} = 3.54$, $p < 0.05$ in (e). (b, d, f) Total number of arm entries. $F < 1$ in (b), $F_{(3,74)} = 2,06$, $p > 0.05$ in (d); $F_{(3,47)} = 1.69$, $p > 0.05$ in (f). In (c, d), NE-100 was injected at 3 mg/kg/day i.p. during 7 days. The number of animals per group was 21-22 in (a, b), 17-20 in (c, d) and 12 in (e, f). *$p < 0.05$, **$p < 0.01$ *vs.* the respective Sc.Aß-treated group; Dunnett's test.

FIGURE 2

Memory impairments after $A\beta_{25-35}$ injection in wild-type, NE-100-treated or σ_1 KO mice: Passive avoidance task





(a, c, e) Step-down latency during passive avoidance retention. Kruskal-Wallis non-parametric ANOVA: $H = 9.82$, $p < 0.05$ in (a), $H = 19.76$, $p < 0.001$ in (c), $H = 8.08$, $p < 0.05$ in (e). (b, d, f) Percentage of animals to criterion. In (c, d), NE-100 was injected at 3 mg/kg/day i.p. during 8 days. The number of animals per group was 23-25 in (a, b), 18-20 in (c, d) and 10-12 in (e, f). *$p < 0.05$, **$p < 0.01$ *vs.* the respective Sc.Aß-treated group; Dunn's test in (a, c, e); Fisher's exact test in (b, d, f).

MATERIAL & METHODS

Animals

Mice were housed with free access to food and water, in a regulated environment (23 ± 1°C, 40-60% humidity) under a 12 h light/dark cycle. OPRS1 mutant mice (+/-) Oprs1$^{Gt(IRESBetageo)33Lex}$ were purchased from the Texas Institute for Genomic Medicine (Houston, Texas). Littermates from heterozygous mating were used. Initially generated by a gene trapping strategy, animals were maintained in a C57BL/6j background. Genotyping was performed by PCR after modification of the manufacturer's instructions with the following primers sequences : wtF 5'-TCTGAGTACGTGCTGCTCTTCG-3' ; wtR 5'-GAAGGAAGGAAATCACTGGTAGG-3' ; koR 5'-ATAAACCCTCTTGCAGTTGCA-3' and PCR reaction parameters: 35 cycles of 94°C (15 s) - 59°C (30 s) - 72°C (40 s). Western blots confirmed that the σ_1 protein was absent in the kidney, liver, cerebellum, cortex and hippocampus of σ_1 KO mice.

Drugs and administration procedures

The amyloid-β[25-35] peptide ($A\beta_{25-35}$) and scrambled β_{25-35} peptide (Sc.Aβ) from Genepep (France) were solubilized in sterile distilled water at 3 mg/ml and stored at 20°C until use. Before injection, peptides were aggregated at 37°C for 4 days. They were injected i.c.v. in 3 µl per mouse. NE-100 was provided by UCB Pharma (Belgium), solubilized in saline and injected once a day at 3 mg/kg between day 1 to 6 after peptide injection.

Spontaneous alternation in te Y-maze

Spatial working memory was examined through the measure of spontaneous alternation performance in the Y-maze. Animals were allowed to explore the Y-shaped maze during 8 min and the number of alterantions (exploration in consecutive entries of the 3 arms) and total number of arms entered was recorded.

Step-through passive avoidance

Contextual long-term memory was measured using the step-through type passive avoidance test. During training, animals were placed in the white compartment of the two-compartment apapratus. The step-through latency to enter the dark side (of the moon) was recorded and mice received an electric shock (0.1 mA, 3 s). During the retention session, performed 24 h after training, the step-through latency to enter the dark side (of the force) and escape latency, to re-exit, were recorded.

Lipid peroxidation measures

Mouse hippocampus was homogenized in cold methanol, centrifuged at 1,000 *g* 5 min and supernatant collected. Homogenate was added to a solution of $FeSO_4$ 1mM, H_2SO_4 0.25 M, xylenol orange 1 mM and incubated for 30 min at RT. 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 RT. Absorbance was measured at 580 nm ($A_{580}2$). Lipid peroxidation was determined as CHP equivalents: CHP eq. = $A_{580}1/A_{580}2$ x [CHP (nmol)] x dilution.

ChAT activity measures

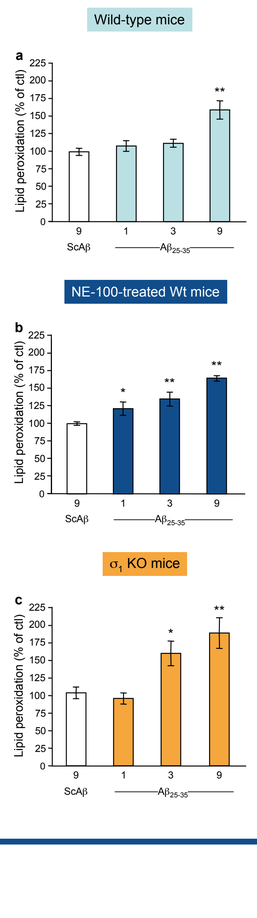
Hippocampi were homogenized in Trton X-100 1%, 10 mM EDTA, 10 mM phosphate buffer (pH 7.4) The incubation mixture contained 50 µL of 10 mM choline bromide, 50 µL of 1 mM physostigmine, 50 µL of 10 mM phosphate buffer (pH 7.4), 150 µL of 0.67 mM [^{3}H]acetyl-CoA (about 220,000 dpm), and 200 µL of mouse hippocampus homogenate. After incubation at 37 °C for 30 min, 2 mL of acetonitrile containing 10 mg of sodium tetraphenylborate (Kalibor) were added, together with 10 mL of liquid scintillator (Microscint 0). The solution was shaken and 4 mL sample of the organic phase was counted using a liquid scintillation counter (Packard, TriCarb 2300 TR). The ChAT activity was expressed as pmol of ACh formed per hour and per mg of tissue.

Western blotting

The hippocampus were disected out and stored at -80◦C. They were homogenized in lysis buffer (Tris-Hcl 125 mM pH 6.8; SDS 4%; glycerol 20%) including protease and phosphatase inhibitors (Roche Diagnostics), heated to 70◦C for 10 min and centrifuged. Protein concentration was determined using BCA assay (Pierce). Proteins, 40 mg per lane, were resolved on a 15% SDS-polyacrylamid gel. Proteins were transferred to a nitrocellulose membrane. Using Snap Id Millipore Machine, Membranes were blocked in 0,005 % non-fat dry milk in TBS 20 mM Tween-20 0.1% (TBS-T), incubated with Bax primary antibody (#2772, Cell Signaling). Membranes were incubated with goat anti-rabbit (A6154, Sigma-Aldrich), diluted 1:1000. After washing for 15 min and immunoreactive bands were visualized with the ECL (Pierce) using a Lumi-Imager F1 Workstation. Then, membranes were stripped using the Stripping Buffer (Pierce) and reprobed with ERK antibody (#43, Cell Signaling), diluted 1:1000 in TBS-T/milk 5%, followed by the goat anti-rabbit-HRP diluted 1:1000 and detection with ECL. Peroxidase activity was quantified ImageJ.

FIGURE 3

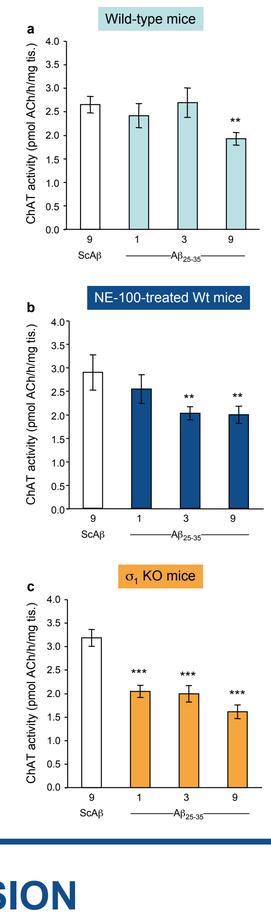
Oxidative stress after $A\beta_{25-35}$ injection in wild-type, NE-100-treated or σ_1 KO mice: lipid peroxidation and Bax protein levels in the hippocampus



Lipid peroxidation levels in hippocampus extracts measured 9 days after peptide ICV injection. ANOVA: $F_{(3,82)} = 10.21$, $p < 0.0001$ in (a), $F_{(3,39)} = 14.29$, $p < 0.0001$ in (b), $F_{(3,43)} = 8.84$, $p < 0.0001$ in (c).
In (b), NE-100 was injected at 3 mg/kg/day i.p. during 9 days. The number of animals per group was 20-21 in (a), 10 in (b) and 10-12 in (c). *$p < 0.05$, **$p < 0.01$ *vs.* the respective Sc.Aß-treated group; Dunn's test.

FIGURE 4

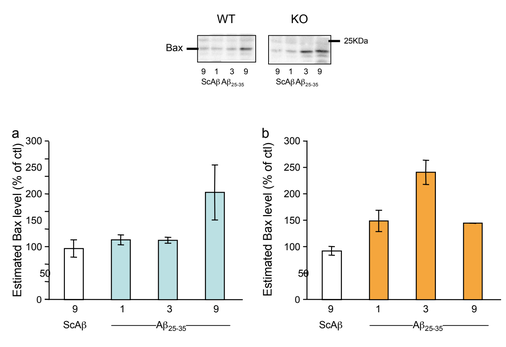
ChAT activity in hippocampus extracts after $A\beta_{25-35}$ injection in wild-type and σ_1 KO mice



ChAT activity levels in hippocampus extracts measured 9 days after peptide ICV injection. ANOVA: $F_{(3,47)} = 5.18$, $p < 0.01$ in (a), $F_{(3,47)} = 5.81$, $p < 0.01$ in (b), $F_{(3,47)} = 39.8$, $p < 0.0001$ in (c).
In (b), NE-100 was injected at 3 mg/kg/day i.p. during 9 days. The number of animals per group was 12. ** $p < 0.01$, *** $p < 0.001$ *vs.* the respective Sc.Aß-treated group; Dunn's test.

FIGURE 5

Bax protein expression after $A\beta_{25-35}$ injection in the hippocampus of wild-type, NE-100-treated or σ_1 KO mice



Bax expression levels were measured by Western blots in hippocampus extracts 9 days after peptide ICV injection. The number of animals per group was 1-4.

DISCUSSION

● In the present study, we present preliminary data showing that inactivation of the σ_1 chaperone protein results in enhanced sensitivity to the toxic effects of an oligomeric $A\beta_{25-35}$ peptide preparation. Two strategies were used: (1) the repeated IP administration of the selective σ_1 antagonist NE-100,[1,2] expected to lead to a long-lasting blockade of the receptor; and (2) mice knockout for the protein.[3] σ_1 KO mice do not show morphological alterations, particularly in the hippocampus or cortex, structures where $A\beta_{25-35}$ peptide dispersed and the toxicity occured the most intensively.[4]

● The $A\beta_{25-35}$ peptide was administered ICV at the 1, 3, 9 nmol doses and short-term (spontaneous alternation) and long-term (passive avoidance) memory deficits were observed one week after the injection of 9 nmol in wild-type animals. Similarly, biochemical markers of the toxicity, the oxidative stress (lipid perodixation), decreased cholinergic tonus (shown by decreased ChAT activity), and increased Bax expression, were measured in the hippocampus at this dose. In NE-100 treated animals, learning deficits and ChAT activity alteration appeared at 3 and 9 nmol and lipid peroxidation was significant at all doses tested. In σ_1 KO mice, learning deficits appeared at almost all doses. ChAT activity was highly significantly decreased at all doses. Lipid peroxidation and Bax expression were significantly increased at 3 and 9 nmol. The increase in lipid peroxidation, induced by 9 nmol of peptide, was also higher in σ_1 KO mice (+60%) as compared with controls (+90%). These data clearly illustrated an enhancement of amyloid toxicity after pharmacologic or genetic invalidation of the σ_1 protein in vivo.

● Pharmacological activation of the protein has been repeatedly shown to be neuroprotective, particularly against amyloid toxicity.[4,5,6] Activation of the σ_1 protein results in facilitation and regulation of Ca^{2+} mobilization at the endoplasmic reticulum, motochondria and plasma membrane, regulating ER stress sensor activity,7 kinases activities[8] and survival cellular fonctions.[9,10] The present data clearly suggest that activation of the σ_1 protein is a required endogenous protection systelm in neurons. The mechanism involved and cellular effects induced by σ_1 protein activation has to be determined, but strategies targeting σ_1 chaperones are clearly pertinent.

REFERENCES

[1]Okuyama et al. (1995) Life Sci 57:PL333-7; [2]Urani et al. (2001) J Pharmacol Exp Ther 298:1669-79; [3]Chevallier et al. (2010) J Psychopharmacol 25:960-75; [4]Villard et al (2009) Neuropsychopharmacology 34:1552-66; [5]Marrazzo et al. (2005) Neuroreport 16:1223-26; [6]Meunier et al. (2006) Br J Pharmacol 149:998-1012; [7]Hayashi & Su (2007) Cell 131:596-601; [8]Morin-Surun et al. (1999) Proc Natl Acad Sci USA 96:8196-9; [9]Meunier, Hayashi (2010) J Pharmacol Exp Ther 332:388-97; [10]Su et al. (2010) Trends Pharmacol Sci 31:557-66.