

Original Research Communication

Physiological Control of Nitric Oxide on neuronal BACE1 Translation by Heme-Regulated Eif2 α Kinase HRI Induces Synaptogenesis

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Abstract

Aims: Hippocampus is the brain center for memory formation, a process that requires synaptogenesis. However hippocampus is dramatically compromised in Alzheimer's disease due to the accumulation of amyloid β -peptide, whose production is initiated by BACE1. It is known that pathological stressors activate BACE1 translation through the phosphorylation of the eukaryotic initiation factor-2 α (eIF2 α) by GCN2, PERK or PKR kinases leading to amyloidogenesis. However BACE1 physiological regulation is still unclear. Since nitric oxide (NO) participates directly in hippocampal glutamatergic signaling we investigated the neuronal role of the eIF2 α kinase HRI, which can bind NO by a heme group, in BACE1 translation and its physiological consequences.

Results: We found that BACE1 is expressed upon glutamate activation being NO the downstream effector by triggering eIF2 α phosphorylation, as it was obtained by western blot and luciferase assay. It is due to the activation of HRI by NO as assayed by western blot and immunofluorescence with a HRI inhibitor and *HRI* siRNA. BACE1 expression was early detected at synaptic spines contributing to spine growth and consolidating the hippocampal memory as assayed with mice treated with HRI or neuronal NO synthase inhibitors.

Innovation: We provide the first description that HRI and eIF2 α are working in physiological conditions in brain under the control of nitric oxide and glutamate signaling, and also that BACE1 has a physiological role in hippocampal function.

Conclusion: We conclude *BACE1* translation is controlled by NO through HRI in glutamatergic hippocampal synapses where it plays physiological functions allowing the spine growth and memory consolidation.

Keywords: Alzheimer's disease; BACE1; eIF2 α ; nitric oxide; HRI; protein translation.

Introduction

Learning and memory are brain functions performed by the hippocampus. These functions are dramatically compromised in Alzheimer's disease (AD) due to the extracellular deposition of the neurotoxic amyloid β -peptide ($A\beta$) in the hippocampus (26).

$A\beta$ derives from the enzymatic cleavage of the amyloid precursor protein (APP) whose C-terminal domain is cleaved by the γ -secretase. The N-terminal domain of APP can be cleaved by the non-amyloidogenic α -secretase (ADAM10) preventing $A\beta$ formation, whereas the amyloidogenic β -secretase, a protease termed β -site APP Cleaving Enzyme 1 (BACE1) (38) initiates the $A\beta$ production.

BACE1 is the rate limiting step in the genesis of $A\beta$ but it also cleaves physiological substrates involved in the regulation of axonal myelination, membrane excitability and neurite length (20, 39), which would provide a physiological explanation for the existence of such enzyme. *BACE1* mouse knockout (KO) presents no serious abnormalities (24), although these animals show alterations in hippocampal synaptic plasticity as well as in performance on tests of cognition and emotion (22).

The limiting step that controls BACE1 expression is translation. *BACE1* mRNA contains a 5' untranslated region (5'UTR) that represses translation under basal conditions (23). *BACE1* 5'UTR is 570 nucleotides long, contains four upstream initiation codons (uAUGs) and possesses

a rich GC content. These features prevent the ribosome from reaching the main open reading frame (ORF) to start *BACE1* protein synthesis. Therefore *BACE1* translation is minimal under basal conditions. Paradoxically, *BACE1* translation is activated when global protein translation is inhibited through the phosphorylation of the eukaryotic initiation factor 2- α (eIF2 α) at Serine 51 (29).

There are four different eIF2 α kinases: the double-stranded RNA-activated protein kinase (PKR), the PKR-like endoplasmic reticulum-related kinase (PERK), the general control nonderepressible-2 kinase (GCN2) and the Heme-Regulated eukaryotic initiation factor eIF2 α kinase (HRI) (9). Some studies show that cellular stress associated with AD neurodegeneration stimulates *BACE1* translation through the activation of a specific eIF2 α kinase and the consequent eIF2 α phosphorylation, resulting in a burst of A β production that would trigger sporadic AD (18, 28, 29).

In the hippocampal synapses glutamate opens the NMDA receptor, producing a calcium influx that activates the nitric oxide (NO) neuronal synthase (nNOS), releasing NO. Since HRI has been previously reported to respond to NO in reticulocytes and erythrocytes (27,37) and there are evidences of HRI function in hippocampus (17) here we addressed the study of the role of NO in the physiological regulation of *BACE1* translation by HRI activation.

Results

Nitric oxide (NO) induces eIF2 α phosphorylation and BACE1 translation at synapses

BACE1 expression is activated by glutamate treatment for 1 h in hippocampal neurons (Fig. 1A). To restrict our study to the synaptic area, we stimulated mouse synaptosomes (Fig. 1B) with glutamate for 1 h (Fig. 1C). We found a significant increase in BACE1 protein (Fig. 1C, D) and phosphorylated eIF2 α (Fig. 1C, E). Co-treatment with 7-NI (a nNOS inhibitor; NOS-i) inhibited this effect (Fig. 1C-E), suggesting that eIF2 α phosphorylation and BACE1 expression were NO-dependent. This was confirmed by treating synaptosomes with increasing concentrations of the NO donor SNP for 1 h (Fig. 1F-H). The same results were obtained with primary cultures of cortical neurons (Fig. S1).

The rapid increase ($\Delta t \leq 1$ h) in BACE1 expression and eIF2 α phosphorylation in response to stimulation with NO suggested a translational activation from a pre-existing *BACE1* mRNA rather than *de novo* transcription of *BACE1* gene. We studied the possible transcriptional effect of NO and glutamate on BACE1 mRNA and we did not observe any BACE1 mRNA increase (Fig. 2A). Furthermore, we challenged SH-SY5Y cells with cycloheximide (a translation inhibitor) to demonstrate the translational regulation of BACE1 upon NO stimulation and as it was expected the effect of NO on BACE1 expression was abolished in the presence of cycloheximide (Fig.

2B). The use of anucleated preparations such as synaptosomes reinforced the view that BACE1 up-regulation was due to a nucleus-independent post-transcriptional event.

BACE1 has 4 AUGs in the 5'UTR (Fig. 3A), which are directed involved in the basal repression of its translation (23). In order to demonstrate that the effect of NO on BACE1 expression was due to a translational mechanism involving eIF2 α phosphorylation, we cloned BACE1 5'UTR upstream of a luciferase reporter gene (Fig. 3A) and confirmed its inhibitory effect over translation. This translational repression was overcome by mutating the 5'UTR (changing its 4 ATG by TTG) or using Sal003, a drug that selectively inhibits eIF2 α dephosphorylation (Fig. 3B). In Figure 3C it is shown that the treatment of the 5'UTR-luciferase construct (5'UTR-luc) transfected cells with high concentrations of SNP for 1 h reproduced the stimulatory effect induced by the mutated 5'UTR over *BACE1* 5'UTR, suggesting that NO stimulates the translation of *BACE1* mRNA through the phosphorylation of eIF2 α .

HRI kinase is required to activate BACE1 translation in response to NO

Among the four existing eIF2 α kinases in the mammalian genome only HRI induces eIF2 α phosphorylation upon direct activation by NO (37). Therefore we studied the expression of *HRI* mRNA and protein in human hippocampus, mouse hippocampal neurons and human neuroblastoma cells. We have obtained that both *HRI* mRNA and protein are present in all the tissues and cells studied (Fig. 4A, B). We also found that HRI colocalized with the post-synaptic marker PSD95 in the synaptic spines (Fig. 4C), as well as p-eIF2 α (Fig. S2).

To study the role of HRI in eIF2 α phosphorylation and BACE1 expression we used both a specific HRI-i (32) and a small interfering RNA (siHRI) knockingdown HRI expression *in vitro* (Fig. S3A). SH-SY5Y cells pre-incubated with the HRI-i failed to respond to NO and did

not overexpress BACE1 nor increase the levels of phosphorylated eIF2 α (Fig. 5A-C). On the other hand the same activation on BACE1 expression was obtained with two different NO donors, CysNO and GSNO (Fig. S4).

Similarly when HRI expression was knocked down by a specific siRNA, NO did not up-regulate BACE1 protein (Fig. 5D). Similar results were also obtained by using Noc 7, another NO donor (Fig. S3B). Consistent with these *in vitro* findings, NOS-i and HRI-i administered chronically to mice for one week induced a significant reduction in the levels of BACE1 and phospho-eIF2 α in the synaptosomes isolated from their hippocampi (Fig. 5E-G).

In order to demonstrate the specificity of the NO-HRI-eIF2 α in our experiments we challenged with the NO donor MEF cells with a Serine 51 to Alanine substitution in the eIF2 α , the phosphorylation residue. Hence, we studied the BACE1 levels after the SNP treatment in the eIF2 α -SS MEF cells (the homozygous wildtype) and in the eIF2 α -AA MEF cells (the homozygous mutant) (Fig. S5A). Since eIF2 α can be also phosphorylated by other kinases (PKR, PERK and GCN2) we have demonstrated in KO MEF cells for each of these kinases that NO induces the expression of BACE1. This expression is depending on HRI as it was also demonstrated with HRI KO cells or by using HRI-i in the other eIF2 α -kinase KO MEF cells (Fig. S5B).

On the other hand, we treated SH-SY5Y cells with increasing concentrations of the cGMP analog dibutyryl-cyclic GMP (dbcGMP) and observed no stimulatory effect on BACE1 expression (Fig. S6), ruling out the possibility of participation of the classical NO/sGC/cGMP pathway in the NO-induced BACE1 up-regulation.

NO induces the translation of an active BACE1 protease

In the Figure 6 we addressed whether the rapid translational activation of BACE1 induced by NO would result in an active BACE1 protease. We stimulated HEK-APP^{sw} cells with SNP for 1 h and collected the medium for A β determination as an indicator of BACE1 activity (Fig. 6A,B). We found that both A β ₁₋₄₀ and A β ₁₋₄₂ isoforms were increased after treatment with increasing concentrations of SNP for 1 h, supporting the fact that BACE1 translation is rendering the active enzyme. To confirm the exclusive role of BACE1 in A β increase induced by NO we stimulated ADAM10 KO MEFs with SNP and observed an increase in APP C-terminal fragment (APP CTF) that correlated with a substantial increase in BACE1 expression (Fig. 6C), suggesting a role of BACE1 protease in the A β increase induced by NO signaling (Fig. 6A,B). Similarly, PS1/PS2 db-KO MEFs responded to SNP with an increase in APP CTF (Fig. S7) that can be due to an increased BACE1 activity over APP. Concomitant with the enhancement in A β production due to BACE1 activity, we observed that the levels of secreted soluble APP α (sAPP α), a product of α -secretase cleavage, decreased gradually after stimulation with SNP in HEK-APP^{sw} cells *versus* the total levels of APP (holo APP) (Fig. 6D).

HRI colocalizes with BACE1 at dendritic spines in response to NO

Synaptic plasticity in the hippocampal glutamatergic neurons occurs at dendritic spines allowing their growth. The NMDA receptors and all the downstream molecules involved in hippocampal function are located in the dendritic spines to allow a rapid expression of the needed proteins. We confirmed the increase of synaptic markers in hippocampal cells in response to the NO donor SNP, a specific regulation that was avoid in the presence of HRI-i (Fig. 7A). We found that BACE1 expression increased at dendritic spines colocalizing partially with PSD95 as we shown by immunofluorescence analysis and Mander's coefficient quantification (Fig. 7B, C). We

also found that the activation of HRI induced spine growth as the ratio spine number/spine size increased after stimulation with SNP as shown in the Figure 7D. This effect was prevented by pre-incubating the cells with the HRI-i but not with the PKR-i (Fig. 7D). Furthermore, a classical antioxidant as reduced glutathione (GSH) did not affect the levels of BACE1 induced by glutamate in hippocampal cells (Fig. S8A). To confirm this lack of activity of oxidative stress in our results we have challenged the cells with the oxidative stressor hydrogen peroxide, an activator of PKR, and did not observe any effect in spine growth (Fig. S8B).

HRI is essential for memory consolidation in mice

To further study the *in vivo* relevance of the NO/HRI pathway in cognitive processing we administered i.p. inhibitors for HRI and NOS during the memory consolidation period as shown in the Figure 8. Firstly we showed by Western Blot from hippocampi of the treated mice the decrease on BACE1 and p-eIF2 α when mice were treated with HRI-i (Fig. 8A). In the cognitive study, we used two paradigms involving hippocampal processing: the object-recognition test (Fig. 8B) and the context-recognition test (Fig. 8C). Mice receiving HRI-i or NOS-i showed a memory impairment in the object-recognition (Fig. 8B), without affecting their exploratory behavior (inset in Fig. 8B). Similarly, context-recognition memory was significantly compromised by both inhibitors (Fig. 8C).

Discussion

Hippocampus is the main center for memory formation, where NO plays a key role in glutamatergic signaling (12, 35, 40). However hippocampus is one of the most affected brain structure in AD because of the high load of A β , due to the deregulation of BACE1 activity, that induces a dramatic memory impairment when aggregates (19, 26). Currently the physiological role of BACE1 in hippocampus is practically unknown. In the present work we report that the enzyme HRI is activated by NO in hippocampal neurons contributing to synaptogenesis through BACE1 expression.

Glutamatergic signaling is characterized for a postsynaptic burst in NO, which acts as a retrograde gaseous neurotransmitter (2) with an important role in long-term potentiation (LTP) and memory formation (12, 35, 40) due to the maintenance of hippocampal glutamatergic release at the presynaptic ending. Among the four existing eIF2 α kinases (9) only HRI induces eIF2 α phosphorylation upon direct activation by NO (37). HRI was first identified in rabbit reticulocytes (27) where it participates in the regulation of hemoglobin synthesis according to heme availability (4). In this context, the lack of heme activates HRI (9) leading to a translational arrest that avoids the synthesis of defective hemoglobin. HRI is activated by NO binding to the heme group in the N-terminus of the kinase (36). The structural details of the NO-mediated activation of HRI have been identified (15) resulting in a mechanism similar to that of the

canonical NO/sGC pathway since both enzymes share a heme group (16). The effector domain of HRI catalyses the eIF2 α phosphorylation. The phosphorylation of eIF2 α leads to an arrest of global protein synthesis. Nevertheless, neuron terminals have highly compartmentalized environments. Therefore, eIF2 α phosphorylation occurs far away from the neuron soma without any consequence in the neuron somatic metabolism, and only altering temporarily the translational profile at synaptic sites, an effect that can be easily delimited by the action of eIF2 α phosphatases. Translational control is a crucial mechanism in highly polarized cells such as neurons, where mRNAs are distributed asymmetrically at different subcellular locations. In neurons, new protein synthesis is needed at active synaptic sites. Translational control of mRNAs located at synaptic sites allows rapid and specific genetic changes in a given synapse, in a process referred as “input specific synaptic remodelling”. However, the nature of the intracellular signals that couple neurotransmitter to the translational machinery is currently unknown. In that regard, we propose that NO can control translational initiation through direct activation of HRI kinase.

Furthermore the phosphorylation of eIF2 α has been shown to be a critical event in long-term memory formation (7). Interestingly HRI has been recently proposed to mediate memory consolidation in mice (17), as we have also found in this work regarding BACE1 activation as a downstream effect of HRI activation. We have found that NO-HRI-eIF2 α signaling induces BACE1 translation. We used SNP as a NO donor because it mimics the NO burst produced by NOS activation however a decrease in BACE1 expression have been previously reported by using the NO donor CysNO (21). These controversial results would be related with the different cell types used in the cited work, since we have also obtained an increase in BACE1 when hippocampal neurons were treated with CysNO.

BACE1 mRNA is present in dendritic spines of hippocampal pyramidal neurons (34) and contains two cytoplasmic polyadenylation elements (CPE), and accessory sequences in its long 3'untranslated region (3'UTR) that target mRNAs to dendrites, where they are translated upon adequate stimulation (14). It allows having a constitutive pool of *BACE1* mRNA at postsynaptic ending while its regulation is carried out at translational level due to eIF2 α kinases (18, 29, 30).

BACE1 translation by NO action produces an active enzyme, which agrees with previous reports showing that basal synaptic activity produces A β (6). We found that HRI controls BACE1 expression and both enzymes are located at dendritic spines. A strict physiological regulation of BACE1 protein expression, whose levels are aberrantly up-regulated in sporadic AD (13), might explain the participation of BACE1 in synaptic plasticity and memory formation as a rapid mechanism to allow the cleavage of APP (25) (Fig. S9A). We hypothesize that the NO-mediated BACE1 increase at synapses is directed to lower the adhesion force of neuritic terminals to the extracellular matrix through the cleavage of APP. Since APP is a cell adhesion molecule (5, 8), localized cleavage of APP would imply a reduction in the dendritic attachment that allows synaptic growth and rearrangement. In fact memory formation requires synaptic structural changes (1). An effect of BACE1 in the shedding of other proteins needed for neuronal function (20, 39), beside APP cleavage, is also completely plausible but far from the main aim of our work. As well as the NO-HRI-eIF2 α pathway in glutamatergic signaling should have other key targets, as the CREB repressor ATF4 (CREB2) (11, 17), which are critical for hippocampal function. In the recent years, an increased number of papers have reported a role for hippocampal BACE1 protein in memory consolidation. Furthermore, here and in a previous work we identified that hippocampal HRI kinase is a necessary player in the consolidation of object recognition memory, a process in which HRI regulates hippocampal BACE1 expression (17).

In pathological events involving oxidative stress such as Alzheimer's disease, two parallel events take place in relation to NO. Firstly, there is a loss in NO bioavailability due to the reaction with superoxide anion to form peroxynitrite (19), therefore its physiological effects in memory formation and neuronal plasticity are dramatically reduced. Secondly, peroxynitrite triggers the initiation of a harmful nitrative cascade (19). In this pathological scenario the tight regulation of BACE1 translation by NO-HRI-eIF2 α is lost but the other eIF2 α kinases (PKR, PERK and GCN2), which are stress sensors, will be permanently activated to induce BACE1 expression in an uncontrolled way yielding to the phosphorylation of eIF2 α , which initiates the amyloid cascade that triggers AD (Fig. S9B).

In conclusion, we propose NO as the physiological activator of BACE1 translation in synapses upon HRI activation, which is critical to allow synaptogenesis and memory consolidation. As the signaling pathway initiated by NO transduces the signal through the phosphorylation of eIF2 α , and eIF2 α phosphorylation is an important homeostatic response to cellular stress, our findings could help understand the opposing effects in which BACE1 is involved.

Innovation

BACE-1 (β -secretase-1) is the key enzyme producing amyloid β -peptide ($A\beta$) that causes Alzheimer's disease (AD) but BACE1 physiological role is practically unknown. BACE1 knock out mice were reported to have problems in memory and learning and here we found that BACE1 is allowing hippocampal spine growth and memory consolidation. Moreover we have found that BACE1 translation in the hippocampal glutamatergic synapses is mediated by the enzyme heme-kinase HRI, upon activation of nitric oxide, which phosphorylates the initiation factor eIF2alpha.

Materials and Methods

Human hippocampal samples

Human hippocampal samples were supplied by the Banc de Teixits Neurològics (Serveis Científico-Tècnics, Hospital Clínic, Universitat de Barcelona). The procedure was approved by the ethics committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. The hippocampus from a healthy aged individual was lysated with a cocktail containing NP40 lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 0.05% aprotinin, 1mM dithioitretol) and protease inhibitors (Complete mini-EDTA free, Roche Diagnostics GmbH, Basel, Switzerland). The mix was mechanically disaggregated and centrifuged at 17,500 x g for 10 min. The supernatant was quantified by the Bicinchoninic Acid (BCA; Pierce BCA Protein assay kit, Thermo Scientific) assay.

Cell cultures

Hippocampal and cortical neurons were isolated from 18 day-old CB1 mouse and Wistar rat embryos. Mice and rats were anesthetized with 20 mg/Kg xilacina plus 100 mg/Kg ketamina and sacrificed. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques-Universitat Pompeu Fabra. Hippocampi were aseptically dissected and trypsinized. Cells were seeded in phenol-red-free Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, USA) plus 10% horse serum into 1% poly-L-Lysine coated coverslips (5×10^4 cells/cover). After 120 min, the medium was removed and neurobasal medium containing 1% B27 supplement (Gibco BRL, Carlsbad, USA) plus antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin) was added. Glial proliferation was avoided by adding 2

μ M cytosine arabinoside (Sigma) at day 3 for 24 h. Cultured hippocampal and cortical neurons were used for the experiments on day 7. Human neuroblastoma cells (SH-SY5Y) were grown with F-12 (Ham) supplemented with 15% fetal bovine serum (FBS) and antibiotics. HEK cells overexpressing the Swedish mutation of APP (HEK-APP^{sw} cells), which enhances its cleavage by BACE1, were grown with DMEM supplemented with 10% FBS and antibiotics. Mouse embryonic fibroblasts (MEFs) that lacked either ADAM 10 (ADAM10 KO MEFs), the α -secretase, were grown with DMEM/F12 containing 10% FBS and antibiotics. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Preparation of cortical and hippocampal synaptosomes

C57 mice were treated intraperitoneally (i.p.) with 50 mg/Kg of nNOS inhibitor (NOS-i; 7-nitroindazole; Sigma-Aldrich) or 10, 50 and 100 mg/Kg HRI inhibitor (HRI-i; N-2,6-dimethylbenzyl-6,7-dimethoxy-2H-benzofurool-3,2-c-pyrazol-3-amine hydrochloride; Janssen Research and Development) and DMSO as vehicle control in a volume of 2 mL/Kg. HRI-i specificity was tested in a commercially-available selectivity panel (EMD Millipore) of over 100 kinase, enzyme, GPCR, and ion channel targets, and showed >100 fold selectivity for HRI kinase versus these pharmacological loci (32). After 24 h mice were anesthetized with 20 mg/Kg xilacina plus 100 mg/Kg ketamina and sacrificed. Cortex and hippocampi were dissected. The procedure was approved by the Ethics Committee of the Institut Municipal d'Investigacions Mediques-Universitat Pompeu Fabra. Samples were homogenized in cold homogenization buffer (5 mM Tris-HCl and 320 mM sucrose) and filtered. The homogenate was centrifuged at 1,000 x

g at 4°C for 10 min. The supernatant was recovered, and sucrose buffer added to a final sucrose concentration of 0.8 M. Samples were centrifuged at 13,000 x *g* for 30 min at 4°C. The supernatant was discarded and the synaptosome layer separated from mitochondria by carefully adding 1 mL of ice-cold 320 mM sucrose buffer and gently shaking. Synaptosome fraction was diluted in Hank's balanced saline solution (HBSS) buffer to a final protein concentration of 1 mg/mL. Protein concentration was determined using Bio-Rad protein assay. The final synaptosome suspension was distributed in 1 mL aliquots to perform the experiments. Synaptosome integrity was assessed by electron microscopy.

HRI mRNA extraction and RT-PCR

RNA extraction (Nucleospin RNA II kit, Macherey-Nagel) from human hippocampus, primary cultures of mouse hippocampal neurons and SH-SY5Y cells was carried out and RT-PCR was performed using SuperScrip-RT system (Invitrogen, Carlsbad, USA). Aliquots of 1 µg cDNA were used as template for PCR. The primers used to amplify HRI from human were: 5'-CCCCGAATATGACGAATCTG-3' and 5'-CAGATTCGTCATATTCGGGC-3'; the primers used to amplify HRI from mouse were: 5'-GAAGTGGGTTTGGTTCATGC-3' and 5'-GCATGAACCAAACCCACTTC-3'. PCR conditions for all transcripts were: 95°C for 3 min; 95°C for 30 s; 60° for 30 s, 72°C for 30 s; 72°C for 7 min; with 35 cycles of amplification. The amplified HRI was resolved in a 2% agarose gel.

HRI immunoprecipitation

200 µg of homogenate human hippocampus, 200 µg of protein from primary cultures of mouse hippocampal neurons and SH-SY5Y cells were used for the IP. The samples were pre-

incubated 30 min at 4°C with protein G (GE Healthcare, UK) previously washed with PBS. Then samples were centrifuged at 10,000 x g for 10 min. Overnight incubation of supernatants with 5 µg of rabbit anti-HRI Ab (Abcam, Massachusetts, USA) was followed by the addition of protein G immobilized on sepharose and shaken for 2 h at RT. HRI was pulled down by centrifugation at 14,000 x g for 10 min and washed thrice.

Cloning of BACE1 5'-untranslated region

Total RNA was extracted from SH-SY5Y cells, and one-step RT-PCR was carried out using kit (Qiagen, Hilden, Germany) with primers designed to amplify BACE1 5'UTR: 5'-GAAGCTTACAAGTCTTTCCGCCTCCCC- 3', 5'- GAAGCTTGGTGGGCCCCGGCCTTC-3'. PCR product, a single band matching the molecular weight of BACE1 5'UTR (~500 nt), was isolated and purified from an agarose gel using the Illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, UK) and stored at -20°C for further uses. The 5'UTR DNA fragment was then inserted into the HindIII site of a modified pGL4.10 [luc2] vector from Promega containing the CMV promoter cloned at BglIII and HindIII sites.

BACE1 mRNA analysis

SH-SY5Y were seeded in 6 well plates and exposed to 100 nM SNP and 10µM glutamate and for 1 h. Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions and reverse transcription PCR was performed using SuperScrip-RT system (Invitrogen, Carlsbad, USA). Quantitative RT-PCR was performed on an ABI Prism 7900HT (Applied Biosystems, Carlsbad, USA) with SYBR-Green (SYBR-Green Power PCR Master Mix, Applied Biosystems) and the following primers: BACE1

5'- CAA TGG ACA GGA TCT GAA AAT GG -3' and 5'- GCC ACT GTC CAC AAT GCT CTT -3'; GAPDH 5' GGA GTC CAC TGG CGT CTT C -3' and 5'- TGG CTC CCC CCT GCA AAT G -3'. GAPDH was used as a control. PCR conditions were: 95°C for 5 min; 95°C for 30 s; 60°C for 30 s, 72°C for 30 s; 72°C for 5 min; with 40 cycles of amplification.

Transient DNA transfection of SH-SY5Y cells and Luciferase assay

SH-SY5Y cells were seeded in 96-well plates at a density of 9,000 cells per well and grown for 12 h. Afterwards, a total of 250 ng of DNA was transfected into each well, adjusting to the following conditions: 250 ng of pcDNA3 plasmid (blanks); 25 ng of Renilla, 25 ng of CMV-Luciferase Vector and 200 ng of pcDNA3 (controls); and 25 ng of Renilla, 25 ng of BACE1-5'UTR CMV-Luciferase construct, 25 ng of BACE1-mutated-5'UTR CMV-Luciferase construct (the mutation consists in the change of the 4 ATG triplets of the 5'UTR by 4 TTG; Genscript) and 200 ng of pcDNA3 (test samples). Cells were transfected using JetPEI transfection reagent (PolyPlus, Illkirch, France) for 2 h. The medium was changed and the cells incubated for 10 h to allow gene expression. Cells were treated with 100 μ M of the inhibitor of p-eIF2 α phosphatase PP1 salubrinal (Sal003; Tocris) or the NO donor sodium nitroprusside (SNP; Sigma) for 1 h. 100 μ M SNP provides a flow of 100 μ M/min of NO (10). Cells were lysed and luciferase and Renilla activities were measured by using the Dual-Glo™ Luciferase Assay System (Promega, Wisconsin, USA) following manufacturer's instructions and luminescence was read using a luminometer (Fluostar OPTIMA, BMG Labtech).

Treatments

Hippocampal neurons, cortical synaptosomes, SH-SY5Y cells, HEK-APPsw cells, ADAM10 KO MEFs, PS1/PS2 db-KO MEFs were treated with glutamate (Sigma), SNP, cycloheximide, noc7, NOS-i, HRI-i or PKR inhibitor (PKR-I; Calbiochem). Cells and synaptosomes were immediately either harvested for WB analysis, fixed with 4% PFA for immunofluorescence or the medium collected for A β quantification and α APPs identification.

Protein identification by Western Blot

Cells and synaptosomes were lysed on ice with a solution containing 1 M Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, pH 7.4 and a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined by Bio-Rad protein assay in the homogenates and in the medium from HEK-APPsw cells. Aliquots of 80 μ g of protein were loaded into 8% (for HRI immunoprecipitated) and 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Darmstadt, Germany). Membranes were blocked in Tween 20-Tris buffer solution (TTBS: 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 5% milk and incubated o.n. at 4°C with 1:1,000 rabbit anti-BACE1 Ab (Chemicon International, Darmstadt, Germany), 1:500 rabbit anti-phospho-eIF2 α (Ser51) Ab (Cell signaling), 1:500 mouse anti-eIF2 α Ab (Cell signaling, Beverly, USA), 1:1000 rabbit anti-HRI, 1:500 rabbit anti-sAPP α (Abcam, Massachusetts, USA), 1:1,000 mouse anti-BACE1 Ab (Cell Signalling; for the work with KO MEFs), 1:10,000 rabbit anti-APP B63 (kindly provided by Dr. Bart de Strooper, Leuven University, Belgium), 1:4,000 mouse anti-actin Ab (Sigma) or 1:2,500 rabbit anti-GADPH Ab (Cell Signalling). Primary Abs were diluted either in 5% skimmed milk-TTBS (anti-BACE1, anti-eIF2 α , anti-HRI, anti-sAPP α and anti-actin) or in 5% bovine serum albumin in TTBS (anti-phospho-eIF2 α).

Peroxidase-conjugated donkey anti-rabbit and anti-mouse Abs (GE Healthcare) were used as secondary Abs at 1:5000 for 1 h at room temperature (RT). Bands were visualized with Super Signal (Pierce, Rockford, IL, USA) and develop with the Quantity One system. Optical density analysis of the bands was carried out by Quantity One software.

Immunofluorescence assays

Murine hippocampal neurons (5×10^4 cells/coverlip) were seeded into 1% poly-L-Lysine. SH-SY5Y cells (4×10^4 cells/coverlip) were seeded on 1.5% gelatine coated 12 mm coverslips. After treatments, cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 and incubated for 2 h at RT in a hydration chamber with 1:100 rabbit anti-BACE1 Ab, 1:100 phospho-eIF2 α (Ser 51) Ab, 1:500 rabbit anti-HRI Ab, 1:200 mouse anti-PSD95 (Abcam) or 1:200 mouse anti- β 3tubulin tuj1 (Covance, San Diego, CA, USA). After primary Ab, cells were incubated with 1:700 Alexa Fluor 555 goat anti-rabbit Ab (Life Technologies, Carlsbad, CA, USA) and/or 1:700 Alexa Fluor 488 goat anti-mouse Ab (Life Technologies) for 1 h at RT. Nuclei were stained with 1:1000 TO-PRO 3 iodide (Sigma, St. Louis, USA) in PBS for 10 min before mounting. Digital images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software. For colocalization experiments primary Ab was tested with its counter secondary Ab to discard any cross reactivity between rabbit and mouse isotypes.

A β measurement

The medium from HEK-APPsw cells was replaced for experimental medium (phenol-red-free DMEM containing 4.5 g/L D-glucose, L-glutamine, 25 mM HEPES) plus antibiotics and

supplemented with 0.2% FBS. After 1 h of SNP treatment, 800 μ L of medium were collected and centrifuged at 16,000 $\times g$ for 5 min to eliminate cellular debris. Solid-phase sandwich ELISA kits containing two highly specific Ab for detection of the A β peptides were used following manufacturer's instructions to measure human A β_{1-40} and A β_{1-42} (IBL Codes, Hamburg, Germany). The absorbance at 450 nm was determined for each sample. Data are expressed as percentages of the value for untreated cells.

Mander's coefficient measurement

Mander's coefficients were calculated in the experiments of colocalization of BACE1 and PSD95 in the synaptic spines from primary cultures of rat embryo hippocampal cells. Mander's coefficients measure the overlap of two different immunofluorescence channels (green and red stainings for BACE1 and PSD95 respectively in our experiments) showing the percentage of molecules stained with different colours that share the same location. Therefore we calculated the overlapping of the green channel (BACE1) with the red channel (PSD95) as previously described (31). It is expressed in arbitrary units from 0 to 1, being 0 a lack of overlapping and 1 the maximum of overlapping.

Object-recognition task. C57 mice were trained and tested in the V-maze for object-recognition memory, as described previously (3). After the training session, mice were injected i.p. with NOS-i (50 mg/Kg), HRI-i (10 or 50 mg/Kg) or vehicle (DMSO) in a volume of 2 mL/Kg. Object-recognition memory was tested 24 h later, and a discrimination index was calculated based on the time of exploration for novel and familiar objects in the test session, as previously described (3). Object recognition exploration times from animals (treated with HRI-i

or NOS-i as indicated above) were studied as described previously (3) to discard behavioral disorders.

Context-recognition task. C57 mice were trained and tested in conditioning chambers (Panlab) as described previously (3). Briefly, after electric foot shock conditioning, mice were injected i.p. with HRI-i (10 or 50 mg/Kg), NOS-i (50 mg/Kg) or vehicle (DMSO) in a volume of 2 mL/Kg, and their freezing behavior was analyzed 24 h later, during a 3 min re-exposure to the same conditioning chamber.

Statistical analysis

Data were expressed as the mean \pm SEM of the values from the number of experiments as indicated in the corresponding figures. Data were evaluated statistically by using the Student's t-test. Memory tests were evaluated statistically by using one-way ANOVA followed by Bonferroni's *post hoc* analysis.

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Author disclosure statement

The authors declare no conflict of interest.

Abbreviations used

Ab = Antibody

A β = Amyloid- β peptide

AD = Alzheimer's disease

ADAM10 = non-amyloidogenic β -secretase

APP = amyloid precursor protein

APP CTF = APP C-terminal fragment

APP^{sw} = Swedish mutation of APP

BACE1 = β -site APP Cleaving Enzyme 1

cGMP = cyclic guanosine monophosphate

eIF2 α = eukaryotic initiation factor 2- α

FBS = fetal bovine serum

GAP = D-glyceraldehyde 3-phosphate

GCN2 = general control nonderepressible-2 kinase

HRI = Heme-Regulated eukaryotic initiation factor eIF2 α kinase

HRI-i = HRI inhibitor

KO = knockout

MEF = mouse embryonic fibroblasts

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NO = nitric oxide

nNOS = neuronal NO synthase

NOS-I = nNOS inhibitor

PERK = PKR-like endoplasmic reticulum-related kinase

PKR = double-stranded RNA-activated protein kinase

PS1/PS2 db-KO mice = presenilin1/presenilin2 double knockout mice

sAPP α = soluble APP α

SIN-1 = 3-morpholino-sydnonimine

siRNA = small interfering RNA

SNP = sodium nitroprusside

o.e. = overexpressing

5'UTR = 5' untranslated region

WT = Wild type

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Figure Legends

Figure 1. NO induces BACE1 expression and eIF2 α phosphorylation. (A) BACE1 (green) and tubulin (red) in primary cultures of mouse hippocampal neurons treated with 10 μ M glutamate for 1 h. (B) Transmission electronic image of mouse cortical synaptosomes (arrows indicate synapses). (C) WB from cortical synaptosomes treated with 10 μ M glutamate or 10 μ M glutamate plus 10 μ M NOS-i for 1 h. (D, E) Densitometric quantifications from WBs as shown in C. Data are the mean \pm SEM values of 4 independent experiments; * $p < 0.005$ vs. control; # $p < 0.05$ vs. glutamate treated synaptosomes. (F) WB from cortical synaptosomes treated with SNP, a NO donor, for 1 h. (G, H) Densitometric quantifications from WBs as shown in F corrected for actin (G) and total eIF2 α (H). Data are the mean \pm SEM values of 4 independent experiments; * $p < 0.05$; ** $p < 0.005$ vs. control.

Figure 2. BACE1 mRNA transcription is not modified by NO or glutamate treatment and BACE translation is regulated by SNP. (A) SH-SY5Y cells were treated with 100 nM SNP or 10 μ M glutamate for 1 h. Cells were lysed and *BACE1* mRNA was quantified by qRT-PCR. Data are the mean \pm SEM values of 6 independent experiments. (B) WB from SH-SY5Y cells treated with 100 nM SNP for 1 hour and 30 minutes pre-treatment with 100 μ M cycloheximide. Densitometric quantifications from WB are shown. Data are the mean \pm SEM values of 4 independent experiments; *** $p < 0.001$ vs control; ## $p < 0.01$ vs SNP.

Figure 3. BACE1 translation induced by NO depends in the 5'UTR de-repression. (A) BACE1 3'UTR contains two cytoplasmic polyadenylation elements (CPE, UUUUUAU) and accessory sequences (AAUAA) that facilitate mRNA localization in dendrites by the CPE-binding protein (CPEB). BACE1 5'UTR was cloned and inserted upstream of a luciferase

reporter gen. (B) BACE1 5'UTR strongly downregulates luciferase signal in transfected SH-SY5Y cells. This repression is abolished when the BACE1 5'UTR is mutated or treated with 100 μ M Sal003. Data are the mean \pm SEM values of 6 independent experiments. ** $p < 0.01$ vs CMV; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ vs BACE1 5'UTR. (C) Treatment with increasing concentrations of SNP increases luciferase signal in SH-SY5Y cells transfected with BACE1 5'UTR but do not have any effect on cells transfected with the mutated BACE1 5'UTR. Data are the mean \pm SEM values of 6 independent experiments. * $p < 0.05$; ** $p < 0.01$ vs controls (0 SNP).

Figure 4: HRI is expressed in brain. (A) RT-PCR of HRI mRNA from human hippocampus, mouse hippocampal neurons and SH-SY5Y cells. (B) WB of HRI protein immunoprecipitated from human hippocampus, mouse hippocampal neurons and SH-SY5Y cells, as shown by WB. (C) Colocalization of HRI (red) with PSD95 (green) in primary cultures of mouse hippocampal neurons.

Figure 5. HRI controls BACE1 expression in response to NO. (A) WB from SH-SY5Y cells pre-incubated with HRI-i for 30 min and then treated with 50 nM SNP for 1 h. (B, C) Densitometric quantifications from WBs as shown in A corrected for actin (B) and total eIF2 α (C). Data are the mean \pm SEM values of 4 independent experiments; * $p < 0.05$; ** $p < 0.01$ vs. control; # $p < 0.05$, ## $p < 0.01$ vs. glutamate-treated synaptosomes. (D) BACE1 expression in SH-SY5Y cells HRI-knocked down with specific *siHRI RNA* and treated with 50 nM SNP. Inset shows GFP efficiency of siRNA transfection. (E) WB from hippocampal synaptosomes from mice treated chronically with HRI-i and NOS-i during 1 week. (F, G) Densitometric

quantifications from WBs as shown in E corrected for GADPH (F) and total eIF2 α (G). Data are the mean \pm SEM values of 3-4 independent experiments; * p <0.01; ** p <0.001 vs. control.

Figure 6. NO induces the expression of active BACE1. (A, B) A β ₁₋₄₀ (A) and A β ₁₋₄₂ (B) production in HEK-APPsw cells treated with increasing concentrations of SNP for 1 h. Data are the mean \pm SEM values of 6 independent experiments; * p <0.05; ** p <0.01; *** p <0.001 vs. control. (C) WB from ADAM10 KO MEFs treated with SNP for 1 h and densitometric quantifications from the WBs. Data are the mean \pm SEM values of 5 independent experiments; * p <0.005; ** p <0.001 vs. control. (D) WB from the extracellular media (for sAPP α) and the total lysed cell (for holo APP) of HEK-APPsw cells treated with increasing concentrations of SNP for 1 h.

Figure 7. NO induces BACE1 expression at the synaptic spines. (A) Representative Western blot of hippocampal primary culture cells treated with 100 nM of SNP for 1 h, with or without HRI-i. Analyzing the levels of the post-synaptic protein PSD95 and pre-synaptic protein Synapsin 1. (B) Colocalization of BACE1 (green) with PSD95 (red) in primary cultures of rat hippocampal neurons and amplified synaptic spines treated with SNP for 1 h. Right panels show magnified pictures of synaptic spines. (C) Mander's coefficient measurement in synaptic spines of primary cultures from mouse hippocampal neurons treated with SNP. Data are expressed as arbitrary units being 1 the maximum of colocalization. Data are the mean \pm SEM values of 6-12 dendrites. * p <0.005 vs. control; ** p <0.05 vs. the respective SNP alone treatment. (D) Quantification of the ratio spine number/spine size in primary cultures of mouse hippocampal neurons pre-incubated with HRI-i or PKR-i for 30 min and treated with SNP for 1 h. Data are the

mean \pm SEM values of 6 independent experiments; * $p < 0.05$ vs. control; # $p < 0.05$ vs. the respective SNP alone treatment.

Figure 8. HRI inhibition impairs memory consolidation. (A) WB from hippocampi from mice treated chronically with HRI-i during 1 week. Densitometric quantifications from WBs of BACE1 and p-eIF2 α corrected for GAPDH and total eIF2 α . Data are the mean \pm SEM values of 5 independent experiments; ** $p < 0.01$ and *** $p < 0.001$ vs. control. (B, C) Memory tests in mice treated with HRI-i and NOS-i after the training period of the memory tasks. Object recognition discrimination indices (B) showed as the mean \pm SEM values of 6 independent experiments. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$ vs. control. Object recognition exploration times from animals injected with HRI-i or NOS-i (inset in B). Data are the mean \pm SEM values of 6 independent experiments. No differences were obtained between the different experimental groups. Context conditioning (C) showed as the mean of freezing times \pm SEM values of 6 independent experiments. * $p < 0.005$; ** $p < 0.0005$; *** $p < 0.00005$ vs. control.

Fig 1

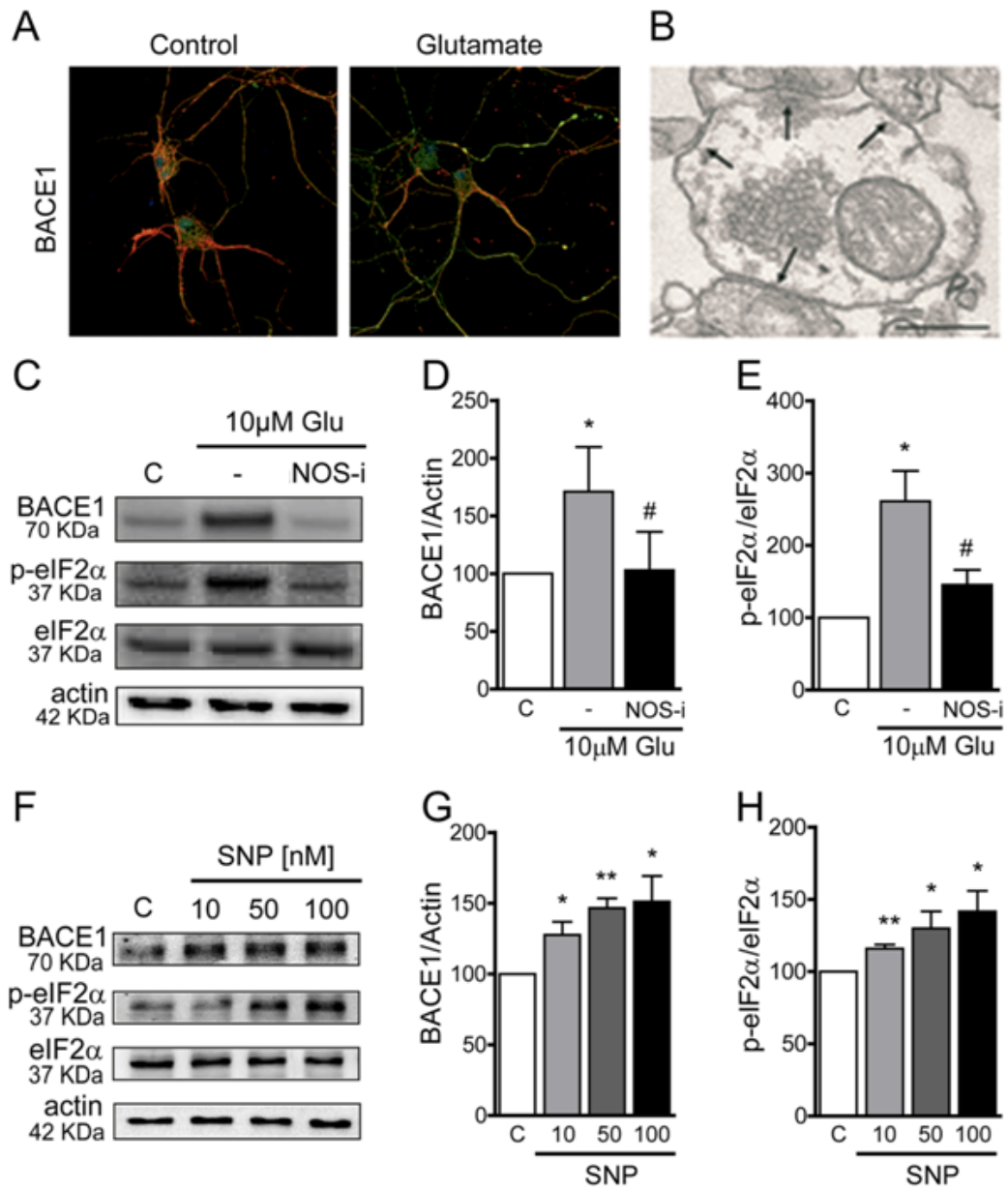


Fig 2

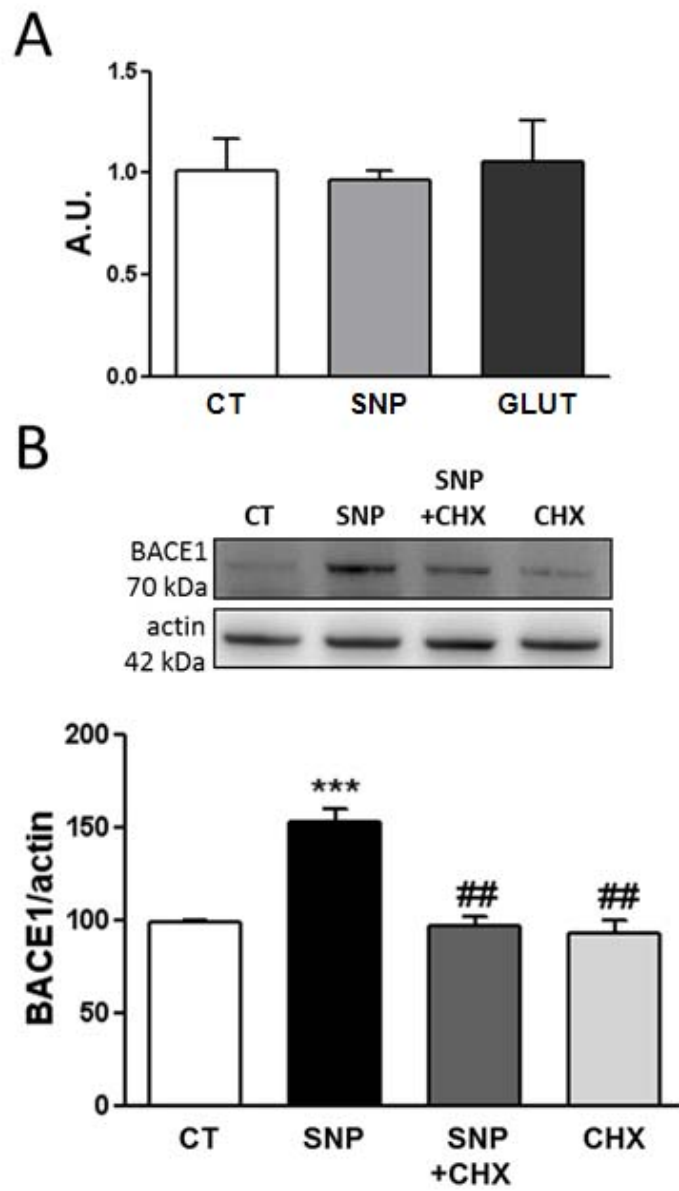


Fig 3

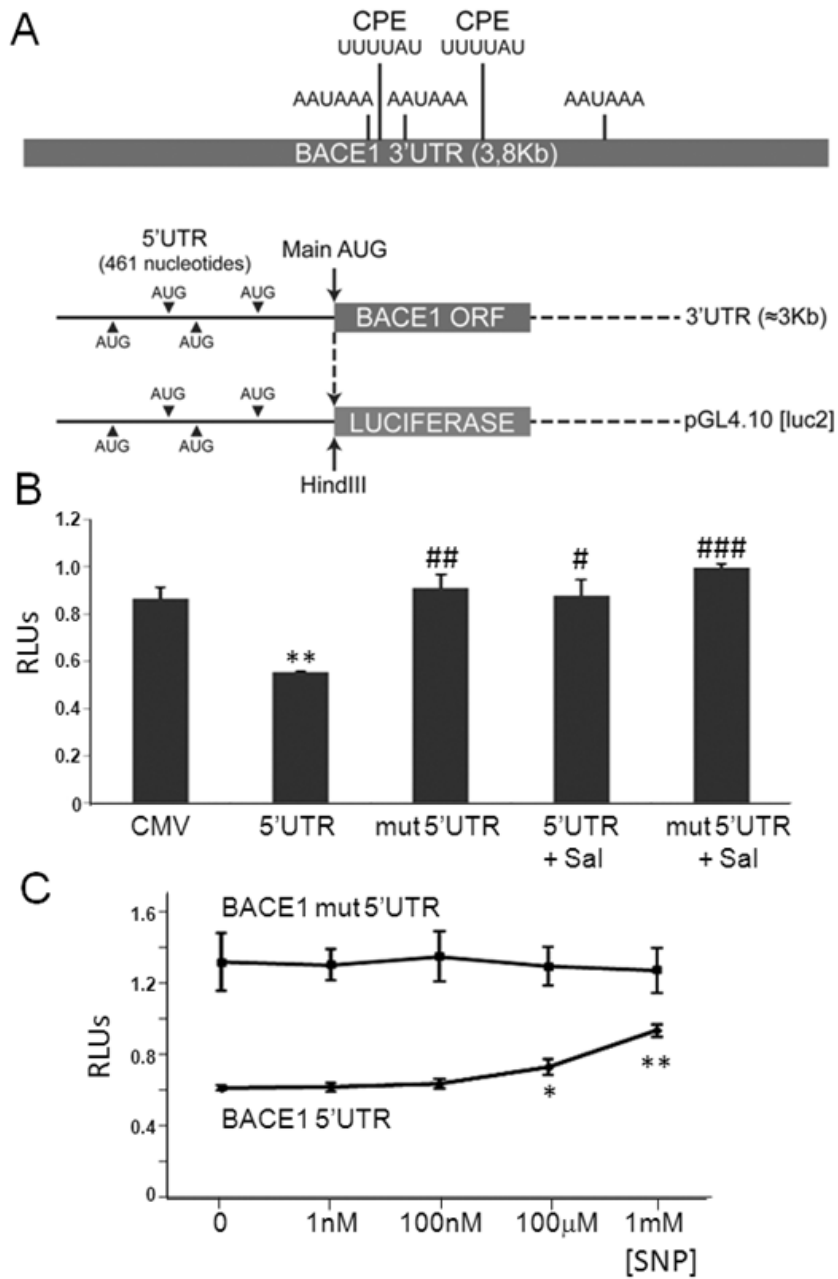


Fig 4

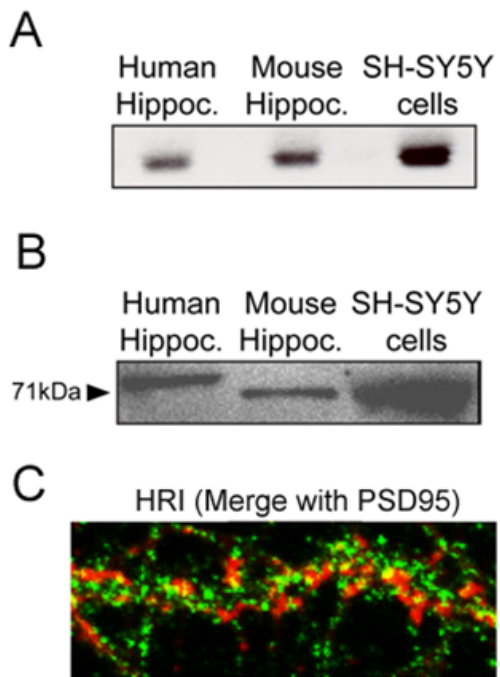


Fig 5

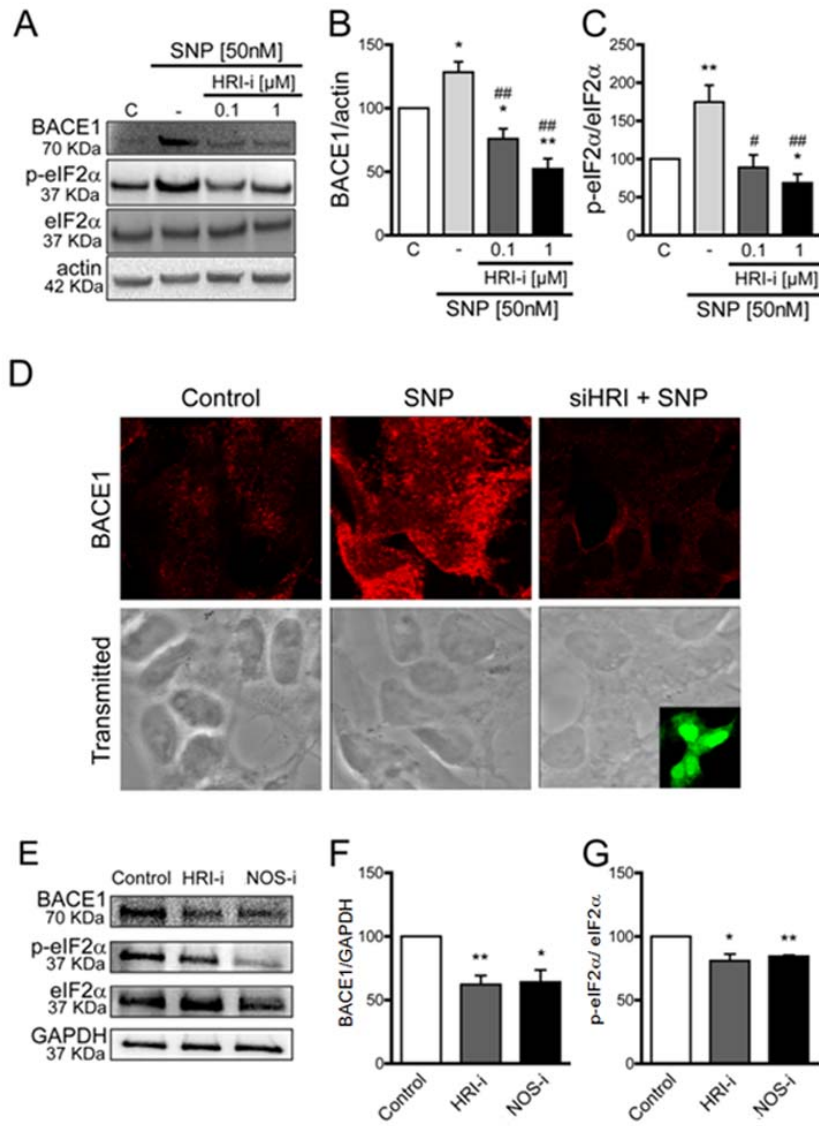


Fig 6

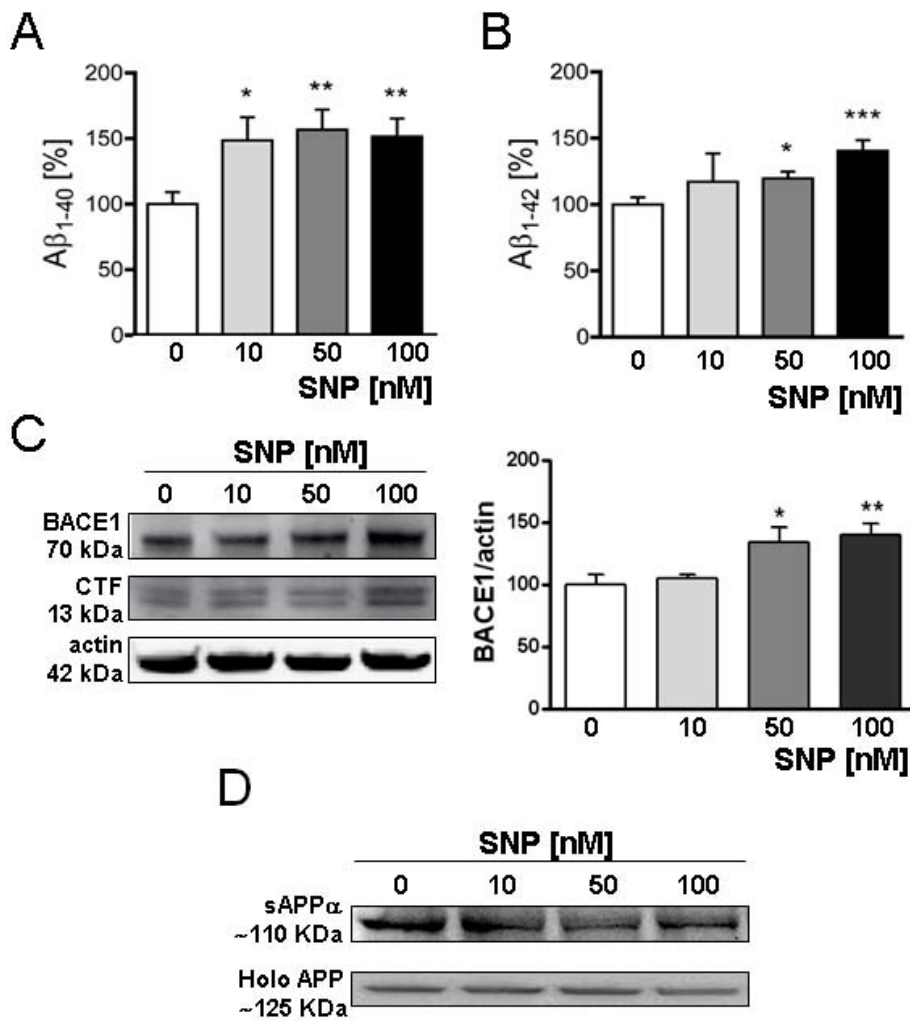


Fig 7

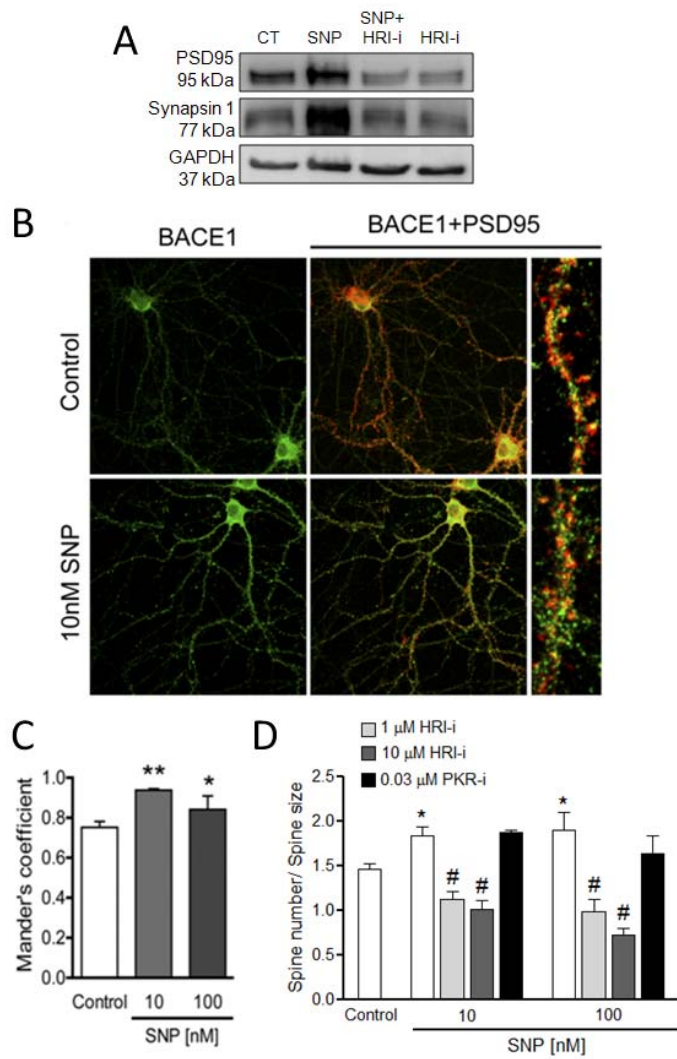


Fig 8

