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The role of NOS in the impairment of spatial memory and damaged neurons in rats injected with amyloid beta 25–35 into the temporal cortex

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ABSTRACT

The $A\beta_{(25-35)}$ fraction mimics the toxic effects of the complete peptide $A\beta_{(1-42)}$ because this decapeptide is able to cause memory impairment and neurodegenerative events. Recent evidence has shown that the injection of $A\beta_{(25-35)}$ into the temporal cortex (TCx) of the rat increases the nitric oxide (NO) pathways with several consequences, such as neuronal loss in rats. Our aim was to investigate the effects of each NOS isoform by the prior injection of NOS inhibitors before the injection of the $A\beta_{(25-35)}$. One month after the treatment, the animals were tested for their spatial memory in the radial maze. The hippocampus (Hp) and TCx were assessed for NO production, nitration of proteins (3-NT), astrocytosis (GFAP), and neuronal loss. Our findings show a significant impairment in the memory caused by $A\beta_{25-35}$ injection. In contrast NOS inhibitors plus $A\beta_{25-35}$ cause a protection yielding a high performance in the memory test and reduction of cell damage in the TCx and the Hp. Particularly, iNOS is the major source of NO and related to the inflammatory response leading to the memory deficits. The inhibition of iNOS is an important target for neuronal protection against the toxicity of the $A\beta_{25-35}$ over the long term.

1. Introduction

Alzheimer's disease (AD) is a frequent neurodegenerative disorder and regarded as a progressive illness for memory loss (Selkoe, 2005). The disease is characterized physiologically by two proteinaceous inclusions in the brain (Hardy and Higgins, 1992), which inclusions are called neuritic plaques (NPs) and neurofibrillary tangles (NFTs) that until now have been characterized postmortem (Selkoe, 1996). The etiology and physiopathology of the disease are not yet fully understood. NFTs are intracellular filaments primarily composed of hyperphosphorylated microtubule-associated tau protein (Novák, 1994). NPs are mainly composed of large extracellular aggregates of the amyloid beta (Aβ) peptides.

Several studies have focused on the Aβ toxicity in models in vivo and in vitro. The Aβ comprises a sequence of 39 to 42 amino acids derived from the transmembrane region of the amyloid-precursor protein (APP) (Hardy and Selkoe, 2002; Turner et al., 2003). It appears that the early and abnormal production of the Aβ-peptides might trigger the start of the neuronal-death processes observed in AD (Thal et al., 2002; Duyckaerts, 2004). Other investigations have shown that the fraction 25–35 of Aβ ($A\beta_{25-35}$) is the toxic functional domain of the full-length $A\beta_{(1-42)}$ (Kubo et al., 2002; Butterfield and Boyd-Kimball, 2005; Gulyaeva and Stepanichev, 2010). Several groups have used this peptide as an experimental model of AD (Maurice et al., 1996; Delobbette et al., 1997; Stepanichev et al., 2004; Cheng et al., 2006, 2009). Recently it has been reported that the $A\beta_{(25-35)}$ could be produced in brains of AD patients by enzymatic cleavage of the naturally occurring Aβ 1–40 (Kubo et al., 2002). Evenmore, Gruden et al. (2004) reported the presence of $A\beta_{(25-35)}$ aggregates, an event that suggest its participation in the pathogenicity of AD (Kaminsky et al., 2010). These are some of the reasons that make the $A\beta_{(25-35)}$ attractive to study in animal models of AD.

Several studies have demonstrated that injection of the $A\beta_{(25-35)}$ into the temporal cortex (TCx) or hippocampus (Hp) results in degeneration of neurons and memory deficits (Perez-Severiano et al., 2004; Limón et al., 2009a; Cuevas et al., 2009; Carrillo-Mora et al., 2010). Our studies have suggested that nitric oxide (NO), as a free radical, could be the molecule implicated in the process of $A\beta_{(25-35)}$ toxicity over the long term with several repercussions on spatial memory (Limón et al., 2009a).

The nitric oxide (NOS) isoform synthases, which are responsible for the synthesis of NO, have been found in the brain. The neuronal NOS (nNOS) was originally detected in different types of neurons of the cerebellum, hypothalamus, striatum, cerebral cortex, and

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hippocampus and the inducible NOS (iNOS) was described in activated macrophages, astroglia, and microglia (Vodovotz et al., 1996). The increase of NO in the chronic progress of AD can contribute to a neurodegenerative disorder through protein nitration by reactive peroxynitrite (Moncada and Bolanos, 2006). This compound can alter membrane and cytosol proteins by an oxidative process, affecting their physical and chemical nature and then serving as an important link in the chain of events leading to the neurodegeneration observed in AD (Koppal et al., 1999).

Earlier studies have shown that the two isoforms of NOS are upregulated after infusion of $A\beta_{(25-35)}$ into rats to produce an increase in the NO levels, which over the long term may result in the impairment of the spatial memory (Limón et al., 2009a). Some studies have associated the loss of neurons from the cerebral cortex with memory impairment (Lüth et al., 2001; Wallace et al., 1997). There is evidence that the use of NOS inhibitors can either delay the onset of or reduce the neurodegeneration.

Recently, our group showed that the injection of $A\beta_{(25-35)}$ into neonatal rats after the previous administration of N_ω-nitro-L-arginine methyl ester (L-NAME), a nonspecific inhibitor of NOS, prevents the deterioration in spatial memory in the adult stage (Díaz et al., 2010).

We need to understand which of the NOS isoenzymes is responsible for the generation of the major amount of NO that contributes to the neurodegeneration. It seems that iNOS could be the principal source of NO in the CTx after the injection of $A\beta_{(25-35)}$, though this is not yet clear.

Several reports indicate that the nNOS appears to be involved in the NO neuroprotective effects in response to the toxicity of $A\beta_{(25-35)}$ (Norris et al., 1996; Lu et al., 2009), whereas iNOS is present in active astrocytes and probably accelerates neuronal death by stimulating the production of NO and the synthesis of proinflammatory cytokine and the tumor necrosis factor-α, which play a crucial role in the $A\beta_{(25-35)}$ neurotoxicity (Akama et al., 1998) and cause damage to the spatial memory. Although these results do not provide direct support to understand what the involvement of iNOS and nNOS is in the toxicity of $A\beta_{(25-35)}$ in vivo, our aim was to investigate the role of both the nNOS and iNOS on the neurodegeneration and impaired spatial memory that is caused by the injection of the $A\beta_{(25-35)}$ into the TCx of rats.

2. Materials and methods

2.1. Reagents

Amyloid-β 25–35 (-GSNKGAIIGLM-) ($A\beta_{(25-35)}$), N_ω-nitro-L-arginine methyl ester (L-NAME), 7 nitroindazole (7-NI), Aminoguanidine (AG), albumin free IgG, and Triton X-100 were all obtained from Sigma Chemical Co. St. Louis, MO. The primary antibodies were the rabbit anti-glial fibrillary acidic protein (GFAP) (Dako, Denmark) and mouse anti-3-Nitrotyrosine (3-NT) (Millipore). The secondary antibodies were Rodamine anti-rabbit and fluorescein isothiocyanate (FITC) anti-mouse (Jackson Immunoresearch Inc West Grove, PA). VectaShield plus 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Lab, Burlingame, CA.

2.2. Animals

Adult male Wistar rats (230 to 250 g) were obtained from Bioterio Claude Bernard BUAP, our animal facilities. Animals were individually housed in a temperature- and humidity-controlled environment in a 12 h:12 h light:dark cycle with free access to food and water. All the procedures described in this study are in accordance with the Guide for the Care and Use of Laboratory Animals of the "Norma Mexicana 062" and strictly according to "the Guidelines for the Use of Animals in Neuroscience Research" from the Society for Neuroscience.

2.3. Drug administration protocol

Eight experimental groups ($n=8$ per group) were designed for the study; Sham (control animals given only vehicle, sterile water), $A\beta_{25-35}$, L-NAME, 7-NI, AG, L-NAME plus $A\beta_{25-35}$, 7-NI plus $A\beta_{(25-35)}$, and AG plus $A\beta_{(25-35)}$. The groups of rats were injected ip with L-NAME (30 mg/kg), 7-NI (25 mg/kg), and AG (100 mg/kg) 30 min before stereotaxic surgery. Doses of the different treatments employed were chosen on the basis of previous reports of Bostanci and Bağirici (2008) and Zhang et al. (2010).

2.4. Preparation of aggregated $A\beta_{(25-35)}$ and surgery

The $A\beta_{(25-35)}$ was dissolved in sterile isotonic saline solution (SSI), pH 7.4, to a final concentration of 100 μM. The $A\beta_{(25-35)}$ solution was then incubated at 37 °C for 36 h. All animals were anesthetized with chloral hydrate (300 mg/kg, ip).

The stereotaxic surgery (Stoelting, IL, USA) was done as follows: One group was assigned to the bilateral injection of 1-μL isotonic saline solution (SSI or vehicle). A second group was used for the injection of 1-μL 100 μM $A\beta_{(25-35)}$ into the TCx (coordinates: A: −4.8 mm from bregma, L: −6.2 mm from midline, V: −5.5 below dura in accordance with Paxinos and Watson (1998)). Injections of $A\beta_{(25-35)}$ or vehicle (1 μL) per side were infused over 5 min with a Hamilton syringe. After surgery, the animals were returned to their cages and monitored during recovery.

2.5. Learning and memory tests

Animals were tested in the eight-arm radial maze ($n=11$ per group) (Olton and Samuelson, 1976; Limón et al., 2009b; Díaz et al., 2010), which was in a room containing many visual cues external to the maze. For this purpose, the body weight of all the animals was controlled for 10 days to achieve 85% of the original weight and this weight was maintained to the end of the behavioural experiments. Before training all animals were habituated to the apparatus and food pellets for 1 day. This 15-min period of habituation was repeated three times a day, at intervals of 1 h.

Twenty days after the injection, the learning was assessed using 20 trials that were made over 2 days. In each trial the animal was placed on a central platform at the middle of the eight-arm radial maze and was allowed to move freely in the maze until the animal made the attempts allowed or the time has elapsed (200 s). Between each trial there was a 50-min interval. Only three of the eight arms contained pellets during the entire test. In the first three trials, each rat was allowed to choose 8 arms. During the later trials the number of attempts was reduced, so that by the second day of training the rat was allowed to choose only three arms for each trial, after which the animal was removed from the maze. The number of correct responses, which were defined as choosing the arm that contained pellets and eating them, assessed the performance of a given animal in each trial. The learning was evaluated using the percentage of correct responses in all the trials.

The memory test was assessed ten days after the learning test was finished. Each rat underwent one trial. At the beginning of the trial the animal was placed on the central platform and was allowed to move freely in the maze. Then the rat was allowed to choose only 3 arms and the animal was allowed to make three test choices. The performance of each animal was assessed in this trial by the percentage of correct responses in the same way as during the training test.

2.6. Nitrite assay

Animals were decapitated after the memory test ($n=7$ per group) and their brains were immediately removed, washed in ice-cold SSI, and the Hp and TCx were dissected out. These were homogenized in 3-mL ice-cold 0.1 M phosphate-buffered saline (PBS), pH 7.4. The

homogenate was centrifuged at 12,500 rpm (4 °C). The supernatant was obtained and stored at −70 °C. Supernatants were used for protein and nitrite level measurements.

Nitrite levels were measured with the Griess method (Green et al., 1982). The Griess reagents were sulfanilamide, glacial acetic acid, N-(1-napthyl) ethylenediamine, and sodium nitrite (Sigma, St. Louis, MO, USA). The reagent, 200 μL, was added directly to an aliquot of the homogenate (200 μL) and incubated under reduced light at 4–8 °C for 10 min. Samples were analyzed at 540 nm in a spectrophotometer. The protein level of each sample was determined using the Lowry method (Lowry et al., 1951). The data were calculated as μM of nitrite per mg of protein.

2.7. Histological examination

After behavioural experiments, the rats ($n=4$ per group) were anesthetized and perfused with 200 mL of 4% paraformaldehyde. The brains were removed and postfixed in the same fixative solution for 48 h and then were embedded in paraffin. Coronal 5-μm thick sections were taken from each brain at the level of the anterior temporal area approximately −3.8 to −6.8 mm from bregma.

2.8. Hematoxylin and eosin (H&E)

Neurodegeneration caused by the injection of Aβ into the TCx was evaluated after staining with hematoxylin and eosin. The neurons were observed through a Leica DM-LS microscope at 40×. Undamaged neurons were recognized as cells with round, blue nuclei and a clear perinuclear cytoplasm. These were evaluated using the criteria formulated by Garcia et al. (1995). Damaged neurons were cells with changed nuclei (pyknosis, karyorrhexis, and karyolysis) and cytoplasmatic eosinophilia or loss of affinity for hematoxylin. The number of undamaged neurons was examined in the TCx and CA1 of the Hp (Kiyota et al., 1991).

The number of neurons were counted in the same region, using an area of 500 μm^2, by using images captured with a digital camera Leica DFC-300FX (Leica Microsystems Digital Imaging, Coldhams Lane, Cambridge, UK) and using Leica software IM1000 (Imagic Bildverarbeitung AG, Leica Microsystems, Heerbrugg, Switzerland). From all pictures we analyzed four serial sections for each rat.

2.9. Immunohistochemistry

Paraffin was removed from the sections (5-mm thick) and they were rehydrated using conventional histological techniques. They were then rinsed with a phosphate-saline buffer (PBS, 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4). Nonspecific binding sites were blocked by incubating in IgG-free 2% bovine serum albumin (BSA, Sigma) for 30 min. Afterwards, specimens were incubated for 10 min with 0.2% Triton X-100 in PBS at room temperature. Then slides were rinsed with PBS. Slides were the incubated overnight at 4–8 °C with a monoclonal mouse antibody anti-3-nitrotyrosine (Millipore) and a polyclonal rabbit antibody anti-glial fibrillary acidic protein (GFAP, Dako, Denmark A/S), all at 1:500 dilution.

The neurons immunostained in the TCx and the CA1 of the Hp were observed through a Leica DM-LS fluorescent microscope at 40× (Leica Microsystems, Wetzlar, GmBH). Fluorochromes were visualized with their specific filters and analyzed in three channels. Antibody labelling was recognized with an isospecific secondary FITC-conjugated antibody and visualized in the green channel. Antibody labelling was also recognized with isospecific secondary rodamine and visualized in the red channel. Slides were counterstained with VectaShield with DAPI (Vector Labs., CA, USA) for nuclei staining (blue channel). The number of immunoreactive cells to GFAP and 3-NT were also counted in the TCx and CA1 subfield of the Hp. The reactive astrocytes and nitrated protein were considered as an indirect index of neurotoxicity. The criteria to define reactive astrocytes and nitrated protein comprised augmented levels of GFAP and 3-NT immunoreactivity. All counting procedures were made in blind by an expert in morphology. The images were projected with a Leica IM1000 version 1.20 release-9 computer-based program (Imagic Bildverarbeitung AG, Leica Microsystems, Heerbrugg, Switzerland).

2.10. Statistical analysis

The results were expressed as the mean ± SE for all experiments. The statistical analysis was done using analysis of variance and multiple comparisons were made using Bonferroni's test, with $P<0.05$ considered significant.

3. Results

3.1. Spatial learning and memory tests

To understand the role of the NOS in the toxicity of $A\beta_{(25-35)}$ rats in the spatial learning and memory in the eight-arm radial maze, we plotted the average of the percentage of correct response using blocks of four trials for the spatial learning and one trial for the spatial memory for the eight groups; vehicle, $A\beta_{(25-35)}$, L-NAME, 7-NI, AG, L-NAME plus $A\beta_{(25-35)}$, 7-NI plus $A\beta_{(25-35)}$, and AG plus $A\beta_{(25-35)}$.

The animal-learning and memory test performance was examined 20 and 30 days after injection of the NOS inhibitors and of the $A\beta_{(25-35)}$ injection into TCx. The correct response of the $A\beta_{(25-35)}$ group was 50% correct response during the learning phase and 56% in the memory test. The vehicle group's correct responses were 70% in learning and 86% in the memory test. These results indicate that the injection of $A\beta_{(25-35)}$ into TCx of rats impairs both spatial learning and memory (Fig. 1). The preadministration of an inhibitor nonspecific to NOS, as the L-NAME, and then the injection of the $A\beta_{(25-35)}$ showed 68% correct responses in the learning test and 82% in the memory test (Fig. 1A and B). The animals treated with 7-NI (an inhibitor of nNOS) before the injection of $A\beta_{(25-35)}$ into the TCx recorded 67% correct responses in the learning test and 61% in the memory test (Fig. 1 C and D). The animals treated with AG (an inhibitor of iNOS) plus $A\beta_{(25-35)}$ recorded 71% correct responses in learning and 80% for the memory test (Fig. 1E and F). These results indicate that the administration of L-NAME and AG before the $A\beta_{(25-35)}$ injection prevents the cognitive impairment. In addition, the groups with L-NAME, 7-NI, and AG alone showed 68%, 65%, and 62% correct responses for learning and 83%, 80%, and 77% for the memory test. The statistical analyses used were a repeated-measure ANOVA, with a significance of $P<0.05$, followed by the Bonferroni posttest.

3.2. Quantification of NO_2^- and the number of cells in the temporal cortex and hippocampus

To assess changes in the NO concentration, we quantified the nitrite (NO_2^-) levels in the homogenized TCx and Hp 30 days after the NOS inhibitor injection and the injection of $A\beta_{(25-35)}$ into the TCx. In Fig. 2A the average of NO_2^- concentrations in the TCx of the $A\beta_{(25-35)}$ group (5.56 ± 0.3 μM/mg protein) and the vehicle group (2.79 ± 0.30 μM/mg protein) are shown. The highest NO_2^- concentration was measured in the $A\beta_{(25-35)}$ group (199%) after 30 days, compared to the vehicle group.

We found that both the L-NAME plus the $A\beta_{(25-35)}$-treated group and AG plus the $A\beta_{(25-35)}$-treated group showed a lower concentration of NO_2^- (3.76 ± 0.19 μM/mg protein and 2.97 ± 0.20 μM/mg protein) compared with the $A\beta_{(25-35)}$-treated group alone, which are decreases of 32% and 46%. The 7-NI plus $A\beta_{(25-35)}$-treated group did not show changes in the NO_2^- levels (4.50 ± 0.34 μM/mg protein) compared with the $A\beta_{(25-35)}$-treated group alone. The L-NAME, 7NI, and AG-treated groups alone do not show a significant difference in the

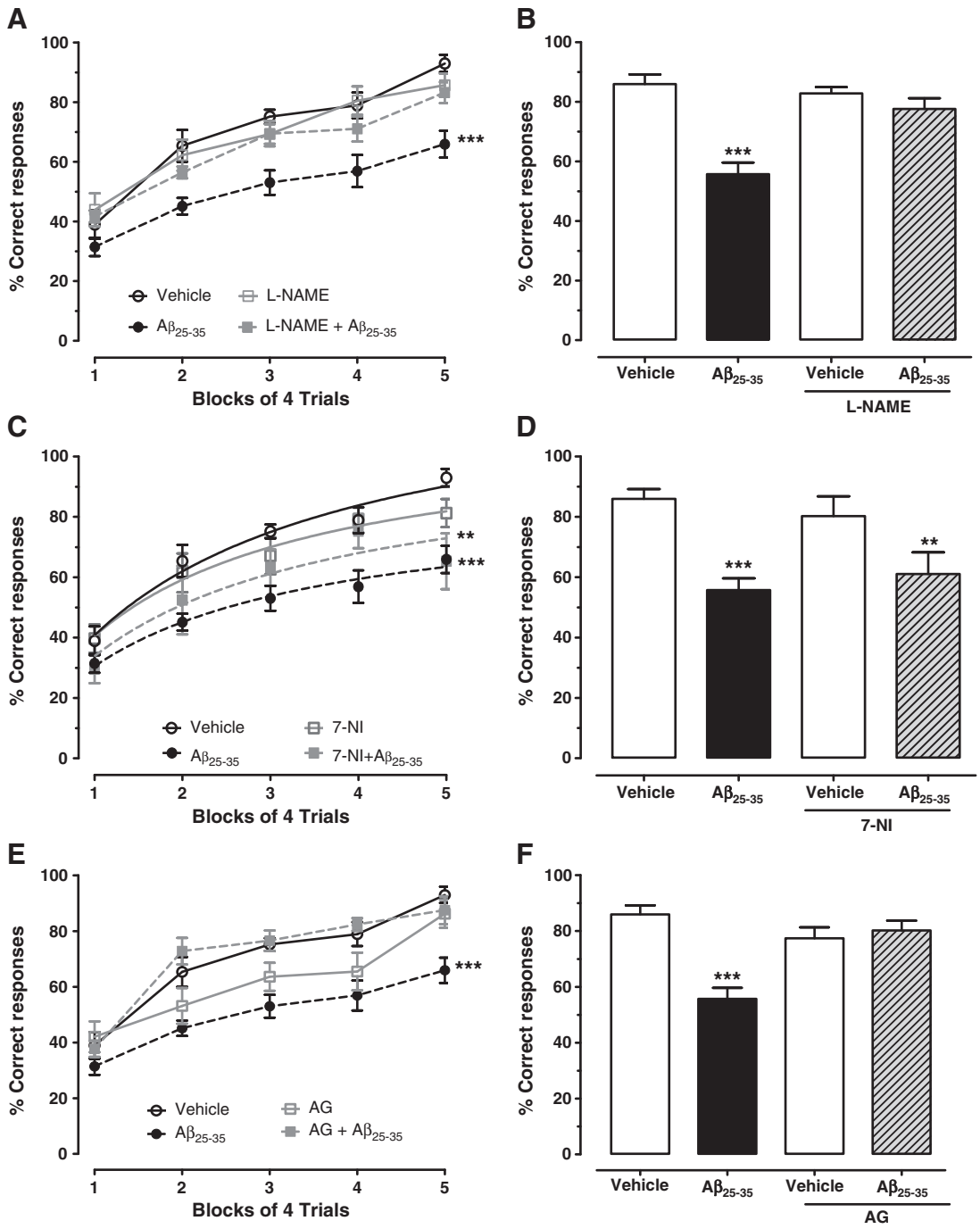


Fig. 1. Effects of L-NAME, 7-NI, and AG on the Amyloid-$\beta_{(25-35)}$-treated rats caused changes in spatial learning and memory. The course of spatial learning and memory development in the eight-radial maze showing the percentage of correct responses of rats injected (ip) with L-NAME (300 mg/kg) in (A) and (B), 7-NI (25 mg/kg) in (C) and (D), and AG (100 mg/kg) in (E) and (F) before the injection of 1 μL $\beta_{(25-35)}$ [100 μM] or 1 μL of vehicle ($n = 11$ per group). The data were given as the mean ± SE. The statistical analysis was made using repeated-measure ANOVA and the Bonferroni posttest for the learning tests and one-way ANOVA and Bonferroni posttest for memory test. ***$P<0.001$ compared to the $A\beta_{(25-35)}$ group.

NO_2^- levels (1.73 ± 0.23 μM/mg protein, 2.28 ± 0.33 μM/mg protein, and 2.30 ± 0.18 μM/mg protein) compared with the vehicle group.

In Fig. 2C is shown the average concentration of NO_2^- in the Hp, where we found an increase of NO_2^- levels in the $A\beta_{(25-35)}$-treated group (4.1 ± 0.19 μM/mg protein) compared with the vehicle group (1.75 ± 0.14 μM/mg protein). This difference was an increase of 234% of the NO_2^- levels. In contrast, preadministration of the $A\beta_{(25-35)}$-treated group with L-NAME and AG caused significant decreases in the NO_2^- levels generated by the $A\beta_{(25-35)}$-treated group alone (1.76 ± 0.20 μM/mg protein and 1.82 ± 0.26 μM/mg protein), with decreases of 40% and 55% as compared to the $A\beta_{(25-35)}$-treated group. The preadministration of the $A\beta_{(25-35)}$-treated group with 7-NI showed no significant difference in the NO_2^- levels compared with the $A\beta_{(25-35)}$-treated group (3.24 ± 0.20 μM/mg protein). The L-NAME, 7NI, and AG-treated groups alone do not show a significant difference in the NO_2^- levels (1.17 ± 0.17 μM/mg protein, 1.04 ± 0.14 μM/mg protein, and 1.49 ± 0.23 μM/mg protein) compared with the vehicle group. The statistical analyses used were a repeated-measure ANOVA, with a significance of $P<0.05$, followed by the Bonferroni posttest.

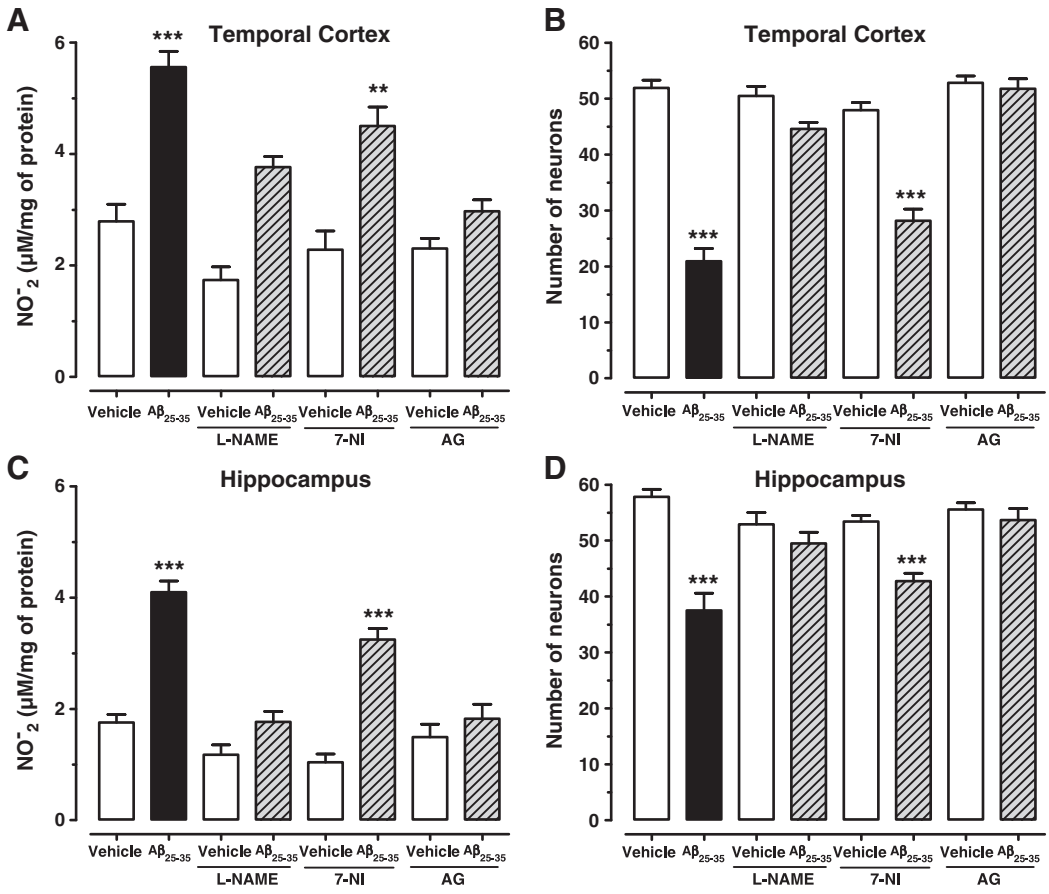


Fig. 2. Effects of the L-NAME, 7-NI, and AG on the Amyloid-$β_{(25-35)}$-treated rats caused changes on the nitric oxide levels and loss of cells in the temporal cortex and the CA1 subfield of the hippocampus of rats. In (A) and (C) the graph shows nitrite levels for the TCx and CA1 subfield of the Hp of the control, $Aβ_{(25-35)}$, L-NAME, L-NAME plus $Aβ_{(25-35)}$, 7-NI, 7-NI plus $Aβ_{(25-35)}$, AG, and AG plus $Aβ_{(25-35)}$ groups. The NO was measured as nitrites by the Griess method and the protein by the Lowry method after the behavioral test was finished. The values are the mean of nitrites [μM]/mg of protein ± SE. The statistical analysis was made using a one-way ANOVA, and a Bonferroni posttest with $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ ($n=7$ per group). In (B) and (D) the brain sections were stained with H&E stain showing the number of neurons in the TCx and Hp of the control, $Aβ_{(25-35)}$, L-NAME, L-NAME plus $Aβ_{(25-35)}$, 7-NI, 7-NI plus $Aβ_{(25-35)}$, AG, and AG plus $Aβ_{(25-35)}$-treated groups observed at 40×. The values are the mean number of neurons ± SE (number of counts/500 μm^2). The statistical analysis was made using a one-way ANOVA and a Bonferroni posttest with $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to the $Aβ_{(25-35)}$ group.

The effects of the preadministration of the $Aβ_{(25-35)}$-treated group with NOS inhibitors on the number of neurons was assessed using hematoxylin and eosin staining. This staining shows damage to the neurons through pyknosis, karyorrhexis, karyolysis, and the cytoplasmic changes of eosinophilia or loss of hematoxylin present in the neurons of the TCx and the CA1 of the Hp (not illustrated).

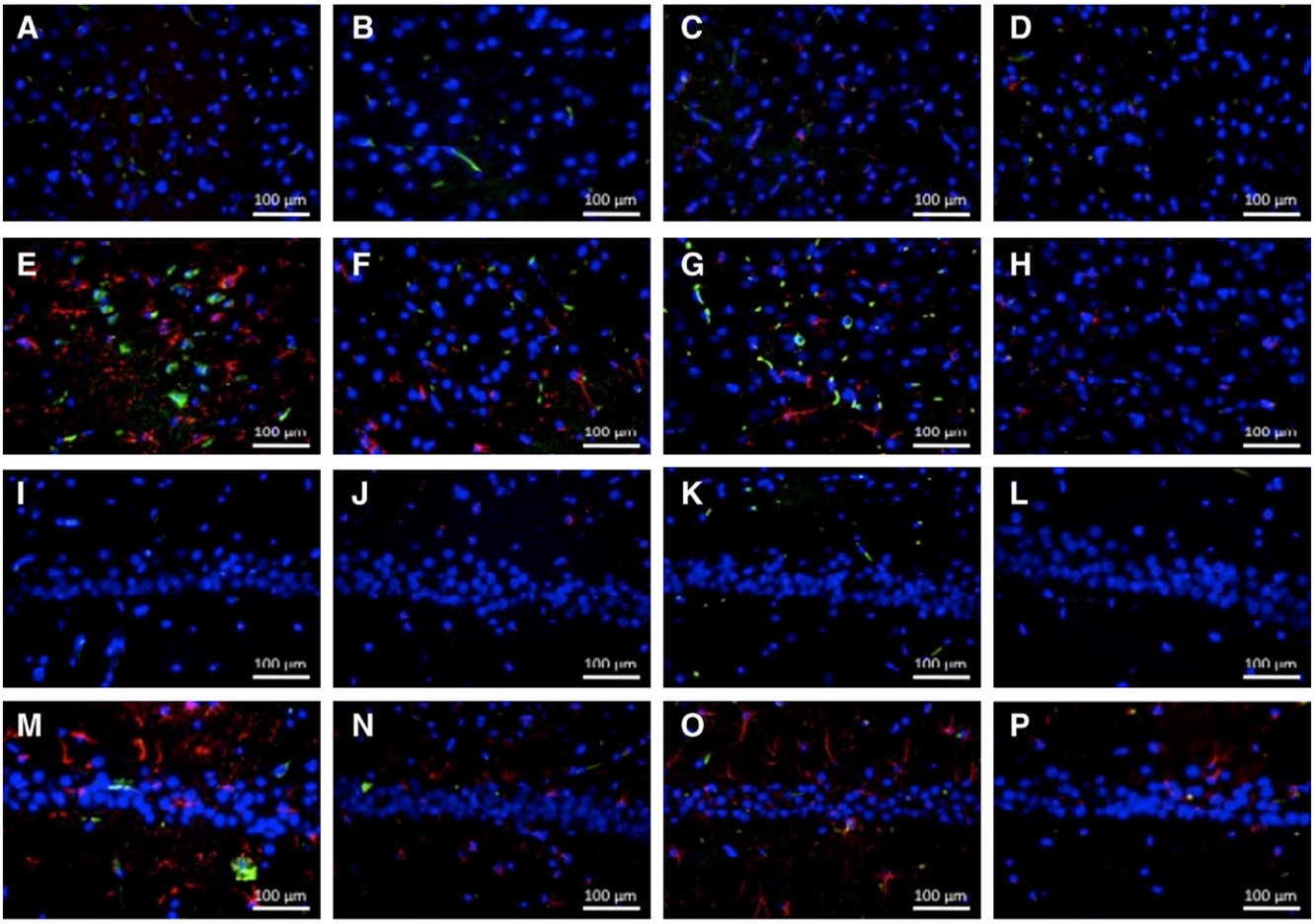
We do show the quantitative data of the neuronal number in the TCx and the CA1 subfield of the Hp. Fig. 2B shows that in the TCx the number of neurons of the $Aβ_{(25-35)}$-treated group is smaller compared with the vehicle group (21 ± 0.3 and 52 ± 1.4 neurons). This is a decrease of 60%. In contrast, in the L-NAME plus $Aβ_{(25-35)}$-treated group and the AG plus $Aβ_{(25-35)}$-treated group the number of neurons is significantly greater compared to the $Aβ_{(25-35)}$-treated group alone (44.5 ± 1.2 and 52 ± 1.7 neurons), which are increases of 138% and 147% compared to the $Aβ_{(25-35)}$-treated group alone.

The 7-NI plus $Aβ_{(25-35)}$-treated group did not show significant changes in the number of neurons (28 ± 2 neurons) compared with the $Aβ_{(25-35)}$-treated group alone, whereas the L-NAME, 7NI, and AG-treated groups alone do not show a significant difference in the number of neurons (50 ± 1.4 neurons, 48 ± 1.4 neurons, and 53 ± 1.2 neurons) compared with the vehicle group.

Fig. 2D shows the average of number of neurons in the Hp. We found the $Aβ_{(25-35)}$-treated groups has a smaller number of neurons compared with the vehicle group (38 ± 3 neurons and 58 ± 1.3 neurons), which was a decrease of 65%. However the preadministrations of the $Aβ_{(25-35)}$-treated group with L-NAME and AG show a significant increase of the number of neurons compared with the $Aβ_{(25-35)}$-treated group alone (50 ± 1.8 neurons and 54 ± 2 neurons), increases of 26% and 39% compared to the $Aβ_{(25-35)}$-treated group alone. The preadministration of the $Aβ_{(25-35)}$-treated group with 7-NI showed no significant difference compared with the $Aβ_{(25-35)}$-treated group (43 ± 1.4 neurons). The L-NAME, 7NI, and AG-treated groups alone do not show a significant difference in the number of neurons (53 ± 2 neurons, 53 ± 1, and 55 ± 2.2 neurons) compared with the vehicle group. The statistical analyses used were a repeated-measure ANOVA, with a significance of $P<0.05$, followed by the Bonferroni posttest.

3.3. The GFAP and 3-NT-immunoreactivity in the temporal cortex and hippocampus

The immunoreactivity for GFAP was assessed in the same rat groups immediately after the memory tests. Immunofluorescence analysis was aimed at estimating the effect of an NOS inhibitor on the formation of reactive nitrogen species (3-NT) and astrocyte reactivity (GFAP), as marked by damaged neurons 30 days after the $Aβ_{(25-35)}$ injection into the TCx (Fig. 3A–P). In the $Aβ_{(25-35)}$-treated group the GFAP and 3-NT immunoreactivity were increased in the TCx as the photomicrographs (Fig. 3A and E) show when compared to vehicle group. In addition, we found that the L-NAME plus $Aβ_{(25-35)}$ and AG plus $Aβ_{(25-35)}$-treated groups showed a low immunoreactivity to GFAP and 3-NT in the TCx (Fig. 3F and H). The L-NAME, 7-NI, and AG groups alone show no significant difference compared to the vehicle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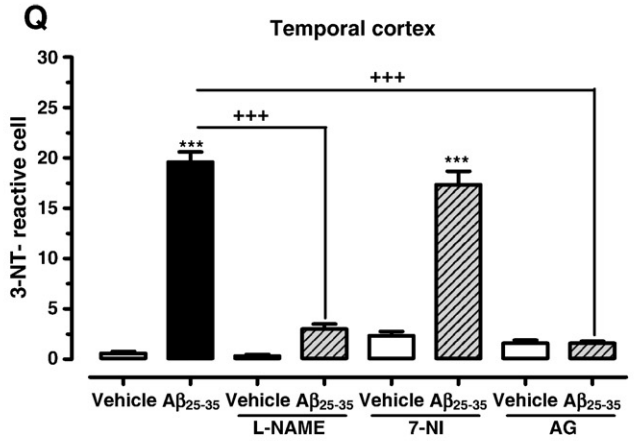
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Temporal cortex
3-NT- reactive cell
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Vehicle
Aβ25-35
L-NAME
7-NI
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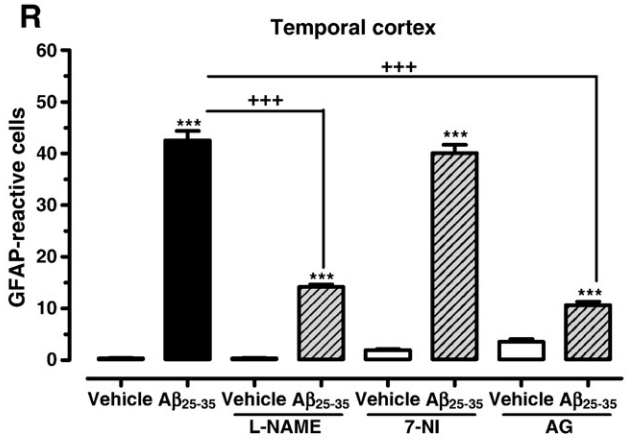
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Temporal cortex
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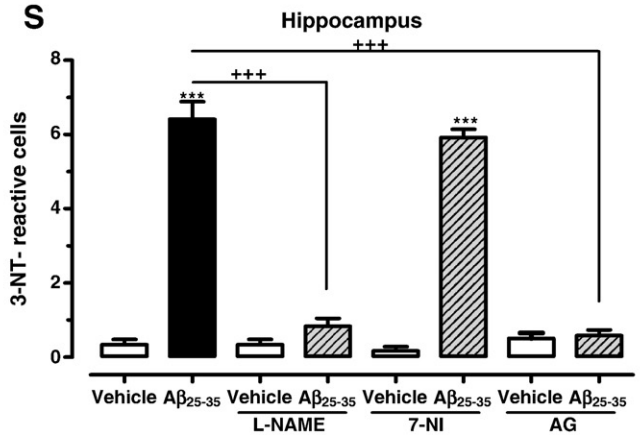
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Hippocampus
3-NT- reactive cells
+++
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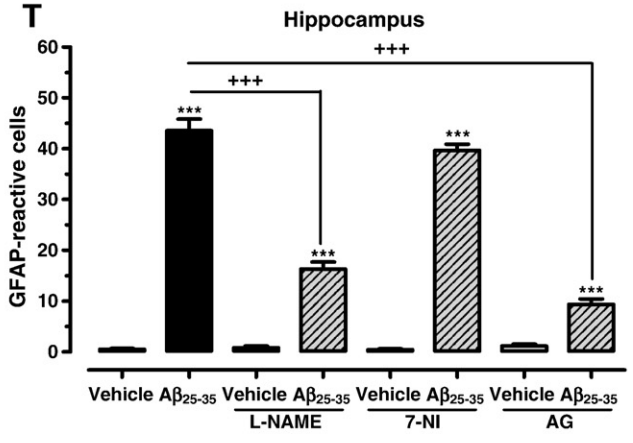
Vehicle
Aβ25-35
L-NAME
7-NI
AG


T
Hippocampus
GFAP-reactive cells
+++
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Vehicle
Aβ25-35
L-NAME
7-NI
AG

group (Fig. 3B–D). The quantitative data of the GFAP- and 3 NT-immunopositive cells in the TCx were for vehicle (0.16 ± 0.01 and 0.58 ± 0.01), $A\beta_{(25-35)}$ (42.6 ± 2.83 and 19.6 ± 0.48), L-NAME plus $A\beta_{(25-35)}$ (14.1 ± 0.5 and 3.0 ± 0.49), 7-NI plus $A\beta_{(25-35)}$ (50.08 ± 1.59 and 17.33 ± 1.2), AG plus $A\beta_{(25-35)}$ (10.67 ± 0.67 and 1.58 ± 0.19), L-NAME (0.33 ± 0.1 and 0.33 ± 0.02), 7-NI (1.91 ± 0.22 and 2.33 ± 0.41), and AG (3.58 ± 0.49 and 1.58 ± 0.31) (see Fig. 3 Q–R).

The GFAP and 3-NT immunoreactivity in the Hp of the $A\beta_{(25-35)}$-treated group is great compared to the control, the L-NAME plus $A\beta_{(25-35)}$, and the AG plus $A\beta_{(25-35)}$-treated groups (Fig.3M, O and P). The 7-NI plus $A\beta_{(25-35)}$ showed no significant difference compared to the $A\beta_{(25-35)}$-treated group alone. The L-NAME, 7-NI, and AG groups alone show no significant difference compared to the vehicle group. The quantitative data of the GFAP- and 3 NT-immunopositive cells in the CA1 subfield of the Hp were for vehicle (0.16 ± 0.01 and 0.38 ± 0.01), $A\beta_{(25-35)}$ (43.6 ± 2.25 and 6.4 ± 0.46), L-NAME plus $A\beta_{(25-35)}$ (16.30 ± 1.36 and 0.83 ± 0.20), 7-NI plus $A\beta_{(25-35)}$ (39.57 ± 1.2 and 5.90 ± 1.2), AG plus $A\beta_{(25-35)}$ (9.33 ± 1.08 and 0.88 ± 0.14), L-NAME (0.83 ± 0.1 and 0.33 ± 0.01), 7-NI (0.41 ± 0.19 and 0.16 ± 0.11), and AG (1.16 ± 0.38 and 0.50 ± 0.15) (Fig. 3S and T). The statistical analyses used were a repeated-measure ANOVA, with a significance of $P<0.05$, followed by the Bonferroni posttest.

4. Discussion

Cognitive dysfunction is a major complication of neurological diseases such as AD, ischemia, and hypoxia, which emphasizes the urgent need to understand the mechanism behind such disorders. The initial origin of AD pathogenesis has not been determined although it has become evident that oxidative stress is implicated in the development of this disease (Butterfield et al., 2001; Butterfield, 2002). The oxidative damage marked by lipid peroxidation, mitochondrial dysfunction, and nucleic acid oxidation are increased in vulnerable neurons of AD (Smith et al., 1997a,b; Butterfield, 2002) and these events are not linked directly to NO. However, recent studies also highlight a role for the NO pathways because NO has remained an important bioregulatory molecule in the SNC that affects several neurophysicological functions in the brain (Thomas et al., 2008). Particularly, the studies postmortem of brains of patients with AD have shown the presence of aberrant NOS reactivity in the pyramidal-like cortical neurons (Lüth et al., 2000), though the role of each isoform of NOS is not yet known in the pathogenesis of AD.

The animal models have led to understanding part of the alterations caused by NOS. An example is the aging rats that showed a high immunoreactivity to nNOS and iNOS in the cerebral cortex (Uttenthal et al., 1998) or the ischemic model that shows high NOS reactivity in the brain (Aguilar-Alonso et al., 2008). Together these models suggest that at least the NOSs, in part, are responsible for the neurodegenerative changes in the rat cortex. In addition, the infusion of Aβ into the rat's brain can increase the presence of nNOS or iNOS over time, as our group has shown (Limón et al., 2009a). These effects have implications in the synthesis of NO, the nitration of proteins, and the inflammatory response that result in impairment of cognitive behaviour over the long term. The results of our present work lead us to understand the role of both NOS (iNOS and nNOS) one month after the injection of $A\beta_{(25-35)}$ into the TCx of rats using specific inhibitors of the NOS. We were interested in blocking each NOS isoform previous to $A\beta_{(25-35)}$ injection to understand the role of each isoform in the $A\beta_{(25-35)}$ toxicity then we used three NOS inhibitors: L-NAME, 7-NI, and AG (Garvey et al., 1994; Wolff and Gribin, 1994; Wolff and Lubeskie, 1995), which were used at the dosage range that are able to cause an efficient blocking of NOS and at the same time show beneficial effects against neurotoxic agents (Takei et al., 2001; Bahremand et al., 2009). Under these conditions we found neuroprotective effects of AG and L-NAME agents in the rats injected with $A\beta_{(25-35)}$ after one month.

Our first aim was to investigate the learning and memory changes over the long term by making a bilateral injection of $A\beta_{(25-35)}$ into the TCx. Animals with or without NOS inhibitors plus $A\beta_{(25-35)}$ were assessed in the radial maze over two days for the learning test and one day for memory. We found that the $A\beta_{(25-35)}$-treated group shows impairment in the spatial learning and memory after one month (57% and 65%). Similarly the 7-NI plus $A\beta_{(25-35)}$ treated group showed impairment in the learning and memory test, which probably means that the nNOS inhibition did not block the toxic effects of $A\beta_{(25-35)}$ on the cognitive process over the long term. In addition, there are reports in animals with brain seizures that show 7-NI does not improve the memory (Vanaja and Ekambaram, 2004). In contrast the animals with L-NAME or AG plus $A\beta_{(25-35)}$ had a good performance during the learning and memory tests. The L-NAME appears to be the better protective agent (64% for the learning and 68% for the memory test) because it is an inhibitor of both nNOS and iNOS, thus blocking the principal sources of NO. The AG produces a protective action against the $A\beta_{(25-35)}$ toxicity also (70% and 71%) as described for the learning and memory processes. This indicates that the iNOS plays an important role in animals with brain seizures caused by the $A\beta_{(25-35)}$ peptide because there is an enhanced expression of iNOS that results in the excessive NO production and cognitive deficits (Togo et al., 2004; Li and Poulos, 2005).

The $A\beta_{(1-42)}$ and $A\beta_{(25-35)}$ peptides are active in reducing the ability of memory retention in rodents trained for several cognitive paradigms (Maurice et al., 1998; Tran et al., 2001; Mazzola et al., 2003). This means that both peptides share similar mechanisms of toxicity, such as oxidative stress, increase of the NO levels, and cause lipid peroxidation (Varadarajan et al., 2000; Sheng et al., 2009; Tran et al., 2001; Mazzola et al., 2003; Gulyaeva and Stepanichev, 2010). However, it appears that each peptide exerts its own toxic action by its different properties. The molecular weight of $A\beta_{(25-35)}$ peptide is about fivefold lower than the full $A\beta_{(1-42)}$, and its toxic effects are greater than the full-length peptide. Some authors suggest that the toxicity of $A\beta_{(25-35)}$ is caused by methionine 35 in the C-terminal because it enhances the rate of oxidative damage. In addition, it appears that the $A\beta_{(25-35)}$ forms aggregates and fibrils more rapidly than the full-length protein, which is an important step in the development of the neurodegenerative process.

Our previous results confirmed that the injection of $A\beta_{(25-35)}$ into the TCx causes an overproduction of NO. In our present experiments the presence of NO again was found higher in the $A\beta_{(25-35)}$-treated group compared to the control group, but the main findings of our work were in the animals with NOS inhibitors (L-NAME or AG) plus $A\beta_{(25-35)}$, which showed low NO levels compared to the $A\beta_{(25-35)}$-treated group after one month (with only a decrease of 32% and 46% in the correct responses). Furthermore, the 7-NI plus $A\beta_{(25-35)}$-treated group had high NO levels in the TCx and Hp, indicating that the nNOS inhibition is not a crucial event for the NO production over the long term in these conditions. Stepanichev et al. (2008) reported that the loss of nNOS immunoreactivity in the neurons coexists with increased enzymatic activity of iNOS to cause oxidative stress during the first week after the injection of the $A\beta_{(25-35)}$. This is because the Aβ toxicity caused

Fig. 3. Effects of the L-NAME, 7-NI, and AG on the Amyloid-β (25-35)-treated rats caused changes in the immunoreactivity of GFAP and 3-NT in the temporal cortex of rats. The photomicrography shows the locations of nitrated protein (green) and GFAP (red) in the TCx (A–H) and CA1 subfield the Hp (I–P) of animals treated with control (A and I), L-NAME (B and J), 7-NI (C and K), AG (D and L), $A\beta_{(25-35)}$ (E and M), L-NAME + $A\beta_{(25-35)}$ (F and N), 7-NI + $A\beta_{(25-35)}$ (G and O), and AG + $A\beta_{(25-35)}$ (H and P) groups. The rats were injected with 1 μL of $A\beta_{(25-35)}$ [100 mM] into the TCx. The nucleus was observed with DAPI staining (blue stain). All stains were observed at 40×. In graph (P and S) are shown the numbers of 3-NT-reactive cells and (R and T) the number of GFAP-reactive cells in the TCx and CA1 subfield the Hp. These evaluations were done 30 days after the administration of the inhibitors and the injection of the $A\beta_{(25-35)}$. The statistical analysis was made using a one-way ANOVA and a Bonferroni posttest with $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to the $A\beta_{(25-35)}$ group ($n=4$ in each group).

vulnerability of nNOS contained in neocortical neurons over the short term, as was suggested by Hartlage-Rübsamen et al. (2001).

The effects of NO production were assessed by the number of neurons in the TCx and CA1 subfield of the Hp for each experimental group. The morphological alterations caused by the injection of the $A\beta_{(25-35)}$ produces severe cellular modifications, an event shown by a reduction in the number of the cells in these regions of the brain. The 7-NI plus $A\beta_{(25-35)}$-treated group had a decrease in the number of neurons whereas the L-NAME or AG plus $A\beta_{(25-35)}$ groups showed no significant loss of neurons in the TCx and Hp. These data largely justified that the 7-NI plus $A\beta_{(25-35)}$-group is the most affected in the neuronal loss and cognitive process in contrast to the L-NAME or AG plus $A\beta_{(25-35)}$ groups, which show the best ameliorative effects, as expected.

The dysfunction in the NO metabolism and the events of toxicity that this molecule enhances has been linked to neurodegeneration (Giasson et al., 2000; Stack et al., 2008), as our results have shown. Several authors have studied the effects of NO in the memory process in models of neurodegeneration, but now it appears that high amounts of nitrites could be a source of damage that promotes the nitrosative stress that enhances protein nitration and neuronal damage to cause memory impairment (Limón et al., 2009a,b; Díaz et al., 2010).

The nNOS and iNOS immunoreactivity have been found over the chronic course of β-amyloid toxicity in the cortex of patients with AD (Fernández-Vizarra et al., 2004; Malinski, 2007). Our results indicate the presence of the NOS affects the protein nitration particularly on nitrotyrosine proteins. We found a major nitration of proteins accompanied by astrocytosis in the TCx and Hp of $A\beta_{(25-35)}$-treated rats compared to the control group. The NOS inhibitors (L-NAME and AG) plus $A\beta_{(25-35)}$ groups did not show the nitrotyrosine immunoreactivity or astrocytosis in the cortex and Hp. These results supported the idea that inhibitors of the NOS (principally iNOS) decrease the NO metabolites, such as NO_3, NO_2, and the nitrotyrosine patterns and the cognitive deficits. The inflammatory response in AD has been reported as an effect of NO production by iNOS activity exacerbating brain inflammation by proinflammatory mediators such as cytokines, acute-phase proteins, and several complement proteins that contribute to neuronal death (Apelt and Schliebs, 2001).

Under conditions of cellular stress, a balance is formed between nitrosylation and oxidation depending on the relative concentration of NO, which we measured as nitrite, a stable metabolite from NO. At low NO fluxes, these reactions would tend to lead to oxidation of substrate, whereas at higher levels of NO they will preferentially turn on the nitrosative effect that culminates in the nitration of proteins on residues of tyrosines. This indicates that high NO levels in which the indirect effects are predicted, in particular those that can be measured in a biological system, will favor nitrosative stress that leads to neuronal death (Thomas et al., 2008).

Several authors have described the trophic action of NO linked with the iNOS activity in models of AD, such as Colton et al. (2008), who showed that NO could be an important factor to protect against neuronal damage in a APPsw/NOS2 mouse compared with APPSw, with results different than in the wild-type rat. This kind of protection by NO is related to the inhibition of caspases and the execution of apoptosis, which appears to be a trophic effect of NO at lower concentrations that causes an improvement of memory. Another consideration in the wild-type rats related to the increase of the NO synthesis may be associated with the protective action to counteract Aβ-toxicity, although the nNOS presence over the long term could be decreased in the neurons probably by oxidative effects and in these conditions the high iNOS activity turns toxic (Good et al., 1996; Smith et al., 1997a,b; Perry et al., 2000; Malinski, 2007). Moreover, our group has shown that the $A\beta_{(25-35)}$ toxicity, after this fraction was administered into the hippocampus of rat pups, enhances the nitrite levels and causes a loss of the memory in the adult, an event that was blocked by the prior L-NAME-treatment plus $A\beta_{(25-35)}$ after 90 days (Díaz et al., 2010). One explanation about the toxic role of NO is the increase of the expression of iNOS by $A\beta_{(25-35)}$, as Stepanichev et al. (2008) reported in the wild-type rat, which contrasts with the knockout models. In addition, the blocking of iNOS is an important tool to show the role of NO in rats that have a progressive response caused by $A\beta_{(25-35)}$ injection, without mutations or modification of any NOS, an event that appears important for the study of a slow progressive neuronal damage in vivo.

Taken together with our results, we can suggest the participation of the iNOS as the major source of NO from the TCx and Hp in the toxicity caused by the injection of $A\beta_{(25-35)}$ peptide in rats is a crucial factor to understand the impairment of the spatial memory.

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