

Adenosine A_{2A} Receptor Blockade Prevents Synaptotoxicity and Memory Dysfunction Caused by β -Amyloid Peptides via p38 Mitogen-Activated Protein Kinase Pathway

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Alzheimer's disease (AD) is characterized by memory impairment, neurochemically by accumulation of β -amyloid peptide (namely $A\beta_{1-42}$) and morphologically by an initial loss of nerve terminals. Caffeine consumption prevents memory dysfunction in different models, which is mimicked by antagonists of adenosine A_{2A} receptors (A_{2A} Rs), which are located in synapses. Thus, we now tested whether A_{2A} R blockade prevents the early $A\beta_{1-42}$ -induced synaptotoxicity and memory dysfunction and what are the underlying signaling pathways. The intracerebral administration of soluble $A\beta_{1-42}$ (2 nmol) in rats or mice caused, 2 weeks later, memory impairment (decreased performance in the Y-maze and object recognition tests) and a loss of nerve terminal markers (synaptophysin, SNAP-25) without overt neuronal loss, astrogliosis, or microgliosis. These were prevented by pharmacological blockade [5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261); 0.05 mg · kg⁻¹ · d⁻¹, i.p.; for 15 d] in rats, and genetic inactivation of A_{2A} Rs in mice. Moreover, these were synaptic events since purified nerve terminals acutely exposed to $A\beta_{1-42}$ (500 nM) displayed mitochondrial dysfunction, which was prevented by A_{2A} R blockade. SCH58261 (50 nM) also prevented the initial synaptotoxicity (loss of MAP-2, synaptophysin, and SNAP-25 immunoreactivity) and subsequent loss of viability of cultured hippocampal neurons exposed to $A\beta_{1-42}$ (500 nM). This A_{2A} R-mediated control of neurotoxicity involved the control of $A\beta_{1-42}$ -induced p38 phosphorylation and was independent from cAMP/PKA (protein kinase A) pathway. Together, these results show that A_{2A} Rs play a crucial role in the development of $A\beta$ -induced synaptotoxicity leading to memory dysfunction through a p38 MAPK (mitogen-activated protein kinase)-dependent pathway and provide a molecular basis for the benefits of caffeine consumption in AD.

Introduction

Alzheimer's disease (AD) is the most common chronic neurodegenerative disease and is clinically characterized by a progressive impairment of cognitive functions such as learning and memory. Although the traditional neuropathologic hallmarks of AD are the presence of neurofibrillary tangles and the accumulation of the senile plaques resulting from β -amyloid peptide ($A\beta$) aggregation, the neurochemical parameter best correlated with memory dysfunction in AD is the levels of soluble $A\beta$, mainly $A\beta_{1-42}$ (Selkoe, 2001). Also, the earliest morphological trait and the best

correlated with initial memory impairment in AD is the loss of synapses in the limbic cortex, namely in the hippocampus (Coleman et al., 2004). In fact, synapses seem to be the primordial target of toxic $A\beta$ oligomers, with the resulting synaptic failure underlying memory impairment in AD (Hardy and Selkoe, 2002; Klein et al., 2004). Thus, the early $A\beta_{1-42}$ -induced synaptotoxicity and associated mechanisms constitute major targets in the development of novel therapeutic strategies for AD.

Adenosine modulates synaptic transmission through inhibitory A_1 or facilitatory A_{2A} receptors (A_{2A} Rs), both of which are predominantly located in synapses, namely in the limbic and neocortex (Fredholm et al., 2005). Given the ability of A_1 Rs to inhibit calcium entry into neurons, glutamate release, and NMDA receptor activation, A_1 Rs have been considered promising candidate targets to prevent neuronal damage. However, their rapid downregulation and functional desensitization after insults limits their neuroprotective potential (de Mendonça et al., 2000). More recently, major interest has been devoted to A_{2A} Rs since their blockade affords neuroprotection against chronic insults in the adult brain (Cunha, 2005; Chen et al., 2007), which also trigger major increases in the extracellular levels of adenosine

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(de Mendonça et al., 2000). This is in notable agreement with the ability of caffeine (a nonselective adenosine receptor antagonist) to protect against cognitive impairment in different animal models, an effect that mainly seems to involve A_{2A} Rs (for review, see Cunha, 2008b; Takahashi et al., 2008). Likewise, caffeine consumption inversely correlates with the incidence of AD (Maia and de Mendonça, 2002) and prevents memory impairment in animal models of AD (Arendash et al., 2006; Dall'Igna et al., 2007), an effect mimicked by selective A_{2A} R antagonists (Dall'Igna et al., 2007). Interestingly, A_{2A} R blockade selectively prevented $A\beta$ -induced, but not scopolamine- or dizocilpine maleate (MK801)-induced, memory impairment (Cunha et al., 2008). Notably, memory impairment by $A\beta$ (but not scopolamine or MK801) involves synaptotoxicity. This suggests that A_{2A} R blockade prevents memory impairment by selectively controlling synaptotoxicity, which would provide a molecular basis to support a neuroprotective action of A_{2A} Rs.

The present study tested the ability of A_{2A} Rs to prevent $A\beta_{1-42}$ -induced synaptotoxicity and memory impairment and investigated the underlying mechanisms. Results show that A_{2A} R blockade (pharmacologic or genetic) prevents $A\beta_{1-42}$ -induced synaptotoxicity and subsequent memory dysfunction by a mechanism involving the control of the p38 mitogen-activated protein kinase (MAPK) pathway.

Materials and Methods

Animals. Wistar rats (8–10 week males) were from Charles River. C57BL/6 mice (8–10 week males), both wild-type (WT) and A_{2A} R knock-out (KO), were generously provided by Jiang-Fan Chen (University of Boston, Boston, MA). Animals were maintained under controlled environment ($23 \pm 2^\circ\text{C}$; 12 h light/dark cycle; *ad libitum* access to food and water) and handled according to European Union guidelines (86/609/EEC). Behavioral experiments were conducted between 10:00 A.M. and 4:00 P.M.

Analysis of β -amyloid peptides and in vivo administration procedures. The β -amyloid (1–42) peptide fragment ($A\beta_{1-42}$) or the nonamyloidogenic reverse peptide $A\beta_{42-1}$ ($A\beta_{42-1}$) was dissolved in water at a concentration of 2.25 mg/ml and 2 nmol in 4 μl was administered intracerebroventricularly, as previously described (Dall'Igna et al., 2007). Control animals were intracerebroventricularly infused with a similar volume of water. Behavioral analysis was performed 2 or 15 d after $A\beta_{1-42}$ or $A\beta_{42-1}$ administration. The selective A_{2A} R antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261) (generously provided by Scott Weiss, Vernalis, Wokingham, UK) was injected intraperitoneally at an efficacious dose (0.05 mg/kg of SCH58261) (Cunha et al., 2006, 2008; Dall'Igna et al., 2007), in saline (0.9% sodium chloride) with 10% dimethylsulfoxide, applied daily starting 30 min before $A\beta_{1-42}$ administration. Control animals were injected intraperitoneally with saline with 10% dimethylsulfoxide.

The qualitative analysis of the oligomerization status of the $A\beta$ peptide solution was evaluated by Western blot analysis using the 6E10 antibody that recognizes different human $A\beta$ homomeric forms, as previously described (Evans et al., 2008). Briefly, 10 μl of the different batches of $A\beta$ solutions was mixed with sample buffer (40% glycerol, 2% SDS, 0.2 M Tris-HCl, pH 6.8, and 0.005% Coomassie blue) and analyzed by electrophoresis (40 mA for 3 h and 30 min) using a tricine running buffer (Gibson et al., 2004). The blots were revealed with Coomassie blue (using a Coomassie blue R-250 solution made of 40% methanol, 10% acetic acid, and 0.1% Coomassie blue R-250 for 30 min, followed by destaining with 40% methanol and 10% acetic acid) or with 6E10 antibody (1:1000 dilution; Covance), as described below (see Western blot analysis).

The $A\beta_{1-42}$ levels in the hippocampus were quantified using two ELISA kits (Invitrogen), one detecting $A\beta_{1-42}$ (and isoforms with lower length) and the other $A\beta_{1-40}$, as previously described (Cao et al., 2009). Briefly, one hippocampus was homogenized in RIPA buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 1% IGEPAL, 0.2% SDS,

and protease inhibitor mixture containing leupeptin, pepstatin A, chymostatin, and aprotinin, all 1 mg/ml from Sigma-Aldrich). The mixture was centrifuged (30 min at $27,000 \times g$) and the supernatant was stored at -80°C until ELISA quantifications, which were performed following the manufacturer's instructions. $A\beta_{1-42}$ levels were estimated by subtracting the estimated amount of $A\beta_{1-40}$ from those of $A\beta_{1-42}$ and were normalized by tissue weight and/or amount of protein, determined with the bicinchoninic acid (BCA) method (Pierce Biotechnology).

The detection of $A\beta$ aggregates in the hippocampus was performed using Congo Red (Puchtler et al., 1985) or Thioflavin-S histochemical analysis (Reyes et al., 2004) of hippocampal sections (see below), as previously described (Melo et al., 2009).

Behavioral analysis. Locomotor activity was monitored in an open-field arena (50 \times 50 cm, divided in four squares of 25 cm for rats, and 30 \times 30 cm, divided in nine squares for mice, respectively), and the exploratory behavior of the animals was evaluated by counting the total number of line crossings and the number of rearings over a 5 min period. Hippocampal-dependent memory performance was assessed by measuring spontaneous alternation performance during 8 min in the Y-maze test, which allows evaluating cognitive searching behavior, although it does not allow isolating memory performance (for review, see Hughes, 2004). The series of arm entries was recorded visually and an alternation was defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as follows: total of alternations/(total arm entries – 2), as previously described (Dall'Igna et al., 2007). Memory performance was also evaluated using the object recognition test consisting of two 3 min sessions (24 h after habituation): the first with two identical objects (training session) and the second (test session, 30 min after) with two dissimilar objects (a familiar and a novel one); recognition object index was calculated by the ratio of the time spent exploring novel object over the total exploration time of both objects, as previously described (Costa et al., 2008b). The experimenter conducting behavioral analysis was blinded to treatment conditions.

Histochemistry and immunohistochemistry. Brain fixation was performed through transcardiac perfusion with 4% paraformaldehyde (in 0.9% sodium chloride and 4% sucrose), as previously described (Cunha et al., 2006). Frozen brain was sectioned (20 μm coronal slices) with a Leica CM1850 cryostat (Leica Microsystems), mounted on slides coated with 2% gelatin with 0.08% chromalin (chromium and potassium sulfate), allowed to dry at room temperature, and stored at -20°C until use.

Neuronal morphology in hippocampal sections was evaluated by cresyl violet staining of Nissl bodies, as previously described (Lopes et al., 2003). Briefly, sections were incubated for 10 min with cresyl violet (Sigma-Aldrich) solution (0.5% in acetate buffer). Sections were then washed twice with acetate buffer, twice in 100% ethanol, cleared with xylene, and mounted with Vector medium (Vector Laboratories). Degenerating neurons were detected using Fluoro-Jade C, which fluorescently labels them independently of the mechanism of cell death (Schmued et al., 2005). We used a 0.0001% solution of Fluoro-Jade C (Histo-Chem), as previously described (Cunha et al., 2006).

Detection of nerve terminals was performed as previously described (Cunha et al., 2006), using immunohistochemical detection of synaptophysin, a protein located in synaptic vesicles (Masliah and Terry, 1993). Immunohistochemistry detection of CD11b (a marker of microglia) (Jensen et al., 1997) and of glial fibrillary acidic protein (GFAP) (a marker of astrocytes) (Pekny and Nilsson, 2005) was performed to evaluate microgliosis and astroglia, respectively. The sections were first rinsed for 5 min with PBS (140 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) and then three times for 5 min with Trizma base solution (TBS) (0.05 M containing 150 mM NaCl, pH 7.2) at room temperature. Sections were then permeabilized and blocked with TBS containing 0.2% Triton X-100 and 10% goat serum during 45 min, incubated in the presence of the mouse anti-synaptophysin antibody (1:500) or rat anti-CD11b (1:600; Serotec) or anti-GFAP-Cy3 (1:500; Sigma-Aldrich) for 72 h at 4°C , rinsed three times for 10 min in TBS, and subsequently incubated with goat anti-mouse or goat anti-rat secondary antibody conjugated with a fluorophore (Alexa Fluor 488; Invitrogen) (1:100) for 2 h at room temperature. After rinsing twice for 10 min in TBS and once for

10 min in distilled water, the sections were dehydrated and passed through xylene before mounting on slides, using Vectashield mounting medium (Vector Laboratories).

All sections were examined under a transmission and fluorescence Zeiss Axiovert 200 microscope, with AxioVision software 4.6 (PG-HITEC).

Assays in hippocampal synaptosomes. Synaptosomes (i.e., enriched nerve terminals) were prepared from the hippocampus using a sucrose/Percoll-based series of centrifugations, as previously described (Rebola et al., 2005). Briefly, the two hippocampi from one animal were homogenized at 4°C in sucrose solution (0.32 M) containing 1 mM EDTA, 10 mM HEPES, 1 mg/ml bovine serum albumin (BSA), and 1 mM dithiothreitol (DTT), pH 7.6, centrifuged at 3000 × g for 10 min at 4°C, the supernatants were collected and centrifuged at 14,000 × g for 12 min at 4°C, and the pellet was resuspended in 1 ml of a 45% (v/v) Percoll solution in Krebs' buffer (140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose, pH 7.4). After centrifugation at 14,000 × g for 2 min at 4°C, the top layer was removed (synaptosomal fraction) and washed in 1 ml of Krebs' buffer. Protein determination was performed with the BCA method.

The redox status of synaptosomes, known to be affected by exposure to β-amyloid peptides (Mattson et al., 1998), was measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich), as previously described (Silva et al., 2007). Synaptosomes were incubated for 2 h at 37°C in Krebs' buffer in the absence or presence of Aβ₁₋₄₂ (500 nM) and/or SCH58261 (50 nM). MTT (0.5 mg/ml) was then added and incubated for 1 h at 37°C in the dark. As MTT is converted to a water-insoluble blue product (formazan) by viable terminals, the precipitated dye can be spectrophotometrically (570 nm) quantified after exposing synaptosomes to isopropanol containing 0.04 M HCl. Values were expressed as the percentage of optical density of control synaptosomes, in the absence of added drugs.

The mitochondrial membrane potential of synaptosomes was measured by a fluorimetric assay adapted and optimized for synaptosomes from a fluorimetric protocol used in isolated brain mitochondria (Oliveira et al., 2007). Synaptosomes were incubated for 2 h at 37°C in Krebs' buffer in the absence or presence of Aβ₁₋₄₂ (500 nM) and/or SCH58261 (50 nM), followed by 1 h incubation with 2 nM tetramethyl rhodamine methyl ester (TMRM⁺) (Invitrogen) and a short-spin centrifugation. The pellet was resuspended in 150 μl of Krebs-HEPES with 2 nM TMRM⁺. The functional assay was performed in a fluorescence spectrometer (Spectra Max Gemini EM; Molecular Devices), using 540 nm excitation and 590 nm emission, with a cutoff of 570 nm, and analyzed with SoftMax Pro V5 (Molecular Devices). The experiment is initiated by measuring a baseline (370 ± 8 fluorescent arbitrary units; n = 8) for 10 min, followed by the simultaneous addition of carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) (2 μM) and oligomycin (1 μg/ml) and sequential measurement during 10 min to establish the new baseline yielding a change of relative fluorescence of 607 ± 28 fluorescent arbitrary units (control, n = 8). The effect of tested drugs was measured as changes in this difference between final and initial baseline and are expressed as the percentage of the difference observed in control conditions.

Primary cultures of neurons. Hippocampal neurons were cultured from 17- to 19-d-old Wistar rat embryos, as previously described (Silva et al., 2007), and plated on poly-D-lysine-coated 16-mm-diameter coverslips or six-well plates at densities of 5 × 10⁴/coverslip (viability and immunocytochemistry assays) or 1 × 10⁶/well (Western blot analysis). Neurons were grown at 37°C in a 5% CO₂ humidified atmosphere in Neurobasal medium with B-27 supplement, glutamate (25 μM), glutamine (0.5 mM), and gentamicin (0.12 mg/ml).

Drug treatments and evaluation of cell death. Aβ₁₋₄₂-induced neuronal damage was evaluated after culturing the neurons for 5–7 d. After 1 week, the culture matures and forms functional synaptic connections, and most of the regions exhibit spontaneous synaptic transmission (Rui et al., 2006). Either Aβ₁₋₄₂ (500 nM) or Aβ₄₂₋₁ (500 nM) were directly added to the medium and incubated for 12–48 h. To test the ability of any drug [SCH58261, 8-Br-cAMP, or N-[2-((o-bromocinamyl)amino)ethyl]-5-isouquinolinesulfonamide (H-89) from Sigma-Aldrich or 4-(4-fluoro-

phenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB202190) from Tocris] to modify the effects of Aβ₁₋₄₂, these drugs were added 15 min before addition of Aβ₁₋₄₂ onward.

Viability assays were performed by double labeling (3 min incubation) with the fluorescent probes Syto-13 (4 μM) and propidium iodide (PI) (4 μg/ml; Invitrogen) followed by fluorescence microscopy cell counting. As previously described (Silva et al., 2007), viable neurons present nuclei homogeneously labeled with Syto-13 (green fluorescent nuclei), whereas apoptotic neurons show condensed and fragmented nuclei labeled with Syto-13 (primary apoptosis) or with Syto-13 plus PI (secondary apoptosis) and necrotic neurons present intact nuclei labeled with PI (red fluorescent nuclei). Each experiment was repeated using different cell cultures in duplicate, and cell counting was performed in at least six fields per coverslip, with a total of ~300 cells. Results are mean ± SEM and statistical significance (p < 0.05) was evaluated by one-way ANOVA followed by Newman-Keuls multiple-comparison test.

Immunocytochemical evaluation of synaptotoxicity. After fixation with 4% paraformaldehyde, cells were permeabilized with PBS with 0.2% Triton X-100 for 2 min and incubated with 3% of BSA in PBS for 30 min for the simultaneous immunocytochemical analysis of a presynaptic marker [synaptophysin or 25 kDa synaptosomal-associated protein (SNAP-25)] and a dendritic marker [microtubule-associated protein-2 (MAP-2)] (Silva et al., 2007). Cells were incubated with rabbit anti-MAP-2 (1:400; Santa Cruz Biotechnology) and mouse anti-synaptophysin (1:200; Sigma-Aldrich) or mouse anti-SNAP-25 (1:200; Sigma-Aldrich) for 1 h. After three washes with PBS, cells were incubated with anti-mouse or anti-rabbit secondary antibody conjugated with a fluorophore (Alexa Fluor 488 and Alexa Fluor 594, respectively; 1:200; Invitrogen). The cells were visualized by confocal microscopy (MRC 600).

Western blot analysis. Cultured hippocampal neurons were washed twice with PBS and gently scraped with ice-cold lysis buffer composed of 25 mM HEPES-Na, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and supplemented with 2 mM DTT, 100 μM phenylmethanesulfonyl fluoride (PMSF), 2 mM orthovanadate, 50 mM sodium fluoride, and a protease inhibitor mixture containing leupeptin, pepstatin A, chymostatin, and aprotinin (1 mg/ml; all from Sigma-Aldrich). The synaptosomal extract from rat or mice was solubilized in 5% SDS supplemented with 2 mM DTT and 100 μM PMSF and rapidly sonicated. After determining the amount of protein using the BCA method, a 1/6 vol of 6× SDS-PAGE sample buffer was added before storage at -20°C. Electrophoresis was performed using a 10 or 7.5% SDS-PAGE gel after loading of different amounts of each sample. Proteins were then transferred to PVDF (polyvinylidene difluoride) membranes (GE Healthcare). Membranes were blocked for 1 h at room temperature with 5% low-fat milk in Tris-buffered saline or 3% bovine serum albumin (depending on the antibodies used), pH 7.6, and containing 0.1% Tween 20 (TBS-T). Membranes were then incubated overnight at 4°C with primary antibodies, namely mouse anti-synaptophysin (1:5000–20,000), mouse anti-SNAP-25 (1:5000–20,000), mouse anti-phospho-c-Jun N-terminal kinase (JNK) (1:1000; Cell Signaling), or mouse anti-phospho-p38 MAPK (1:1000; Cell Signaling). After washing with TBS-T, membranes were incubated either with anti-mouse or anti-rabbit IgG secondary antibodies (1:10,000–20,000 in TBS-T; Invitrogen). After washing, membranes were revealed using an ECF kit (GE Healthcare) and visualized in a VersaDoc 3000 (Bio-Rad). The membranes were then reprobed and tested for α-tubulin immunoreactivity using a mouse anti-α-tubulin antibody (1:10,000–20,000; Zymed), as previously described (Rebola et al., 2005). To determine phosphorylation ratio of p38 and JNK, the membranes were reprobed with rabbit anti-total JNK/SAPK (stress-activated protein kinase) or rabbit anti-p38 MAPK total (both 1:1000; Cell Signaling).

HPLC quantification of adenosine levels in the incubation medium. After addition at time 0 of Aβ₁₋₄₂ (500 nM), cultured neurons were maintained at 37°C in a 5% CO₂ humidified atmosphere with 1.2 ml of medium, and samples (125 μl) were collected from the incubation medium after 0, 3, 12, 24, and 48 h. Each sample was filtered through 0.22 μm filters (Millex-GV from Millipore; Interface) and stored at -20°C until analysis by reverse-phase HPLC, as previously described (Cunha and Sebastião, 1993). The quantification of adenosine was

achieved by calculating the peak area and then converting to concentration values (correcting the change of incubation volume over time) by calibration with known standards (0.03–3 μ M).

Statistical analysis. Results are presented as mean \pm SEM. Data were analyzed with one-way ANOVA and Newman–Keuls multiple-comparison test (unless otherwise stated), using a significance level of 0.05.

Results

Characterization of $A\beta_{1-42}$ -induced memory impairment and morphological modifications

Western blot analysis of the $A\beta_{1-42}$ solutions used in this study showed that they were mainly constituted by monomers (4 kDa) and oligomers constituted by up to four monomers (Fig. 1A). The intracerebroventricular administration of $A\beta_{1-42}$ (2 nmol) led to an accumulation of $A\beta_{1-42}$ in the hippocampus (91.1 ± 25.1 pg/mg of protein; $n = 4$; $p < 0.05$), indicating that $11.3 \pm 2.9\%$ of the total amount of administered $A\beta_{1-42}$ accumulated in hippocampal tissue after 2 d. The hippocampal $A\beta_{1-42}$ levels decreased over time (Fig. 1B), since only 26.6 ± 8.9 pg/mg of protein ($n = 4$) was detected after 15 d ($p < 0.05$; $F = 12.39$ compared with $A\beta_{1-42}$ levels at 2 d).

The intracerebroventricular administration of $A\beta_{1-42}$ (2 nmol) caused a time-delayed (within 2 weeks) memory impairment, in agreement with previous reports (Dall'igna et al., 2007; Cunha et al., 2008), whereas it failed to affect memory performance within 2 d, as evaluated both in the Y-maze (Fig. 1D) or the object recognition test (Fig. 1E), without changes in locomotor activity 2 or 15 d after $A\beta_{1-42}$ administration (data not shown). The control peptide ($A\beta_{42-1}$; 2 nmol) changed neither Y-maze behavior nor locomotor activity ($n = 4$) (data not shown). This indicates that $A\beta_{1-42}$ might trigger a cascade of events leading to a delayed rather than acute perturbation of memory performance, which likely results from the action of soluble forms of $A\beta_{1-42}$ since we only found soluble $A\beta_{1-42}$ and no evidence of the presence of $A\beta$ aggregates 15 d after the intracerebroventricular administration of $A\beta_{1-42}$ (Fig. 1C).

Histological analysis of hippocampal sections, 2 weeks after the injection of $A\beta_{1-42}$, revealed a preservation of cresyl violet staining of Nissl bodies (Fig. 2A, showing CA3, which is identical with CA1) and absence of neuronal loss evaluated by Fluoro-Jade C, which is indistinguishable from control rats (Fig. 2B). Furthermore, there was no evidence of microgliosis (evaluated by CD11b immunoreactivity) or astrogliosis (evaluated by GFAP immunoreactivity), neither after 15 d (data not shown) nor after 2 d (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) of $A\beta_{1-42}$ administration. Further excluding acute toxic effects of $A\beta_{1-42}$ administration, there was no difference of cresyl violet or Fluoro-Jade C staining 2 d after the injection of $A\beta_{1-42}$ or vehicle (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). However, immunohistochemical analysis revealed a decrease in the synaptic marker synaptophysin in hippocampal sections obtained from rats 15 d after $A\beta_{1-42}$ injection (Fig. 2C), which was confirmed by quantitative

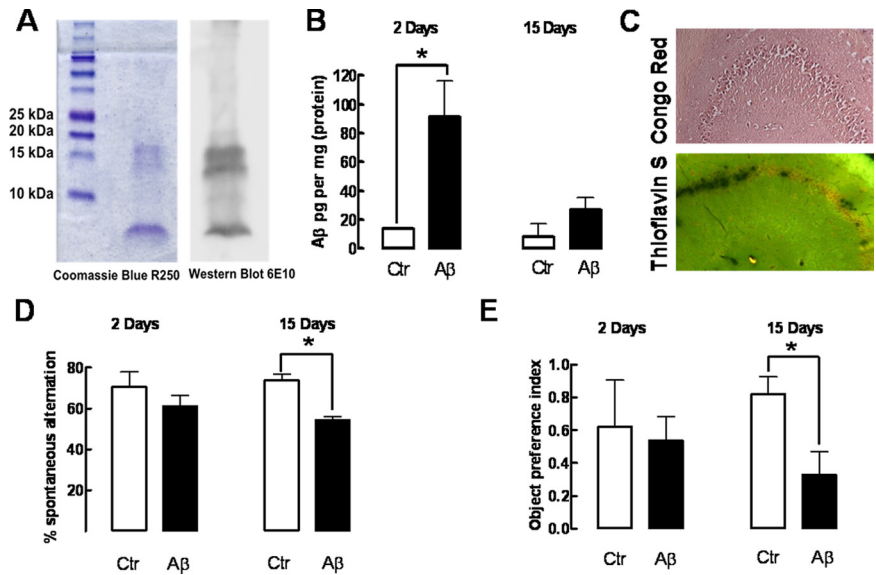


Figure 1. Intracerebroventricular administration of soluble β -amyloid peptides leads to an accumulation of soluble but not aggregated forms of $A\beta$ in the hippocampus, causing delayed memory impairment without evident acute effects. The Coomassie R-250 staining and 6E10 antibody-based Western blot analysis of the two different batches of $A\beta_{1-42}$ used showed that they were mainly constituted by monomers and oligomer containing up to four monomers (A). Rats were treated with $A\beta_{1-42}$ (2 nmol, i.c.v.) or water (control), which accumulated in the hippocampus after 2 and 15 d (B), as measured by ELISA ($n = 4$ rats treated with water and $n = 6$ treated with $A\beta_{1-42}$). Congo Red and Thioflavin S staining (C) failed to reveal the presence of $A\beta$ aggregates in hippocampal sections collected 15 d after $A\beta_{1-42}$ administration (images representative of 3 animals). D, Spontaneous alternation in the Y-maze test of control and $A\beta_{1-42}$ -treated rats after 2 or 15 d ($n = 6$ animals treated with water and $n = 9$ treated with $A\beta_{1-42}$). E, Object recognition index in the object recognition test of control and $A\beta_{1-42}$ -treated rats after 2 or 15 d ($n = 4$ animals treated with water and $n = 6$ –7 animals treated with $A\beta_{1-42}$). Data in bar graphs are mean \pm SEM; * $p < 0.05$.

Western blot analysis. As illustrated in Figure 2D, synaptophysin immunoreactivity was lower ($-25.7 \pm 4.3\%$; $n = 7$; $p < 0.001$) in hippocampal membranes collected from rats 15 d after $A\beta_{1-42}$ administration when compared with controls. In contrast, the nonamyloidogenic $A\beta_{42-1}$ peptide failed to modify synaptophysin immunoreactivity (data not shown).

Pharmacological blockade of adenosine A_{2A} receptor protects from $A\beta_{1-42}$ -induced synaptotoxicity and memory impairment

We then tested whether the blockade of A_{2A} Rs prevented the loss of synaptic markers and memory impairment observed 2 weeks after the intracerebroventricular administration of $A\beta_{1-42}$. For that purpose, we used a selective A_{2A} R antagonist (SCH58261) in a dose (0.05 mg/kg, i.p.) that has previously been shown to preserve memory performance without peripheral or locomotor effects (Dall'igna et al., 2007; Cunha et al., 2008). As illustrated in Figure 2C, SCH58261 (0.05 mg/kg) completely prevented the decrease of synaptophysin immunoreactivity caused by $A\beta_{1-42}$. In fact, synaptophysin immunoreactivity in hippocampal sections was indistinguishable in control conditions and in $A\beta_{1-42}$ -injected rats that were treated daily with SCH58261 (Fig. 2C). Accordingly, Western blot analysis confirmed that the decrease in synaptophysin density on $A\beta_{1-42}$ injection was prevented by SCH58261 ($p < 0.001$) (Fig. 2D). In parallel, SCH58261 was also able to significantly ($p < 0.001$) prevent the decreased Y-maze spontaneous alternation on $A\beta_{1-42}$ injection. In contrast, SCH58261 did not modify synaptophysin immunoreactivity (Fig. 2D) or spontaneous alternation in control rats (Fig. 2E) nor did it affect locomotion in control or $A\beta_{1-42}$ -treated rats (data not shown).

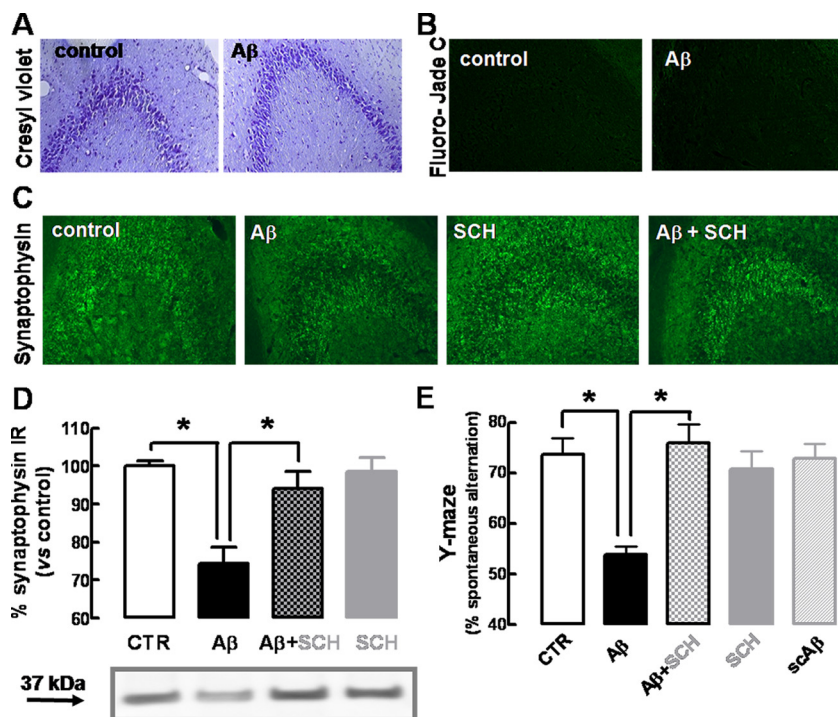


Figure 2. β-Amyloid administration causes a selective synaptotoxicity and memory dysfunction, which is prevented by blockade of adenosine A_{2A} receptors. Rats were treated with Aβ₁₋₄₂ (2 nmol, i.c.v.) or water (control). The A_{2A}R antagonist SCH58261 (0.05 mg/kg, i.p.) was administered daily starting 30 min before Aβ, and rats were behaviorally analyzed after 15 d. **A, B**, Cresyl violet staining of Nissl bodies (**A**) and Fluoro-Jade C staining of neuronal death (**B**) in hippocampal sections from control and Aβ₁₋₄₂-injected rats. **C, D**, Immunohistochemical labeling with anti-synaptophysin in hippocampal sections from rats injected with water (control), Aβ₁₋₄₂ (Aβ), SCH58261 (SCH), and Aβ plus SCH (images representative of 5 experiments) (**C**) and quantification by Western blot analysis (**D**) of synaptophysin immunoreactivity in hippocampal membranes from these different experimental groups (data are mean ± SEM from 7 experiments; **p* < 0.05). **E**, Spontaneous alternation in the Y-maze test of the same groups of rats, as well as rats injected with the nonamyloidogenic scrambled Aβ₁₋₄₂ peptide (scAβ) (data are mean ± SEM from 9 rats; **p* < 0.001).

Genetic inactivation of A_{2A} receptor abolishes Aβ₁₋₄₂-induced synaptotoxicity and memory deficits

The memory impairment and loss of synaptic markers observed in rats could also be reproduced on Aβ₁₋₄₂ administration in wild-type (C57BL/6) mice. In fact, 2 weeks after the intracerebroventricular administration of Aβ₁₋₄₂ (2 nmol), WT mice displayed a decreased memory performance, measured as a decreased (−23.0 ± 1.7%; *n* = 7; *p* < 0.001) spontaneous alternation in the Y-maze (Fig. 3A), without modification of locomotor activity (Fig. 3B), and a decreased density of two synaptic markers, synaptophysin (−26.7 ± 3.7%; *n* = 4; *p* < 0.001) and SNAP-25 (−25.8 ± 2.3%; *n* = 4; *p* < 0.001) (Fig. 3C), when compared with vehicle-injected (i.e., control) mice. Furthermore, the histological analysis of hippocampal sections of Aβ₁₋₄₂-treated WT mice showed the absence of the following: neuronal loss evaluated by Fluoro-Jade C, microgliosis evaluated by CD11b immunoreactivity, and astrogliosis evaluated by GFAP immunoreactivity (Fig. 3D, showing CA1 area, with similar results obtained for CA3 area) (data not shown).

To confirm the key role of A_{2A}Rs in controlling Aβ₁₋₄₂-induced loss of synaptic markers and memory impairment, we tested the effects of Aβ₁₋₄₂ in A_{2A}R KO mice. The A_{2A}R genetic inactivation in KO mice led to a decrease in the number of crossings (30 ± 7; *n* = 14; *p* < 0.05) and rearings (7 ± 2; *n* = 14; *p* < 0.05) when compared with WT mice; however, this does not affect the Y-maze alternation, on comparison of saline-injected WT and KO mice (Fig. 3B). As shown in Figure 3, Aβ₁₋₄₂ admin-

istration induced neither loss of synaptic markers nor memory impairment in A_{2A}R KO mice. Indeed, Aβ₁₋₄₂-injected A_{2A}R KO mice did not display a decrease of spontaneous alternation in the Y-maze (Fig. 3A) or a decrease in the density of the synaptic markers, synaptophysin or SNAP-25 (Fig. 3C). Furthermore, vehicle- or Aβ₁₋₄₂-injected A_{2A}R KO mice did not display cell death, microgliosis, or astrogliosis (Fig. 3D).

Blockade of A_{2A} receptors prevents Aβ₁₋₄₂-induced dysfunction of purified nerve terminals

The observations that Aβ₁₋₄₂ triggered an A_{2A}R-sensitive selective loss of synaptic markers prompted the hypothesis that this A_{2A}R-sensitive Aβ₁₋₄₂-induced toxicity could be replicated in enriched nerve terminals (synaptosomes). Previous studies have already reported that exposure of synaptosomes to β-amyloid peptides triggers mitochondrial dysfunction (Mattson et al., 1998), which has been argued to be a key feature of Alzheimer's disease (Moreira et al., 2006). Accordingly, synaptosomes exposed for 2 h to 500 nM Aβ₁₋₄₂ display a decrease (−8.3 ± 3.6% compared with control; *n* = 4; *p* < 0.001) in MTT reduction (Fig. 4A), which measures the redox status of synaptosomes, indicative of synaptosomal viability (Mattson et al., 1998; Silva et al., 2007). Furthermore, a decrease in TMRM⁺ accumulation, indicative of decreased mitochon-

drial membrane potential (−11.5 ± 2.5%; *n* = 8; *p* < 0.05) in Aβ₁₋₄₂-treated synaptosomes was also observed (Fig. 4B).

On blockade of A_{2A}Rs with SCH58261 (50 nM), there was a prevention of the Aβ₁₋₄₂-induced disruption of the functionality (Fig. 4A) and mitochondrial membrane potential of synaptosomes (Fig. 4B), whereas SCH58261 was devoid of effects in control synaptosomes (i.e., not treated with Aβ₁₋₄₂) or treated with the nonamyloidogenic Aβ₄₂₋₁ peptide (data not shown).

Blockade of A_{2A} receptor protects hippocampal neurons from Aβ₁₋₄₂-induced toxicity

To investigate the mechanism involved in the A_{2A}R-mediated control of Aβ₁₋₄₂-induced neurotoxicity, we used a cell culture model, namely, primary cultures of hippocampal neurons. Cultured hippocampal neurons were exposed for 12, 24, and 48 h to 500 nM Aβ₁₋₄₂, and neuronal death was analyzed by double labeling with Syto-13 and PI (Fig. 5A, B). After 12 h of exposure to Aβ₁₋₄₂, hippocampal neurons did not present any significant decrease (−1.0 ± 1.0%; *n* = 5; *p* > 0.05) of either cell viability (Fig. 5A) or number of apoptotic-like neurons (Fig. 5B) when compared with control neurons (either not exposed to Aβ₁₋₄₂ or exposed to the nonamyloidogenic Aβ₄₂₋₁ peptide). In fact, a decrease of cell viability (−9.0 ± 2.0%; *n* = 5; *p* < 0.001) was only observed 24 h after Aβ₁₋₄₂ exposure (Fig. 5A), which was accompanied by an increased number of apoptotic-like neurons (5 ± 1%; *n* = 5; *p* < 0.001) (Fig. 5B). This Aβ₁₋₄₂-induced neuronal death was larger after 48 h of exposure to Aβ₁₋₄₂, as evaluated by

the decreased number of viable neurons ($-12.3 \pm 3.7\%$; $n = 5$; $p < 0.001$) (Fig. 5A) and the increased number of apoptotic-like neurons ($9 \pm 2\%$; $n = 5$; $p < 0.001$) (Fig. 5B), indicating a time-dependent evolving profile of $A\beta_{1-42}$ -induced neurodegeneration. As occurred *in vivo* and in native brain preparations, this $A\beta_{1-42}$ -induced neurotoxicity was prevented by the $A_{2A}R$ antagonist, SCH58261 (50 nM), which did not affect neuronal viability in control neurons (Fig. 5C,D).

We next investigated whether the exposure of cultured neurons to $A\beta_{1-42}$ caused an initial synaptotoxicity preceding neuronal death. Since we observed that neurons incubated for 12 h with $A\beta_{1-42}$ did not display loss of viability or damage, we evaluated whether $A\beta_{1-42}$ -induced synaptotoxicity would be present after 12 h of exposure to $A\beta_{1-42}$, by evaluating the double staining of MAP-2 and synaptophysin or SNAP-25. As shown in Figure 6 (and in supplemental Fig. 2, available at www.jneurosci.org as supplemental material), there was a retraction of MAP-2-labeled segments and a decrease in the number of synaptophysin-immunoreactive spots after 12 h of exposure to $A\beta_{1-42}$ (i.e., at the time when neuronal damage is not yet present) (Fig. 5). To quantify this $A\beta_{1-42}$ -induced synaptotoxicity, we used Western blotting analysis, which showed a decrease in the density of synaptophysin ($-30.3 \pm 7.5\%$; $n = 6$; $p < 0.05$) and SNAP-25 ($-37.0 \pm 6.6\%$; $n = 6$; $p < 0.05$) on exposure to $A\beta_{1-42}$. As occurred *in vivo*, this initial and evolving $A\beta_{1-42}$ -induced synaptotoxicity in neuronal cultures was also prevented by $A_{2A}R$ blockade with the selective $A_{2A}R$ antagonist, SCH58261 (50 nM) (Fig. 6A; supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

This observation that SCH58261 prevents $A\beta_{1-42}$ -induced neurotoxicity but is devoid of effects in controls suggests that the levels of extracellular adenosine might be increased on exposure to $A\beta_{1-42}$, which is in accordance with the general concept that noxious stimuli are expected to increase the extracellular levels of adenosine (Fredholm et al., 2005). As predicted, incubation of hippocampal neurons with $A\beta_{1-42}$ (500 nM) caused a $>100\%$ increase of the extracellular concentration of adenosine (104.7 ± 38.8 nM; $n = 5$; $p < 0.05$) after 3 h that is persistent until 48 h of incubation (Fig. 6C).

Signaling pathways involved in the neuroprotection afforded by A_{2A} receptor blockade against $A\beta_{1-42}$ -induced neurotoxicity

Since one of main transducing systems operated by $A_{2A}R$ s involves cAMP/protein kinase A (PKA) pathway (Fredholm et al., 2005), we investigated whether the neuroprotective effects af-

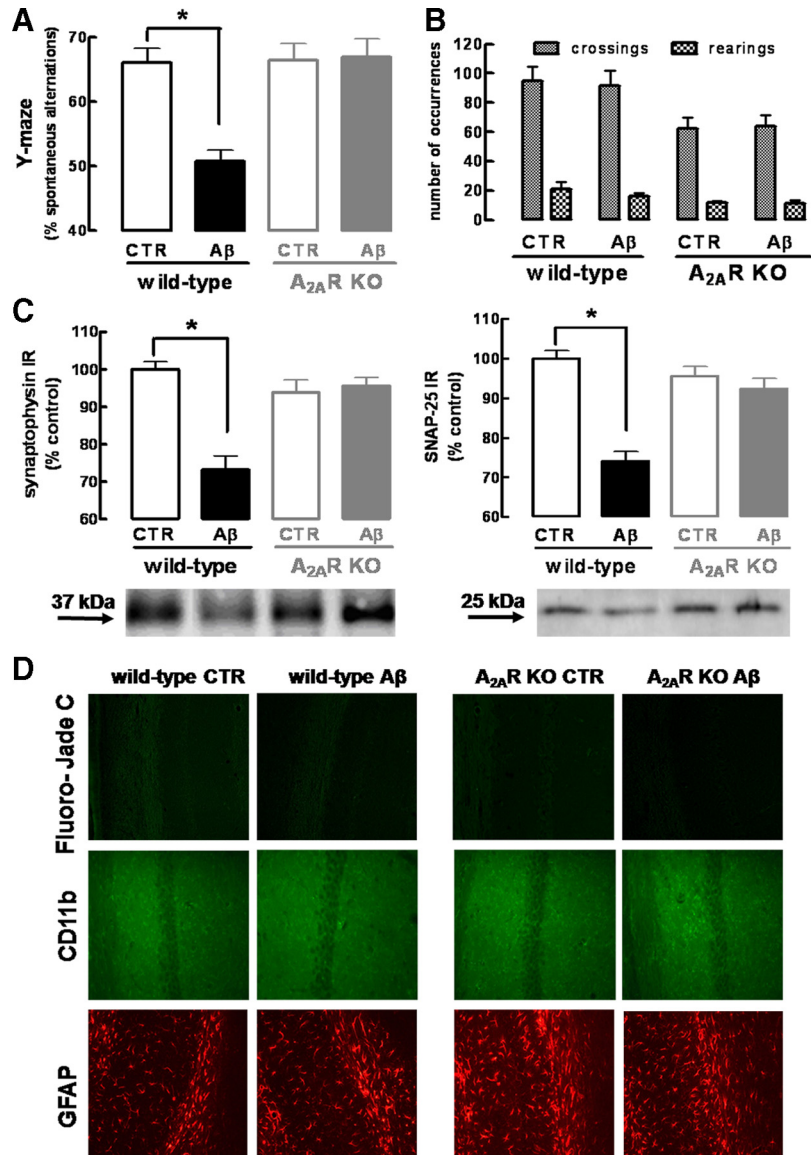


Figure 3. Genetic inactivation of adenosine A_{2A} receptors prevents β -amyloid-induced synaptotoxicity and memory impairment. Wild-type C57BL/6 or $A_{2A}R$ KO mice were treated with $A\beta_{1-42}$ (2 nmol, i.c.v.) or water [control (CTR)] and analyzed after 15 d. **A, B**, Spontaneous alternation in the Y-maze test (**A**) and spontaneous locomotion evaluated in an open-field arena (**B**) (data are mean \pm SEM of $n = 7$ mice per experimental group; $*p < 0.001$). **C**, Western blot comparing synaptophysin and SNAP-25 immunoreactivity in hippocampal membranes obtained from wild-type or $A_{2A}R$ KO mice injected with water (CTR) or $A\beta_{1-42}$ (data are mean \pm SEM of $n = 4$ mice per experimental group; $*p < 0.001$). **D**, Fluoro-Jade C staining of neuronal death, CD11-b immunohistochemistry evaluating microglia, and GFAP immunohistochemistry evaluating astroglia in hippocampal sections from wild-type or $A_{2A}R$ KO mice injected with water (CTR) or $A\beta_{1-42}$ (images representative of $n = 4$ mice per experimental group).

forded by SCH58261 involved this pathway. As observed in the Figure 7A, the manipulation of the cAMP/PKA pathway influences $A\beta_{1-42}$ -induced neurotoxicity, as described by others (Parvathani et al., 2000; Gong et al., 2004; Shrestha et al., 2006). In fact, the activation of PKA with the cell-permeable cAMP analog 8-Br-cAMP (200 μ M) attenuated $A\beta_{1-42}$ -induced neurotoxicity, an effect prevented by the PKA inhibitor H-89 (1 μ M) (Fig. 7A). However, the neuroprotection by SCH58261 persisted even in the presence of H-89, ruling out the participation of cAMP/PKA pathway in the neuroprotection resulting from the blockade of $A_{2A}R$ s (Fig. 7A).

It is also suggested that deregulation of the MAPK pathways, namely of JNK and p38 MAPK family of proteins, might play a role in the intracellular mechanisms of neurodegeneration, in

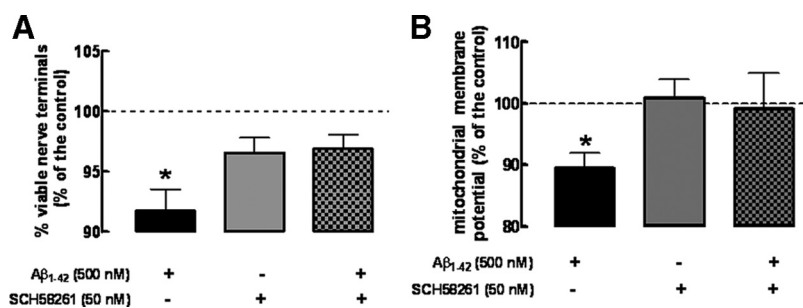


Figure 4. Exposure to Aβ₁₋₄₂ directly decreases the function of rat hippocampal synaptosomes, which is prevented by blockade of adenosine A_{2A} receptors. Synaptosomes were incubated for 2 h with 500 nM Aβ₁₋₄₂ or Krebs' buffer, in the absence or presence of the A_{2A}R antagonist, SCH58261 (50 nM), added 15 min before. **A**, Synaptosomal viability was measured using the MTT assay (data are mean ± SEM of n = 4; *p < 0.05). **B**, Measurement of mitochondrial membrane potential Δ (difference between the final and initial baseline) using the TMRM⁺ indicator after adding FCCP and oligomycin (data are mean ± SEM of n = 8; *p < 0.05).

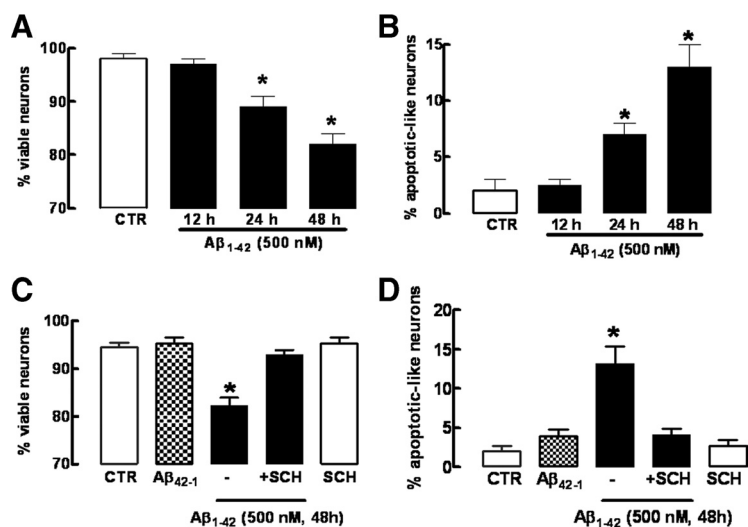


Figure 5. Temporal analysis of neuronal death caused by Aβ₁₋₄₂ and neuroprotection by blockade of adenosine A_{2A} receptors. Hippocampal neurons were preincubated with the A_{2A}R antagonist SCH58261 (50 nM) 15 min before addition of 500 nM Aβ₁₋₄₂. Neurons were double labeled with Syto-13 and PI probes. Viable neurons presented green nuclei stained with Syto-13, whereas apoptotic neurons presented shrinkage nuclei stained with PI and Syto-13. **A, B**, Aβ-induced neuronal death is time dependent. **C, D**, Blockade of A_{2A}R with SCH58261 prevents neuronal death on 48 h of incubation with Aβ. A total of ~300 cells per coverslip was counted. Results are means ± SEM of duplicate coverslips from five independent hippocampal cultures; *p < 0.05.

particular in Aβ₁₋₄₂-induced neurotoxicity (Troy et al., 2001; Minogue et al., 2003; Wang et al., 2004b; Zhu et al., 2005; Muñoz et al., 2007), and A_{2A}Rs can also signal through the MAPK pathway (for review, see Fredholm et al., 2005). To test the involvement of JNK and p38 MAPK in the A_{2A}R-mediated protection against Aβ₁₋₄₂-induced neurotoxicity, we first investigated the time course of Aβ₁₋₄₂-induced activation of p38 MAPK and JNK (evaluated as their degree of phosphorylation) to determine the time points at which this process occurs (data not shown). It was found that, after 2 h of incubation with Aβ₁₋₄₂ (500 nM), there was an increase of JNK (69 ± 21%; n = 6; p < 0.01) (Fig. 7B) and p38 MAPK (41 ± 15%; n = 7; p < 0.05) phosphorylation (Fig. 7C). At this time point, A_{2A}R blockade with SCH58261 (50 nM) increased the Aβ₁₋₄₂-induced JNK phosphorylation (210 ± 74%; n = 6; p < 0.01), whereas it abolished the Aβ₁₋₄₂-induced p38 MAPK phosphorylation (Fig. 7B, C). Confirming the key role of p38 MAPK in the Aβ₁₋₄₂-induced neurotoxicity (Zhu et al., 2005; Muñoz et al., 2007; Origlia et al., 2008), we found that the p38

MAPK inhibitor SB202190 (200 nM) prevented the Aβ₁₋₄₂-induced loss of neuronal viability and increased number of apoptotic-like neurons (Fig. 7D).

Discussion

The present results provide the first demonstration that blockade of a membrane receptor enriched in hippocampal synapses, namely, A_{2A}Rs, abolishes the loss of nerve terminal markers (i.e., synaptotoxicity) triggered by Aβ to culminate in memory dysfunction, the two cardinal features of early phases of AD. These results are relevant for the following three different reasons: (1) they provide evidence that control of a presynaptic modulation system that prevents synaptotoxicity also prevents memory dysfunction, strengthening the hypothesis that synaptic dysfunction is a precocious core modification of AD; (2) they provide additional evidence that A_{2A}Rs, the density of which is increased in AD (Albasanz et al., 2008), are a novel promising target to control AD; (3) they provide a clear demonstration that neuroprotection afforded by A_{2A}R blockade is independent of cAMP/PKA transducing system and results suggest that it is instead mediated by p38 MAPK.

We now observed that intracerebroventricular administration of soluble forms of Aβ₁₋₄₂ (Resende et al., 2008) caused a delayed loss of memory performance only after 15 d that was selectively associated with loss of synaptic markers. In fact, the only morphological change found in the hippocampus of Aβ-injected rodents displaying memory deficits was the loss of synaptic markers, whereas neither overt neuronal damage nor astrogliosis nor microgliosis were observed, neither 15 nor 2 d after Aβ₁₋₄₂ administration. Accordingly, in cultured neurons (in which peripheral, vascular, glial, or immune influences are absent), we also found that exposure to Aβ caused first a synaptotoxicity (Roselli et al., 2005; Calabrese et al., 2007; Shankar et al., 2007; Evans et al., 2008), which is only later followed by overt neuronal damage. Further strengthening that Aβ causes direct effects on nerve terminals, we showed that Aβ indeed directly impairs synaptosomal function, as observed by others (Mattson et al., 1998; Arias et al., 2002). Together, these observations indicate that Aβ, which can bind to synaptic proteins (Lacor et al., 2007) and accumulates synaptically in AD patients (Takahashi et al., 2002; Gylys et al., 2004; Fein et al., 2008), causes a primordial synaptotoxicity that precedes overt neuronal damage, as occurs in different transgenic animal models of AD (Hsia et al., 1999; Mucke et al., 2000; Oddo et al., 2003; Wu et al., 2004; Jacobsen et al., 2006) and in frontal cortical and hippocampal regions early in AD (Scheff et al., 2006, 2007). It should be stressed that we only obtained evidence that Aβ₁₋₄₂ caused loss

of synaptic markers, modification of the viability of nerve terminals (synaptosomes), and degeneration of synapses, which we collectively called synaptotoxicity; however, it remains to be determined to what extent this synaptotoxicity relates to the known $A\beta$ -induced functional impairment of hippocampal synapses (Venkitaramani et al., 2007).

This tight relationship between synaptotoxicity and memory dysfunction is further strengthened by the key observation of the present study [i.e., that blockade of A_{2A}Rs (pharmacological or genetic inactivation) simultaneously prevents synaptotoxicity and memory impairment caused by $A\beta$ administration]. Furthermore, the initial synaptotoxicity that precedes overt neuronal damage on exposure of cultured neurons to $A\beta$ was also prevented by A_{2A}R blockade. Finally, the direct $A\beta$ -induced impairment of nerve terminal function was also prevented by A_{2A}R blockade. All these observations are in agreement with the predominant synaptic localization of A_{2A}Rs in cortical regions (Rebola et al., 2005). These synaptic A_{2A}Rs play a key role controlling NMDA-dependent synaptic plasticity (Rebola et al., 2008), which is severely hampered early in AD (Roselli et al., 2005; Shankar et al., 2007; Venkitaramani et al., 2007). Thus, synaptic A_{2A}Rs normalize the function of these glutamatergic synapses (for review, see Cunha, 2008a), which are dysfunctional in AD (Bell et al., 2007), and their blockade prevents synaptotoxicity caused by different stimuli (Cunha et al., 2006; Silva et al., 2007) that leads to subsequent overt neurodegeneration on stressful conditions (Silva et al., 2007). This implies that the ability of A_{2A}Rs to control memory impairment should be particularly evident when synaptotoxicity is involved. Accordingly, we have previously shown that A_{2A}R blockade can prevent memory impairment caused by $A\beta$, which we now show to involve synaptotoxicity, but are ineffective in controlling acute memory dysfunction caused by pharmacological manipulation of the cholinergic or glutamatergic systems (Cunha et al., 2008), which is reversible and does not involve synaptotoxicity. Overall, this supports the notion that prevention of synaptic impairment on A_{2A}R blockade may underlie the ability of A_{2A}R antagonists to prevent $A\beta$ -induced memory impairment, which illustrates that control of synaptic dysfunction may be a relevant strategy to alleviate memory dysfunction associated with neurodegenerative conditions (Coleman et al., 2004; Wishart et al., 2006).

This putative relevance of targeting A_{2A}Rs to control memory impairment associated with neurodegenerative conditions is strongly supported by the ability of caffeine to counteract the development of neurodegenerative conditions and, in particular, the development of cognitive deficits (for review, see

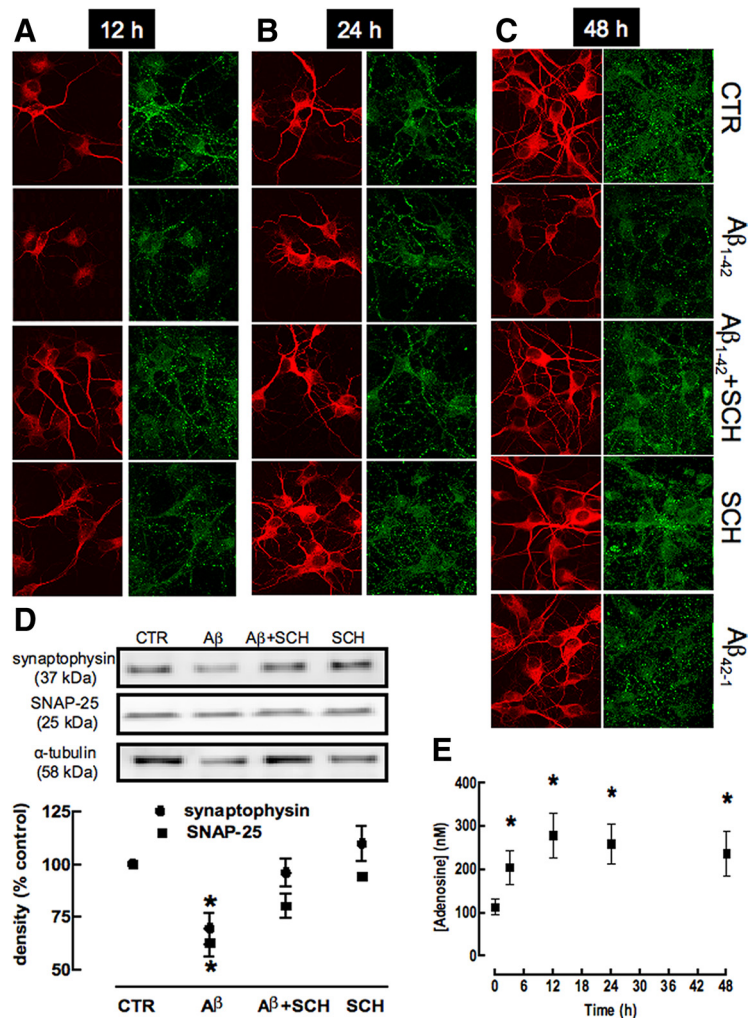


Figure 6. Temporal analysis of synaptotoxicity caused by $A\beta_{1-42}$ and neuroprotection by blockade of adenosine A_{2A} receptors. Hippocampal neurons were preincubated with the A_{2A}R antagonist SCH58261 (50 nM) 15 min before addition of 500 nM $A\beta_{1-42}$. Hippocampal neurons were double-labeled for MAP-2 (red) and synaptophysin (green) after 12 h (**A**), 24 h (**B**), and 48 h (**C**) of incubation and analyzed by confocal microscopy. Magnification, 400 \times . $A\beta_{1-42}$ causes a decrease of MAP-2 and synaptophysin immunoreactivities at all type points, which is prevented by SCH58261 and is not mimicked by the nonamyloidogenic scrambled peptide $A\beta_{2-1}$. **D**, Western blot analysis (15 μ g of protein loaded in each lane) quantifying the loss of synaptophysin and SNAP-25 immunoreactivities in cultures treated with $A\beta$, which is prevented by SCH58261 (data are mean \pm SEM of 6 independent cultures; * p < 0.05). **E**, Time course analysis of the extracellular levels of adenosine (quantified by HPLC) in hippocampal neurons incubated with $A\beta$ (data are mean \pm SEM of 5 independent cultures; * p < 0.05).

Cunha, 2008b; Takahashi et al., 2008). In fact, although it is doubtful that caffeine is a cognitive enhancer, its long-term consumption is clearly associated with decreased memory impairment caused by different perturbing conditions (Cunha, 2008b; Takahashi et al., 2008) such as on aging (Ritchie et al., 2007; Costa et al., 2008a) or Alzheimer's disease (Maia and de Mendonça, 2002; Eskelinen et al., 2009). The only known mechanisms of action of nontoxic doses of caffeine are the antagonism of adenosine receptors (Fredholm et al., 1999). Animal studies indicate that the ability of chronic caffeine consumption to prevent memory deterioration caused by different insults is mimicked by antagonists of A_{2A}Rs rather than A₁Rs (for review, see Cunha, 2008b; Takahashi et al., 2008). Accordingly, we have previously shown that the beneficial effects of caffeine on $A\beta$ -induced neurotoxicity and memory impairment are mimicked by antagonists of A_{2A}Rs but not of A₁Rs (Dall'Igna et al., 2003, 2007). Thus, it is tempting to

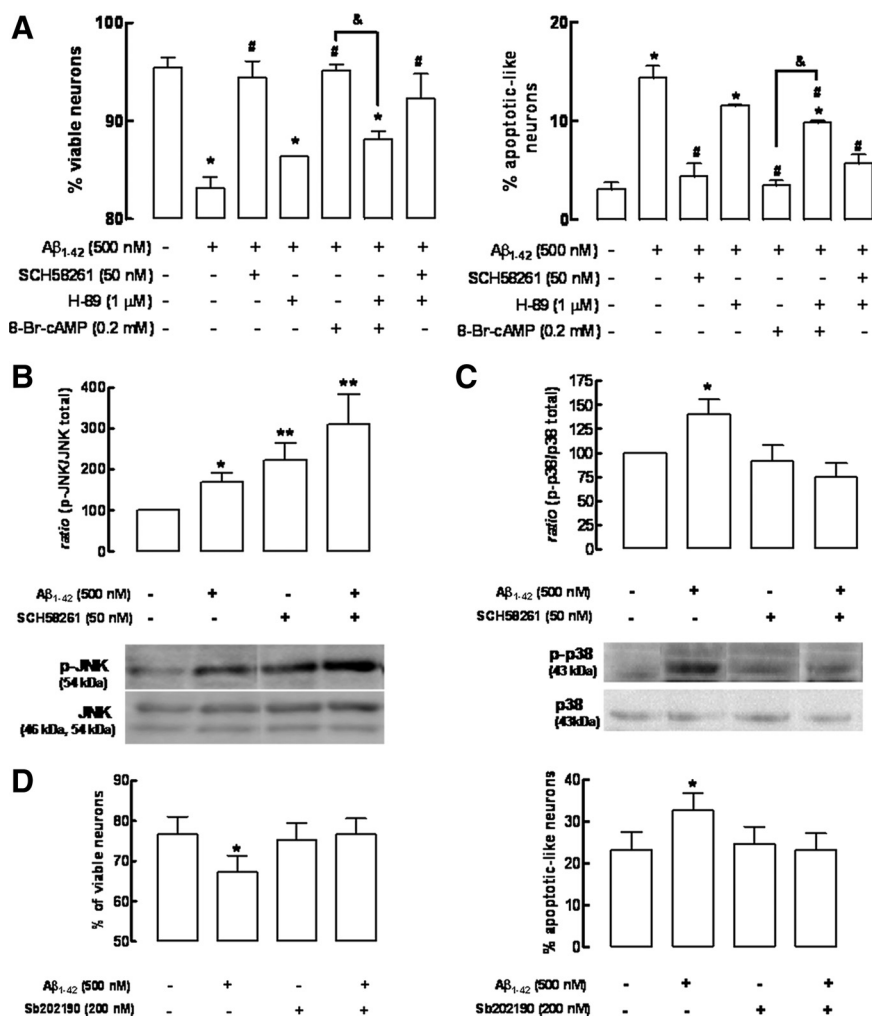


Figure 7. The neuroprotection afforded by blockade of adenosine A_{2A} receptors against A β ₁₋₄₂-induced neurotoxicity involves the p38 MAPK rather than the cAMP/protein kinase A signaling pathway. Hippocampal neurons were preincubated with the A_{2A}R antagonist SCH58261 (50 nM) or with the cAMP analog 8-Br-cAMP (200 μ M) 15 min before addition of 500 nM A β ₁₋₄₂. All inhibitors tested were added 30 min A β ₁₋₄₂. **A**, Neuroprotection by SCH58261 does not involve the cAMP/PKA signaling pathway since the PKA inhibitor H-89 (1 μ M) prevents the neuroprotection afforded by 8-Br-cAMP, but fails to modify the neuroprotection afforded by SCH58261, as evaluated after 24 h of exposure to A β ₁₋₄₂ (**p* < 0.05 vs control; #*p* < 0.05 vs A β ₁₋₄₂; &*p* < 0.05 vs A β ₁₋₄₂ + 8-Br-cAMP). **B**, **C**, A β ₁₋₄₂ triggered the activation of JNK (**B**) and p38 MAPK (**C**), evaluated by their degree of phosphorylation after 2 h, and SCH58261 enhanced JNK phosphorylation, whereas it blocked p38 MAPK phosphorylation (data are mean \pm SEM from 6 independent cultures; **p* < 0.05 vs control; ***p* < 0.05 vs effect of A β). **D**, The p38 MAPK inhibitor SB202190 prevents neuronal death induced by A β ₁₋₄₂ (data are mean \pm SEM from 5 independent cultures; **p* < 0.05 vs control).

propose that the promising beneficial effects of caffeine consumption as a strategy to prevent the burden of AD might be related to the synaptoprotective effect afforded by A_{2A}R blockade. This proposal does not exclude other possible concurring mechanisms by which caffeine may afford protection in AD, such as control of A β production (Arendash et al., 2006), control of the disruption of the blood–brain barrier (Chen et al., 2008), or control of neuroinflammation (Angulo et al., 2003). Thus, although the present data combining the use of fractionated nerve terminals, cultured neurons, and *in vivo* models strongly argue for the predominant importance of synaptic A_{2A}Rs in controlling A β -induced neurotoxicity, it does not exclude the possibility that other mechanisms may also contribute for neuroprotection against A β -induced neurotoxicity and memory impairment.

Finally, this study demonstrates that neuroprotection resulting from A_{2A}R blockade does not involve the cAMP/protein kinase

nase A transducing system but instead depends on control of p38 MAPK. In fact, the mechanisms by which A_{2A}Rs impact on neurodegeneration are still unresolved (for discussion, see Cunha, 2005; Chen et al., 2007). For historical reasons, there is a general consensus that A_{2A}Rs signal through activation of the adenylate cyclase/cAMP/PKA pathway (Fredholm et al., 2005). However, this is unlikely to be the relevant transducing system related to A_{2A}R control of neurodegeneration since enhanced cAMP levels afford neuroprotection against A β -induced neurotoxicity (Parvathani et al., 2000; Gong et al., 2004; Shrestha et al., 2006), whereas it is A_{2A}R blockade (expected to decrease cAMP levels) that affords neuroprotection. Accordingly, neuroprotection afforded by A_{2A}R blockade against A β -induced neurotoxicity was insensitive to the PKA inhibitor H-89, which prevented neuroprotection afforded by enhanced cAMP levels. Other transducing pathways have been documented to control degeneration in AD models, namely, the MAPK pathways (Zhu et al., 2005; Muñoz et al., 2007; Origlia et al., 2008), and, accordingly, we confirmed that A β triggered activation of both JNK and p38 MAPKs. Interestingly, we observed that A_{2A}R blockade prevented the A β -induced activation of p38, whereas it enhanced JNK phosphorylation, an aspect that needs attention in view of the association between JNK activation and neurodegeneration (Wang et al., 2004a). Given that inhibition of p38 activation is sufficient to prevent A β -induced neurotoxicity, as also observed by others (Zhu et al., 2005; Muñoz et al., 2007; Origlia et al., 2008), this indicates that A_{2A}Rs signal through p38 MAPK to control neurodegeneration. Indeed, previous studies have documented the ability of A_{2A}Rs to control MAPK pathways in a cAMP-independent

manner (Schulte and Fredholm, 2003; Fredholm et al., 2005; Gsandtner et al., 2005), and it has previously been suggested that the control by A_{2A}Rs of the ischemia-induced brain damage was related to the ability of A_{2A}R antagonists to blunt the ischemia-induced accumulation of phosphorylated forms of p38 (Melani et al., 2006). Thus, the present results indicate that A_{2A}Rs control A β -induced neurotoxicity through control of p38 MAPK phosphorylation. However, this conclusion derives solely from *in vitro* studies and remains to be confirmed *in vivo*.

In summary, the present observations that blockade of A_{2A}Rs prevents the early synaptotoxicity in both *in vitro* and *in vivo* models pertinent to AD, strengthen the interest of exploring the prophylactic and therapeutic potential of A_{2A}R antagonists, which are about to be introduced into clinical practice as novel antiparkinsonian drugs (Schwarzschild et al., 2006).

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