Alzheimer’s disease (AD) is characterized by memory impairment, neurochemically by accumulation of β-amyloid peptide (Aβ) and morphologically by an initial loss of nerve terminals. Caffeine consumption prevents memory dysfunction in different models, which is mimicked by antagonists of adenosine A2A receptors (A2ARs), which are located in synapses. Thus, we now tested whether A2AR blockade prevents the early Aβ1-42-induced synaptotoxicity and memory dysfunction and what are the underlying signaling pathways. The intracerebral administration of soluble Aβ1-42 (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 A2AR in mice. Moreover, these were synaptic events since purified nerve terminals acutely exposed to Aβ1-42 (500 nM) displayed mitochondrial dysfunction, which was prevented by A2AR blockade. SCH58261 (50 nM) also prevented the initial synaptic toxicity and memory dysfunction (loss of MAP-2, synaptophysin, and SNAP-25 immunoreactivity) and subsequent loss of viability of cultured hippocampal neurons exposed to Aβ1-42 (500 nM). This A2AR-mediated control of neurotoxicity involved the control of Aβ1-42-induced p38 phosphorylation and was independent from cAMP/PKA (protein kinase A) pathway. Together, these results show that A2ARs play a crucial role in the development of Aβ-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.
(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-malate (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$\beta_{42}$-induced synaptotoxicity and memory impairment and investigated the underlying mechanisms. Results show that A$_{2A}$R blockade (pharmacologic or genetic) prevents A$\beta_{42}$-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 British Columbia, Vancouver, BC, Canada) and were maintained under controlled environment (22 ± 2°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$\beta_{42}$) or the nonamyloidogenic reverse peptide A$\beta_{40}$ (A$\beta_{42}^{-}$) was dissolved in water at a concentration of 2.25 mg/ml and 2 nmol in 4 µl was administered intracerebroventriculectomically, as previously described (Dall’Igna et al., 2007). Control animals were intracerebroventriculectomically infused with a similar volume of water. Behavioral analysis was performed 2 or 15 d after A$\beta_{42}$ or A$\beta_{42}^{-}$ administration. The selective A$_{2A}$R antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-$d$]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), in saline (0.9% sodium chloride) with 10% dimethylsulfoxide, applied daily starting 30 min before A$\beta_{42}$ administration. Control animals were injected intraperitoneally with saline with 10% dimethylsulfoxide, applied daily starting 30 min before A$\beta_{42}$ administration. Control animals were injected intraperitoneally with saline with 10% dimethylsulfoxide, applied daily starting 30 min before A$\beta_{42}$ administration.

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 (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 a Coomassie blue R-250 solution made of 40% methanol and 10% acetic acid) or with 6E10 antibody (1:1000 dilution; Covance), as described below (see Western blot analysis).

The A$\beta_{42}$ levels in the hippocampus were quantified using two ELISA kits (Invitrogen), one detecting A$\beta_{42}$ (and isoforms with lower proportion) and the other A$\beta_{40}$, 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% IGEPA), 0.2% SDS, and protease inhibitor mixture containing leupeptin, pepstatin A, chymostatin, and aprotonin, all 1 mg/ml from Sigma-Aldrich). The mixture was centrifuged (30 min at 27,000 × g) and the supernatant was stored at −80°C until ELISA quantifications, which were performed following the manufacturer’s instructions. A$\beta_{42}$ levels were estimated by subtracting the estimated amount of A$\beta_{40}$ from those of A$\beta_{42}$, 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 (Puchelt 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 × 50 cm, divided in four squares of 25 cm for rats, and 30 × 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 habituation sessions: 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 were 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 three times with acetate buffer twice in 100% ethanol, twice in xylene, and mounted with Vector medium (Vector Laboratories). Deenerating 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 astocytes) (Pekny and Nilsson, 2005) was performed to evaluate microgliosis and astrogliosis, respectively. The sections were first rinsed for 5 min with PBS (140 mM NaCl, 3 mM KCl, 20 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$) and then three times for 5 min with Trizma base solution (TBS) (0.05 µM 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...
As previously described (Silva et al., 2007), viable neurons present nuclei homogenously 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-synaptophysin SNAP-25 (1:200; Sigma-Aldrich) or mouse anti-synaptophysin SNAP-25 (1:200; Sigma-Aldrich) or mouse anti-synaptophysin 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; 1:2000; 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, 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 1/10 vol of 6X 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 antibodies used) and stored at −20°C. Membranes were then incubated overnight at 4°C with primary antibodies, namely mouse anti-synaptophysin (1:5000–20,000), mouse anti-synaptophysin 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 ECFC 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 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 (Milllex-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 μM).

Statistical analysis. Results are presented as mean ± 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 Aβ1-42-induced memory impairment and morphological modifications

Western blot analysis of the Aβ1-42 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 Aβ1-42 (2 nmol) led to an accumulation of Aβ1-42 in the hippocampus (91.1 ± 25.1 pg/mg of protein; n = 4, p < 0.05), indicating that 11.3 ± 2.9% of the total amount of administered Aβ1-42 accumulated in hippocampal tissue after 2 d. The hippocampal Aβ1-42 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 Aβ1-42 levels at d 2).

The intracerebroventricular administration of Aβ1-42 (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 modify synaptophysin immunoreactivity (data not shown). The control peptide (Aβ2-12; 2 nmol) changed neither Y-maze behavior nor locomotor activity (n = 4) (data not shown). This indicates that Aβ1-42 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 Aβ1-42 since we only found soluble Aβ1-42 and no evidence of the presence of Aβ aggregates 15 d after the intracerebroventricular administration of Aβ1-42 (Fig. 1C).

Histological analysis of hippocampal sections, 2 weeks after the injection of Aβ1-42, 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 Aβ1-42 administration. Further excluding acute toxic effects of Aβ1-42 administration, there was no difference of cresyl violet or Fluoro-Jade staining 2 d after the injection of Aβ1-42 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 Aβ1-42 injection (Fig. 2C), which was confirmed by quantitative Western blot analysis. As illustrated in Figure 2D, synaptophysin immunoreactivity was lower (−25.7 ± 4.3%; n = 7; p < 0.001) in hippocampal membranes collected from rats 15 d after Aβ1-42 administration when compared with controls. In contrast, the nonamyloidogenic Aβ42-1 peptide failed to modify synaptophysin immunoreactivity (data not shown).

Pharmacological blockade of adenosine A2A receptor protects from Aβ1-42-induced synaptotoxicity and memory impairment

We then tested whether the blockade of A2A-Rs prevented the loss of synaptic markers and memory impairment observed 2 weeks after the intracerebroventricular administration of Aβ1-42. For that purpose, we used a selective A2A 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 Aβ1-42. In fact, synaptophysin immunoreactivity in hippocampal sections was indistinguishable in control conditions and in Aβ1-42-injected rats that were treated daily with SCH58261 (Fig. 2C). Accordingly, Western blot analysis confirmed that the decrease in synaptophysin density on Aβ1-42 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 Aβ1-42 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 Aβ1-42-treated rats (data not shown).
Genetic inactivation of A2A 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, i.c.v.) or water (control), the A2AR antagonist SCH58261 (0.05 mg/kg, i.p.) was administered daily starting 30 min before Aβ1, 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 (Aβ1), SCH58261 (SCH), and Aβ1 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).

Blockade of A2A receptor protects hippocampal neurons from Aβ1-42-induced toxicity

To investigate the mechanism involved in the A2AR-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 Aβ1-42 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 ± 3.7%; n = 5; p < 0.001) (Fig. 5A) and the increased number of apoptotic-like neurons (9 ± 2%; n = 5; p < 0.001) (Fig. 5B), indicating a time-dependent evolving profile of Aβ1–42-induced neurodegeneration. As occurred in vivo and in native brain preparations, this Aβ1–42-induced neurotoxicity was prevented by the A2AR 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 Aβ1–42 caused an initial synaptotoxicity preceding neuronal death. Since we observed that neurons incubated for 12 h with Aβ1–42 did not display loss of viability or damage, we evaluated whether Aβ1–42-induced synaptotoxicity would be present after 12 h of exposure to Aβ1–42, 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 Aβ1–42 (i.e., at the time when neuronal damage is not yet present) (Fig. 5). To quantify this Aβ1–42-induced synaptotoxicity, we used Western blotting analysis, which showed a decrease in the density of synaptophysin (−30.3 ± 7.5%; n = 6; p < 0.05) and SNAP-25 (−37.0 ± 6.6%; n = 6; p < 0.05) on exposure to Aβ1–42. As occurred in vivo, this initial and evolving Aβ1–42-induced synaptotoxicity in neuronal cultures was also prevented by A2AR blockade with the selective A2AR antagonist, SCH58261 (50 nM) (Fig. 6A; supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

This observation that SCH58261 prevents Aβ1–42-induced neurotoxicity but is devoid of effects in controls suggests that the levels of extracellular adenosine might be increased on exposure to Aβ1–42, 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 Aβ1–42 (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 A2AR receptor blockade against Aβ1–42-induced neurotoxicity**

Since one of main transducing systems operated by A2ARs involves cAMP/protein kinase A (PKA) pathway (Fredholm et al., 2005), we investigated whether the neuroprotective effects afforded by SCH58261 involved this pathway. As observed in the Figure 7A, the manipulation of the cAMP/PKA pathway influences Aβ1–42-induced neurotoxicity, as described by others (Parvathenani 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 μM) attenuated Aβ1–42-induced neurotoxicity, an effect prevented by the PKA inhibitor H-89 (1 μM) (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 A2ARs (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
independent hippocampal cultures; *p < 0.05).

**Figure 4.** Exposure to Aβ1-42 directly decreases the function of rat hippocampal synaptosomes, which is prevented by blockade of adenosine A2A receptors. Synaptosomes were incubated for 2 h with 500 nM Aβ1-42 or Krebs’ buffer, in the absence or presence of the A2A 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 Δψ (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 A2A receptors. Hippocampal neurons were preincubated with the A2A 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** and **B.** Aβ1-42-induced neuronal death is time dependent. **C** and **D.** Blockade of A2A 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.

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, A2A receptors, 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 synaptoxic dysfunction is a precocious core modification of AD; (2) they provide additional evidence that A2A receptors, 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 A2A 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 astroglial nor microglialosis 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 causes first a synaptotoxicity (Roselli et al., 2005; Cabreese 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 synapsosomal 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 synthetically in AD patients (Takahashi 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 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 A2A receptors 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 A2A-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, A2A 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).
of synaptic markers, modification of the viability of nerve terminals (synaptosomes), and degeneration of synapses, which we collectively call synaptotoxicity; however, it remains to be determined to what extent this synaptotoxicity relates to the known Aβ-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 A2A Rs (pharmacological or genetic inactivation) simultaneously prevents synaptotoxicity and memory impairment caused by Aβ administration]. Furthermore, the initial synaptotoxicity that precedes overt neuronal damage on exposure of cultured neurons to Aβ was also prevented by A2A R blockade. Finally, the direct Aβ-induced impairment of nerve terminal function was also prevented by A2A R blockade. All these observations are in agreement with the predominant synaptic localization of A2A Rs in cortical regions (Rebola et al., 2005). These synaptic A2A 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 A2A 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 A2A Rs to control memory impairment should be particularly evident when synaptotoxicity is involved. Accordingly, we have previously shown that A2A R blockade can prevent memory impairment caused by Aβ, 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., 2006; Silva et al., 2007) that relates to the known A1Rs (for review, see Cunha, 2008b; Takahashi et al., 2008). A1Rs (Dall'Igna et al., 2003, 2007). Thus, it is tempting to
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 A₂A R blockade. This proposal does not exclude other possible concurring mechanisms by which caffeine may afford protection in AD, such as control of Aβ 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 A₂A Rs in controlling Aβ-induced neurotoxicity, it does not exclude the possibility that other mechanisms may also contribute for neuroprotection against Aβ-induced neurotoxicity and memory impairment.

Finally, this study demonstrates that neuroprotection resulting from A₂A R blockade does not involve the cAMP/protein kinase A transducing system but instead depends on control of p38 MAPK. In fact, the mechanisms by which A₂A 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 A₂A 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 A₂A R control of neurodegeneration since enhanced cAMP levels afford neuroprotection against Aβ-induced neurotoxicity (Parvathenani et al., 2000; Gong et al., 2004; Shrestha et al., 2006), whereas it is A₂A R blockade (expected to decrease cAMP levels) that affords neuroprotection. Accordingly, neuroprotection afforded by A₂A R blockade against Aβ-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 Aβ triggered activation of both JNK and p38 MAPKs. Interestingly, we observed that A₂A R blockade prevented the Aβ-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 Aβ-induced neurotoxicity, as also observed by others (Zhu et al., 2005; Muñoz et al., 2007; Origlia et al., 2008), this indicates that A₂A Rs signal through p38 MAPK to control neurodegeneration. Indeed, previous studies have documented the ability of A₂A 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 A₂A Rs of the ischemia-induced brain damage was related to the ability of A₂A R antagonists to blunt the ischemia-induced accumulation of phosphorylated forms of p38 (Melani et al., 2006). Thus, the present results indicate that A₂A Rs control Aβ-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 A₂A 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 A₂A R antagonists, which are about to be introduced into clinical practice as novel antiparkinsonian drugs (Schwarzchild et al., 2006).
References


