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RESEARCH PAPER

Brain-derived neurotrophic factor mediates neuroprotection against Aβ-induced toxicity through a mechanism independent on adenosine 2A receptor activation

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Abstract

Brain-derived neurotrophic factor (BDNF) promotes neuronal survival through TrkB-FL activation. The activation of adenosine A_{2A} receptors ($A_{2A}R$) is essential for most of BDNF-mediated synaptic actions, such as synaptic plasticity, transmission and neurotransmitter release. We now aimed at evaluating the $A_{2A}R$ influence upon BDNF-mediated neuroprotection against $A\beta_{25-35}$ toxicity in cultured neurons. Results showed that BDNF increases cell survival and reduces the caspase-3 and calpain activation induced by amyloid-β (Aβ) peptide, in a mechanism probably dependent on PLCγ pathway. This BDNF-mediated neuroprotection is not affected by $A_{2A}R$ activation or inhibition. Moreover neither activation nor inhibition of $A_{2A}R$, *per se*, significantly influenced Aβ-induced neuronal death on calpain-mediated cleavage of TrkB induced by Aβ. In conclusion, these results suggest that, in opposition to the fast synaptic actions of BDNF, the neuroprotective actions of this neurotrophin against a strong Aβ insult do not require the activation of $A_{2A}R$.

Keywords

Adenosine, amyloid-β, BDNF, TrkB, calpain, caspase-3

History

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Introduction

Amyloid-β (Aβ) peptide is the main component of amyloid plaques present in the brain of Alzheimer's disease (AD) patients (Glenner & Wong, 1984). Consequently, multiple studies have been conducted to clarify the role of Aβ in neurodegeneration associated to AD. Indeed, data has shown that Aβ has vast toxic effects that ultimately culminate in synaptic dysfunction and neuronal death (Pike et al., 1995; Walsh et al., 2002).

In the brain of AD patients, the levels of the brain-derived neurotrophic factor (BDNF) and of its full length TrkB receptor (TrkB-FL) are decreased comparing to age matched controls (Allen et al., 1999; Connor et al., 1997; Ferrer et al., 1999; Holsinger et al., 2000; Peng et al., 2005). BDNF is an important mediator of endogenous neuroprotection through the activation of TrkB-FL, which is known to regulate neuronal survival, differentiation, and plasticity (Huang & Reichardt, 2001). On contrary, the truncated TrkB receptor, which is a dominant-negative modulator of TrkB-FL (Eide et al., 1996), is increased in AD patients brain (Ferrer et al., 1999). Given the impairment on BDNF signaling in AD, the administration of this neurotrophin directly into the brain was considered a possible therapeutic approach. Indeed, *in vivo* administration of BDNF to animals models of AD and Parkinson's disease (PD), produced beneficial effects with improved synaptic and cognitive function and reduced neurodegeneration (Lu et al., 2013; Rodrigues et al., 2014). However, despite the encouraging results from pre-clinical studies, the results from the BDNF-based clinical trials conducted so far (four in amyotrophic lateral sclerosis and one in diabetic neuropathy) have shown inconclusive results. BDNF-based therapies have been hampered by multiple technical difficulties, such as the low penetrance of BDNF through blood–brain barrier and its rapid *in vivo* clearance with consequent low half-life time in plasma or CSF (Lu et al., 2013). Given these issues, an effort has been made to find small molecules that can activate directly the TrkB-FL, or that can indirectly boost BDNF actions in the brain. It has been shown, at molecular level, that activation of $A_{2A}R$ is able to transactivate a pool of immature TrkB receptors in the absence of BDNF (Lee & Chao, 2001; Rajagopal et al., 2004), and to induce the translocation of TrkB into lipid rafts microdomains in the membrane (Assaife-Lopes, 2013). Functionally, it has been shown that most BDNF-mediated synaptic actions, such as in synaptic plasticity, transmission and neurotransmitter release, are fully dependent on $A_{2A}R$ activation (Diógenes et al., 2004; Fontinha et al., 2008; Jerónimo-Santos et al., 2014; Pousinha et al., 2006; Rodrigues

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et al., 2014; Tebano et al., 2008). However, some BDNF-mediated actions are not dependent on $A_{2A}R$ activation (Sebastião et al., 2011). In this work, we evaluated whether the neuroprotective effect of BDNF upon Aβ-induced neuronal death was affected by pharmacological modulation of $A_{2A}R$.

Experimental procedure

Materials

Unless stated otherwise all reagents were purchased from Sigma (St. Louis, MO). Culture reagents and supplements were from Gibco (Paisley, UK). Recombinant human-met-BDNF was provided by Regeneron Pharmaceuticals (Tarrytown, NY). CGS21680, SCH58261, U73122, U0126 and LY294002 were purchased from Tocris Bioscience (Bristol, UK). The $A\beta_{25–35}$ peptide was purchased from Bachem (Bubendorf, Switzerland) and the stock solutions were performed in MilliQ water to a final concentration of 1 mg/mL which gives rise to a heterogeneous population exhibiting protofibrilar and fibrilar Aβ species, as previously shown (Kemppainen et al., 2012). The anti-pan-TrkB mouse monoclonal antibody (1:1500), raised against the extracellular domain of human TrkB (aa. 156–322), was purchased from BD Bioscience (Franklin Lakes, NJ). The anti-Trk-FL rabbit polyclonal antibody (1:2000), raised against the C-terminus (C-14), the anti-αII-spectrin (C-3) mouse monoclonal antibody (1:2500), raised against human αII-spectrin (aa. 2368–2472), and the anti-pan-Caspase-3 (H-277) rabbit polyclonal antibody (1:1000) and the IgG-horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The anti-GAPDH (6C5) mouse monoclonal antibody (1:5000) was purchased from Thermo Fisher Scientific (Waltham, MA) and the α-tubulin rabbit polyclonal antibody (1:5000) was purchased from Abcam (Cambridge, UK).

Primary neuronal cultures and drug incubations

Neurons were isolated from fetuses of 18-day pregnant Sprague–Dawley females. The fetuses were collected in Hanks' balanced salt solution (HBSS-1) and brains were rapidly removed. The cerebral cortices were isolated and mechanically fragmented. Further tissue digestion was performed with 0.025% (wt/vol) trypsin solution in HBSS without Ca^{2+} and Mg^{2+} (HBSS-2) for 15 min at 37 °C. After trypsinization, cells were washed and resuspended in neurobasal medium supplemented with 0.5 mM L-glutamine, 25 mM glutamic acid, 2% B-27 and 25U/mL penicillin/streptomycin. Cells were plated at a density of 7×10^4 cells/cm^2, on 10 μg/ml poly-D-lysine-coated dishes, and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Incubations with BDNF (20 ng/mL) and Aβ peptide (25 μM) were performed at 7 DIV for 24 h. In the experiments where inhibitors, agonists or antagonists were used, the drugs were applied 30 min before Aβ or BDNF treatment. All the data were collected at 8 DIV.

Western-blot

For neuronal cultures, cells were washed with ice cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$ and 1.5 mM KH_2PO_4, pH 7.4) and lysed with 1% NP-40 lysis buffer containing (in mM): 50 Tris-HCl (pH 7.5), 150 NaCl, 5 ethylenediamine tetra-acetic acid (EDTA), 2 dithiothreitol (DTT) and protease inhibitors (Roche, Penzberg, Germany). In experiments were rat hippocampi were homogenized, it was used a Radio-Immunoprecipitation Assay (RIPA) buffer containing: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethylenediamine tetra-acetic acid (EDTA), 0.1% SDS and 1%Triton X-100 and protease inhibitors cocktail (Roche, Penzberg, Germany). Cell lysates or homogenates were clarified by centrifugation (16,000 *g*, 10 min) and the amount of protein in the supernatant was determined by Bio-Rad DC reagent. All samples were applied with same amount of total protein and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (GE Healthcare, Buckinghamshire, UK). Membranes were stained with Ponceau S solution to check for protein transference efficacy. After blocking with a 5% non-fat dry milk solution in TBS-T (20 mM Tris base, 137 mM NaCl and 0.1% Tween-20), membranes were incubated with the primary (overnight at 4 °C) and secondary antibodies (1 h at room temperature). Finally, immunoreactivity was visualized using ECL chemiluminescence detection system (Amersham-ECL Western Blotting Detection Reagents from GE Healthcare, Buckinghamshire, UK) and bands intensities were quantified by *ImageJ 1.45* software. The band intensities were normalized to the correspondent α-tubulin or GAPDH bands (loading control).

Cell death evaluation

Viability of 8 DIV primary cortical neurons cells was evaluated by the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide (Sigma, St. Louis, MO). MTT was added to the original culture medium and incubated for 3 h. After incubation, the converted die was solubilized with dimethyl sulfoxide (DMSO, Merck, Kenilworth, NJ). Absorbance of converted die was measured at 570 nm with background subtraction at 650 nm.

To specifically evaluate the degree of cell death induced by apoptosis it was performed a terminal transferase-mediated dUTP-fluorescein nick end-labeling (TUNEL) assay. Briefly, cells were fixed in methanol for 10 min at 4 °C, and fluorescein-nucleotide residues were added to the 3′-OH ends of double- or single-stranded DNA by terminal deoxynucleotidyl transferase. Reactions were performed according to manufacturer's recommendations (Roche, Penzberg, Germany) and the specimens were then directly analyzed by epifluorescence microscopy. Three random microscopic fields per sample of approximately 30 nuclei were counted with DAPI staining and mean values expressed as the percentage of apoptotic nuclei. Moreover, the activity of caspase-3, a major player in the apoptotic process, was also evaluated by enzymatic cleavage of p-nitroanilide chromophore (pNA) from the substrate *N*-acetyl-Asp-Glu-Val-Asp (DEVD)-pNA (Sigma, St. Louis, MO). The proteolytic reaction was preceded in lysis buffer containing 50 μg of total protein from cell lysates and 50 μM DEVD-pNA. The reaction mixtures were incubated at 37 °C for 1 h, and the

release of pNA was determined by measuring absorbance at 405 nm using a 96-well plate reader. The protein levels of active caspase-3 (17-kDa) were also evaluated by western-blot using a pan-caspase-3 antibody. Extension of neuronal degeneration was also evaluated by the αII-spectrin breakdown, a cytoskeletal protein that is cleaved by calpains and caspase-3 forming, respectively, a 150 and 120 kDa spectrin-breakdown products (SBDPs).

Statistical analysis

The data are expressed as mean ± SEM of the n number of independent experiments (from different cultures). To perform multiple comparisons between the means of more than two conditions a one-way ANOVA followed by a Bonferroni post-test was performed. Values of $p < 0.05$ were considered to represent statistically significant differences. *Prism GraphPad* software (La Jolla, CA) was used for statistical analysis.

Results

BDNF increases cell viability against Aβ by reducing the activation of caspase-3 and calpain

In this work, we evaluated the protective effect of BDNF against Aβ toxicity by using the MTT and TUNEL assays and by evaluating caspase and calpain activation. Our results showed that cell viability, evaluated by the metabolism of MTT, decreased in 8 DIV primary cortical neurons incubated with $A\beta_{25-35}$ (25 μM) by approximately 50%, when compared to control ($p < 0.001$, $n = 6$, Figure 1A). Remarkably, when neurons were co-incubated with BDNF (20 ng/ml) and $A\beta_{25-35}$ (25 μM), cell viability was restored to almost control levels ($p < 0.05$, $n = 5$). These results are similar to those obtained by Arancibia et al. (2008), showing that BDNF protects neurons against 48 h of Aβ incubation using the MTT assay. Moreover, results from MTT assays also showed that BDNF is neuroprotective against Aβ in PC12 cells

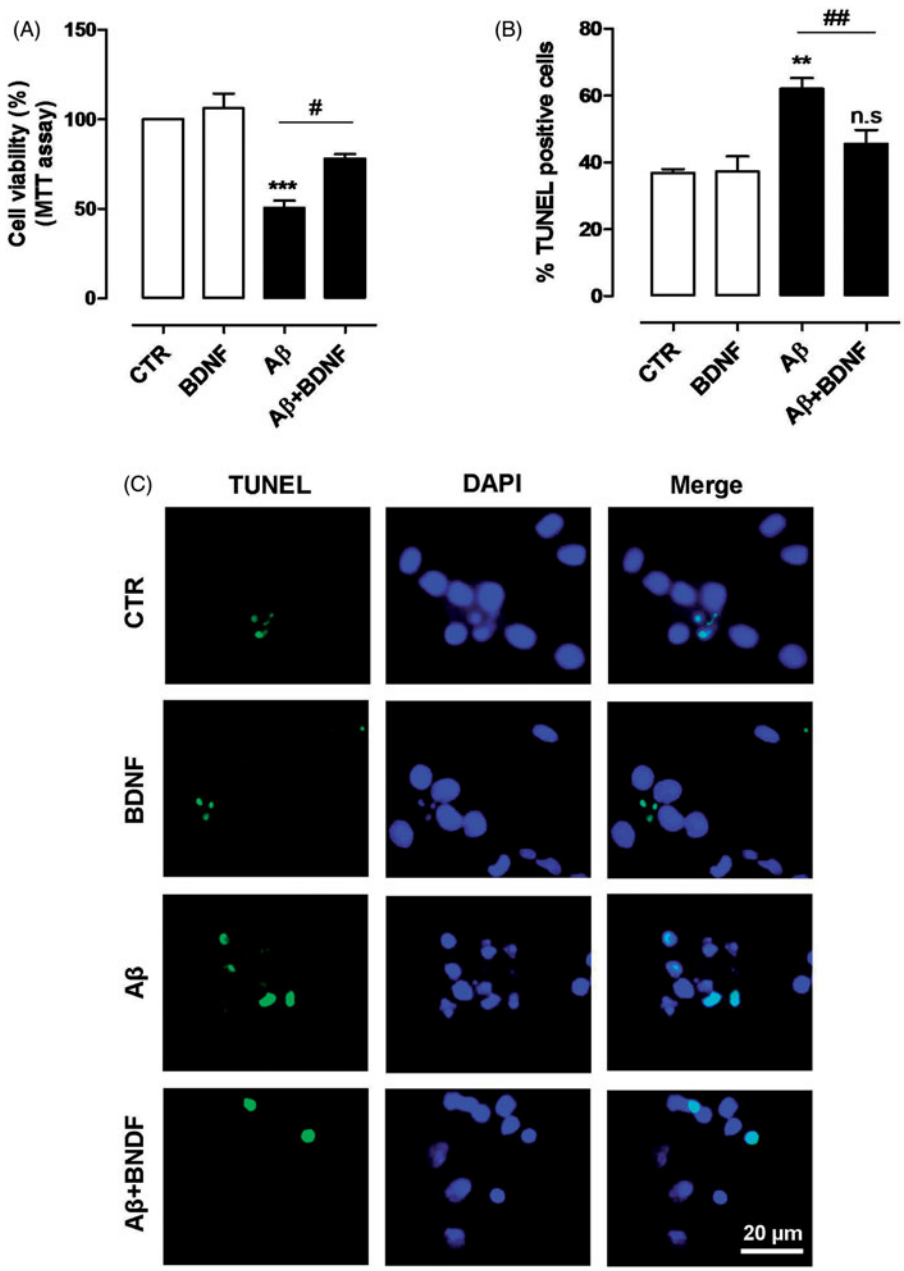

Figure 1. BDNF is neuroprotective against Aβ. (A) Percentage of viable neurons, capable of performing the MTT metabolism. ***$p < 0.001$ when comparing to control and #$p < 0.05$ when comparing to Aβ ($n = 6$ and $n = 5$, respectively, ANOVA followed by Bonferroni post-test). (B) Percentage of apoptotic TUNEL-positive cells. **$p < 0.01$ and n.s. (not significant) when comparing to control and ##$p < 0.01$ when comparing to Aβ ($n = 6$, ANOVA followed by Bonferroni post-test). (C) Representative images of TUNEL-positive cells (green) and total cells (DAPI) untreated or treated with $A\beta_{25-35}$, in the presence of absence of BDNF. Scale bar: 20 μm. Data represented are mean ± SEM in n independent experiments.

(Sun et al., 2012). Accordingly, the percentage of TUNEL-positive apoptotic cells increased approximately 68%, from 37% to 62% stained cells upon $A\beta_{25–35}$ treatment ($p<0.01$, $n=6$, Figure 1B and C). The percentage of TUNEL-positive cells was reduced to 46% when cells were co-incubated with BDNF ($p<0.01$, $n=6$). Thus, these results suggest that BDNF is capable of efficiently increase neuronal viability induced by $A\beta_{25–35}$.

We also evaluated the activation of caspase-3, which is a central mediator of apoptotic cell death, and calpains activation, that plays a central role in necrotic cell death (Wang, 2000). Indeed, calpains rather than caspases have a prominent role in *in vivo* excitotoxic neuronal death (Higuchi et al., 2005). Moreover, calpains also play a central role in the execution of apoptotic cellular death (Momeni, 2011; Smith & Schnellmann, 2012). Accordingly, multiple evidences have shown the involvement of calpains in several neurodegenerative processes (Camins et al., 2006), and its inhibition has shown beneficial effects including in the Aβ-induced neurodegeneration in rats *in vivo* (Granic et al., 2010). Thus, in neurons under degenerative conditions, caspases and calpains contribute to cellular architecture derangement and functional loss (Wang, 2000).

Incubation of 7 DIV cortical neurons with $A\beta_{25–35}$ (25 μM) for 24 h induced a robust activation of caspase-3, as evaluated by enzymatic activity assays and by western-blot (2.5-fold increase in caspase-3 activity and five-fold increase in active 17-kDa caspase-3 protein levels, respectively, when compared to control, $p<0.001$, $n=5$, Figure 2). Conversely, Aβ induced a three-fold increase in the levels of the caspase-3 specific 120 kDa αII-spectrin breakdown product, SBDP120 ($p<0.001$, $n=9$, Figure 3C and D). As expected, Aβ also induced a very robust increase in SBDP150 levels indicating the activation of calpains ($p<0.001$, $n=9$, Figure 3B and D). When BDNF (20 ng/mL) was simultaneously incubated with $A\beta_{25–35}$ (25 μM), the caspase-3 activity and protein levels were significantly reduced by $39\pm7\%$ and $41\pm8\%$, respectively, when compared to $A\beta_{25–35}$ ($p<0.01$, $n=5$, Figure 3). Moreover, BDNF significantly reduced the Aβ-induced formation of SBDP120 and SBDP150, by $57\pm8\%$ and $51\pm6\%$, respectively ($p<0.001$ when compared to Aβ, $n=9$, Figure 3B–D). As a consequence of the reduction of both calpain and caspase-3 activity, BDNF also reduced the Aβ-induced breakdown of αII-spectrin by $56\pm11\%$ ($p<0.01$, $n=9$, Figure 3A and D). The caspase-3 and αII-spectrin levels were not affected by BDNF alone when compared to control (Figures 2 and 3).

To evaluate the contribution of each canonical signaling pathways of BDNF/TrkB to the observed neuroprotective effect, we pre-treated the neurons with selective inhibitors of ERK1/2 (U0126, 10 μM), PI3K (LY294002, 10 μM) or PLCγ (U73122, 4 μM), for 30 min prior to Aβ or BDNF incubation. The results from αII-spectrin breakdown show that the inhibition of ERK and PI3K did not influence the BDNF-mediated neuroprotection against Aβ, which indicates that those signaling pathways are not required for its protective effect (Figure 4, $n=5$) suggesting that PLCγ pathway might be involved. However, the inhibition of this pathway, by U73122, induced spectrin breakdown similarly to Aβ thus occluding the effect of Aβ when incubated simultaneously (Figure 4, $n=5$). Thus, although the abolishment of BDNF effect only observed by PLCγ inhibition, the fact that U73122 *per se* induces cell toxicity masking Aβ toxicity, precludes the

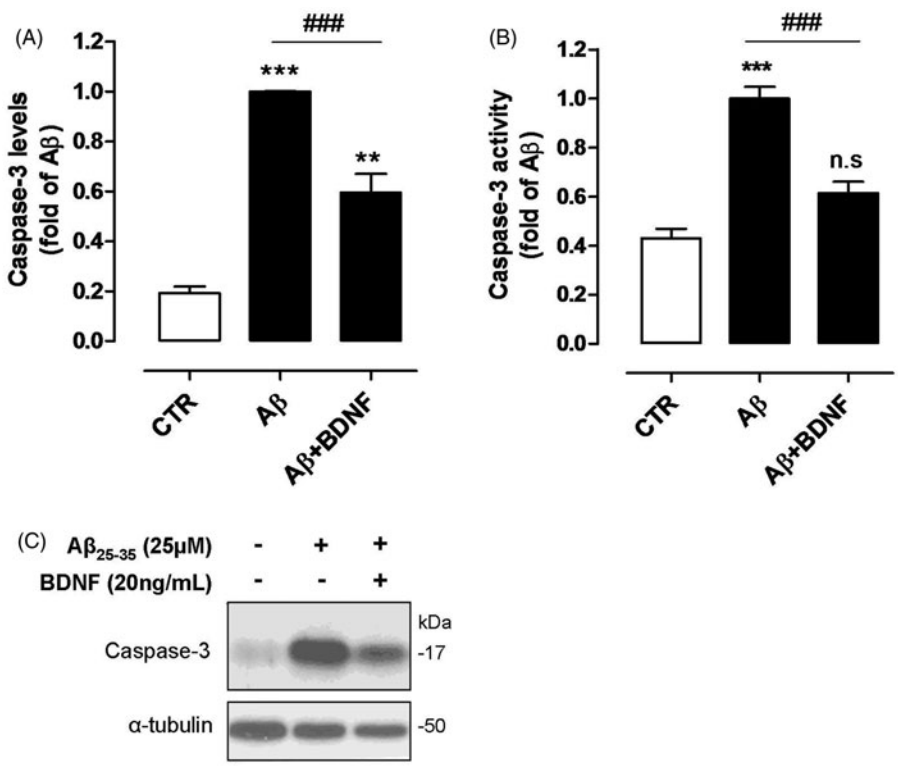

Figure 2. BDNF reduces the caspase-3 activation induced by Aβ. (A) Quantification of active Caspase-3 protein levels and (B) Caspase-3 activity, in 8 DIV cortical neurons non-treated or treated with $A\beta_{25–35}$ (25 μM) for 24 h in the absence or presence of BDNF (20 ng/mL). Data is normalized to Aβ condition given the very low, almost undetectable, caspase activation in control condition. ***$p<0.001$, **$p<0.01$, n.s (not significant) when comparing to control and ###$p<0.001$ when compared to Aβ ($n=5$, ANOVA followed by Bonferroni post-test) (C) Representative western-blot used in (A), showing the protein levels of active Caspase-3 (17-kDa). α-Tubulin was used as a loading control. Data represented are mean ± SEM of n independent experiments.

final conclusion that this pathway is the mediator of BDNF neuroprotective effect against Aβ.

Together, these results show that BDNF significantly reduces the activation of caspase-3 and calpain induced by Aβ, in a mechanism probably dependent on PLCγ activity.

Neuroprotective effect of BDNF does not require $A_{2A}R$ activation

To address if the activation of $A_{2A}R$ is required for BDNF-mediated neuroprotection, as it is verified for most synaptic actions mediated by this neurotrophin, 7 DIV cortical neurons were incubated with $A\beta_{25–35}$ (25 μM) and BDNF (20 ng/ml) for 24 h, in the presence or in the absence of the $A_{2A}R$ antagonist, SCH58261 (100 nM), or the $A_{2A}R$ agonist, CGS21680 (10 nM). Results show that neither SCH58261 nor CGS21680, added 30 min prior of Aβ and BDNF, influenced the BDNF-mediated reduction of caspase-3 activity and levels upon Aβ toxicity (Figure 5A and B, $n = 6$). Regarding αII-spectrin breakdown, similar results were obtained. Indeed, the reduction of αII-spectrin cleavage and correspondent reduction on SBDP150 and SBDP120 formation (cleavage product mediated by calpains and caspases, respectively) induced by BDNF, in cells incubated simultaneously with Aβ, was not influenced by the presence of both $A_{2A}R$ antagonist and agonist (Figure 6, $n = 6$). *Per se*, the incubation with SCH58261 (100 nM) or CGS21680 (10 nM) did not influence the Aβ-induced activation of caspase-3 ($n = 4$, $p > 0.05$, Figures 5 and 6C) or calpain ($n = 6$, Figure 6A, B and D).

Aβ-induced TrkB truncation is not influenced by $A_{2A}R$ activation

As previously shown, Aβ induces a calpain-mediated truncation on TrkB-FL receptor, with concomitant formation of an intracellular fragment (TrkB-ICD) and an increase on truncated receptor (TrkB-Tc) levels (Jerónimo-Santos et al., 2015). As we showed above, $A_{2A}R$ do not influence calpain activation induced by Aβ. However, given that $A_{2A}R$ activation is able to induce the translocation of TrkB receptors into lipid rafts microdomains (Assaife-Lopes et al., 2013), we considered noteworthy to test the hypothesis that $A_{2A}R$ might protect TrkB receptors from calpain-mediated cleavage, by allocating them in a different membrane subdomain.

To test this hypothesis, the levels of TrkB-FL, TrkB-Tc and TrkB-ICD were analyzed in 8 DIV cortical cultures treated with $A\beta_{25–35}$ (25 μM), in the presence or absence of SCH58261 (100 nM) or CGS21680 (10 nM).

The results show that, upon Aβ incubation the $A_{2A}R$ agonist, did not protect the TrkB-FL receptor from the Aβ-induced calpain-mediated cleavage ($p < 0.05$, $n = 6$, Figure

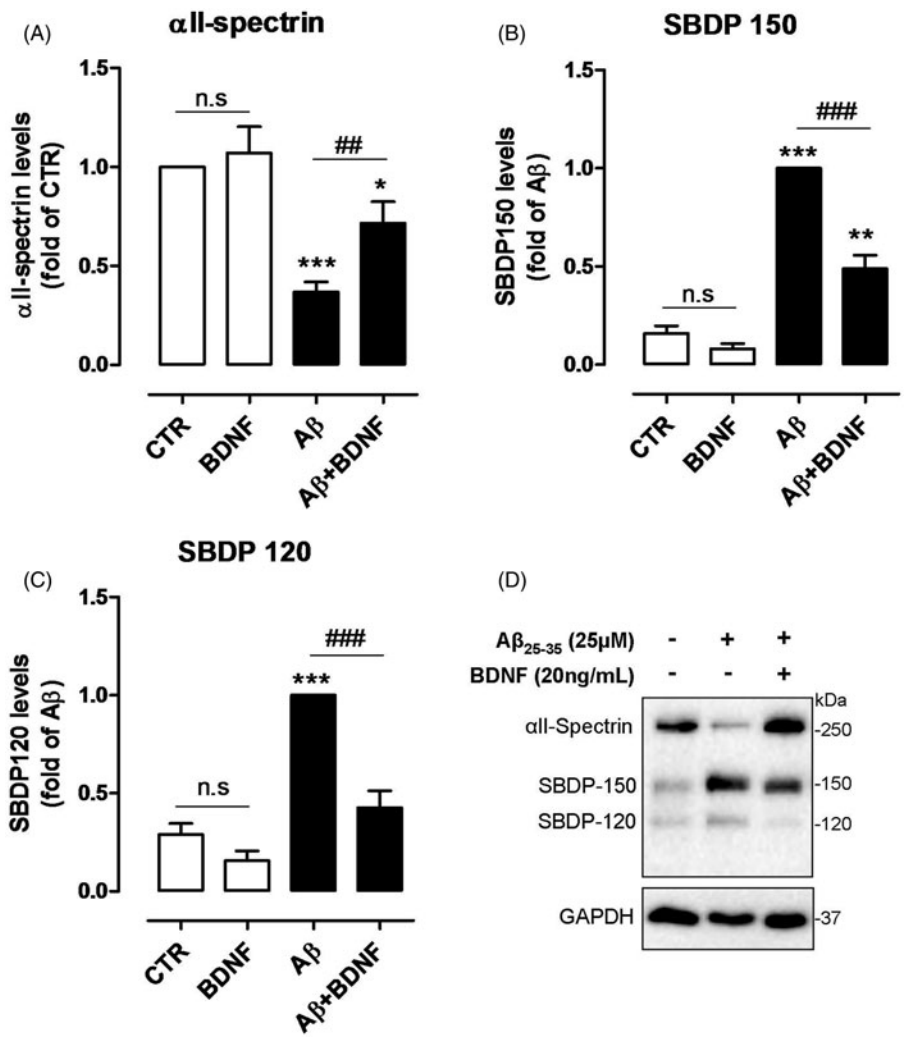

Figure 3. BDNF reduces the αII-spectrin breakdown induced by Aβ. (A) Quantification of αII-spectrin breakdown with consequent formation of (B) SBDP150 and (C) SBDP120 breakdown products. SBDP150 and SBDP120 levels are normalized to Aβ condition given their almost undetectable amount in control conditions. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s (not significant) when comparing to control and ##$p < 0.01$ and ###$p < 0.001$ when comparing to Aβ ($n = 9$, ANOVA followed by Bonferroni post-test) (D) Representative western-blot used in (A–C), showing the protein levels of αII-spectrin, SBDP150 and SBDP120. GAPDH was used as a loading control. Data represented are mean ± SEM of n independent experiments.

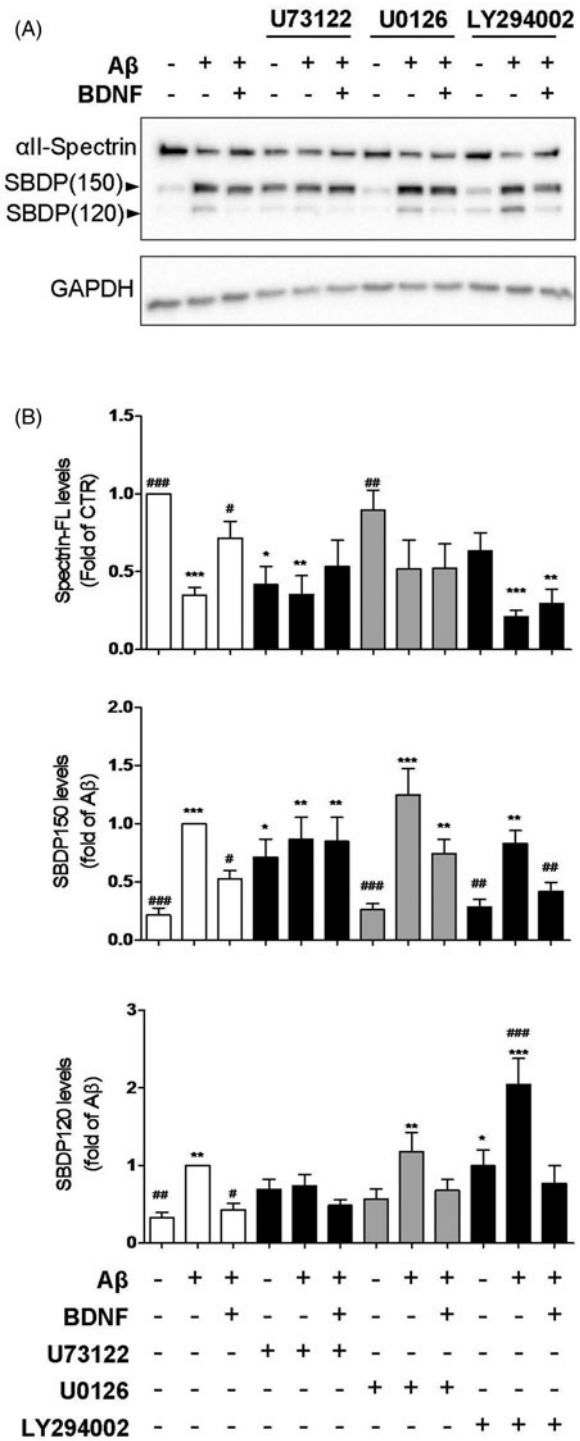

Figure 4. Inhibition of PLCγ abolishes the BDNF protection against Aβ toxicity. (A) Representative western-blot showing the protein levels of αII-spectrin, SBDP150 and SBDP120. GAPDH was used as a loading control. (B) Quantification of αII-spectrin, SBDP150 and SBDP120 levels for the indicated drug incubations. ***$p<0.001$, **$p<0.01$, *$p<0.05$, n.s (not significant) when comparing to control and ##$p<0.01$ and ###$p<0.001$ when comparing to Aβ ($n=5$, ANOVA followed by Bonferroni post-test). Data represented are mean ± SEM of n independent experiments.

7A–D). Conversely, the $A_{2A}R$ antagonist did not change the magnitude of TrkB cleavage induced by Aβ ($p<0.05$, $n=6$, Figure 7A–D). The incubation with the $A_{2A}R$ agonist, or antagonist, for 24 h did not significantly change TrkB expression ($p>0.05$, $n=6$, Figure 7A and D).

In addition, we also evaluated if BDNF can prevent the Aβ-induced TrkB truncation. The results show that 24 h of BDNF (20 ng/mL) incubation on neuronal cultures prevent the Aβ-induced formation on TrkB-ICD and TrkB-Tc ($p<0.05$, $n=6$, Figure 7B–D), an effect unaltered by the presence of the $A_{2A}R$ agonist or antagonist ($p>0.05$, $n=6$, Figure 7B–D). However, the BDNF incubation also strongly reduced the levels of TrkB-FL, even in the absence of Aβ ($p<0.001$, $n=6$, Figure 7A and D).

Discussion

The results described here show that BDNF exerts protective effects by reducing the caspase-3 and calpain activation upon $A\beta_{25-35}$ toxicity, an effect independent on $A_{2A}R$ activation, as illustrated in Figure 8.

The BDNF effects are widely described in several *in vitro* and *in vivo* models of neurodegenerative disorders, as Parkinson's and Alzheimer's disease (Lu et al., 2013; Rodrigues et al., 2014). In particular, BDNF is able to increase cell viability of neurons incubated with toxic concentrations of Aβ peptide (Arancibia et al., 2008), as also observed in the present work through MTT and TUNEL assays. By evaluating the levels of caspase-3 and calpain activation, both proteases involved in cellular death, this work reinforces the BDNF neuroprotective effects upon Aβ toxicity. Accordingly, BDNF incubation on Aβ-treated cortical neurons promotes a robust reduction in the conversion of pro-caspase-3 into active caspase-3 and in the caspase-3 activity. Moreover, BDNF reduces the breakdown of αII-spectrin, a neuronal cytoskeletal protein highly susceptible to neurodegenerative insults, and consequently, it reduces the formation of the calpain- and caspase-3-specific spectrin breakdown products (SBDP150 and SBDP120, respectively) in the Aβ-treated neurons.

Caspases and calpains may act synergistically to induce neuronal death. It is known that: (1) both calpains and caspases share multiple common substrates; (2) calpains can cleave a variety of caspases leading to their activation, or inhibition; (3) caspases can cleave calpastatin, an endogenous calpain inhibitor, leading to calpain activation (Fifre et al., 2006; Raynaud & Marcilhac, 2006; Wang, 2000; Wei et al., 2008). Our results are in accordance to published data showing that Aβ triggers the activation of both calpains and caspases in septal cultured neurons (Wei et al., 2008). Therefore, caspases and/or calpains inhibitors can markedly protect cultured neurons against Aβ-induced toxicity. However the effects of calpain and caspases inhibitors are not additive, suggesting that other pathways might be involved (Wei et al., 2008). In addition to what had been already seen that BDNF can block caspase-3 activation in neurons submitted to different types of insults, such as radiation or hypoxia-ischemia (Han et al., 2000; Kim & Zhao, 2005), here, we show that BDNF reduces the Aβ-induced activation of both caspase-3 and calpain. Interestingly, the results here presented suggest the protection mediated by BDNF against Aβ-peptide is dependent on PLCγ signaling pathway, and not on PI3K and ERK/MAPK pathways. The major signaling pathways activated by the Trk receptors are; (1) PI3K; (2) MAPK and (3) PLCγ pathways, and their

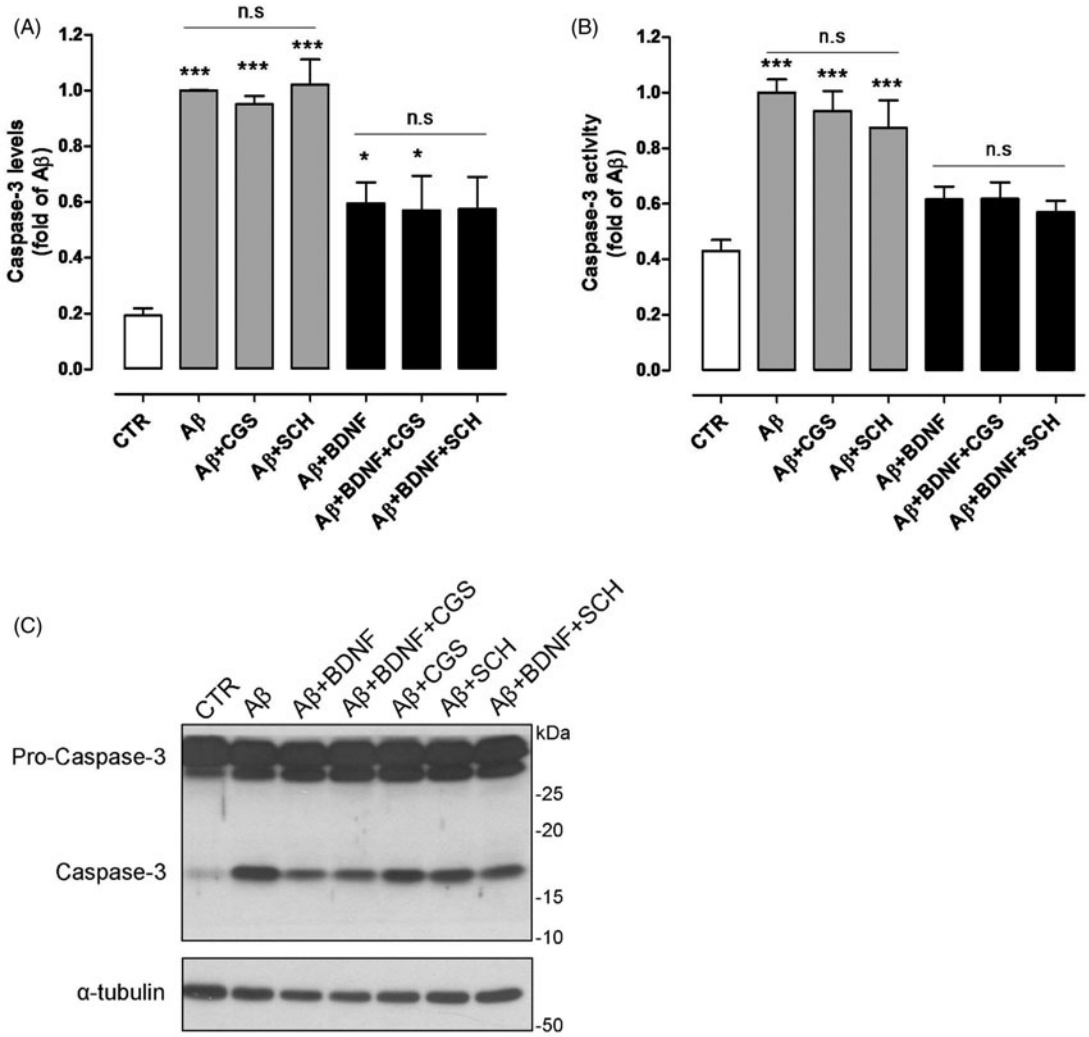

Figure 5. $A_{2A}R$ does not influence BDNF-mediated reduction in Aβ-induced caspase-3 activation. (A) Quantification of active caspase-3 protein levels and (B) Caspase-3 activity, in 8 DIV cortical neurons non-treated or treated with $A\beta_{25–35}$ (25 μM) for 24 h in the absence or presence of BDNF (20 ng/mL) and SCH58261 (100 nM) or CGS21680 (10 nM). Data is normalized to Aβ condition given the very low, almost undetectable, caspase activation in control condition. $^{***}p < 0.001$, $^{*}p < 0.05$, when comparing to control and n.s (not significant) when compared between the conditions indicated by the horizontal line ($n = 6$, ANOVA followed by Bonferroni post-test) (C) Representative western-blot used in (A), showing the protein levels of the unprocessed pro-caspase-3 (32 kDa) and the active caspase-3 (17-kDa). Note that the order of the conditions is different from (A). α-tubulin was used as a loading control. Data represented are mean ± SEM of n independent experiments.

downstream effectors. These include PI3K stimulation of Akt, Ras stimulation of mitogen-activated protein MAPK cascades and PLC-γ dependent generation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Segal & Greenberg, 1996). It is known that BDNF can activate PI3K/Akt signaling pathway, which in turn can halt apoptosis through phosphorylation and inhibition of pro-apoptotic proteins such as Bad and caspase-9 (Zhou et al., 2000). Importantly, other member of neurotrophin family, the neurotrophin-3 (NT-3), also protects cortical neurons from Aβ-induced toxicity by inhibiting caspase-3, -8 and -9, in an Akt-dependent and ERK/MAPK-independent way. In particular, NT-3 incubation activates Akt, which in turn induces the expression of NAIP-1, a member of the inhibitors of apoptosis proteins (IAPs), which can directly inhibit caspase activation (Lesne et al., 2005). Interestingly, our data suggests that the neuroprotective effect of BDNF against Aβ toxicity is mediated by PLCγ pathway. In line with this data, it was shown that, upon a toxic glutamate insult, the protective effect of BDNF is mainly mediated by the PLCγ signaling pathway (Melo et al., 2013). Moreover, this specific signaling pathway has been implicated in other neuroprotection mechanisms (Hayashi et al., 2009; Lakk et al., 2015; Vasefi et al., 2013).

Strikingly, BDNF and EGF can activate m-calpain in a MAPK dependent way (Zadran et al., 2010). However, although calpains could play multiple biological roles upon physiological activation, when overactivated, these enzymes, promote neuronal death and contribute to neurodegeneration. Thus, upon physiological conditions the BDNF-mediated activation of m-calpain might be relevant for some BDNF actions, while upon pathological conditions, the BDNF might normalize calpain activity by reducing its excessive activation, and preventing the neurodegenerative process.

The actions of BDNF upon synaptic transmission and plasticity are well characterized as being dependent on $A_{2A}R$ receptor activation (Diógenes et al., 2004, 2007, 2011, 2014; Fontinha et al., 2008; Jerónimo-Santos et al., 2014; Pousinha et al., 2006; Rodrigues et al., 2014; Tebano et al., 2008).

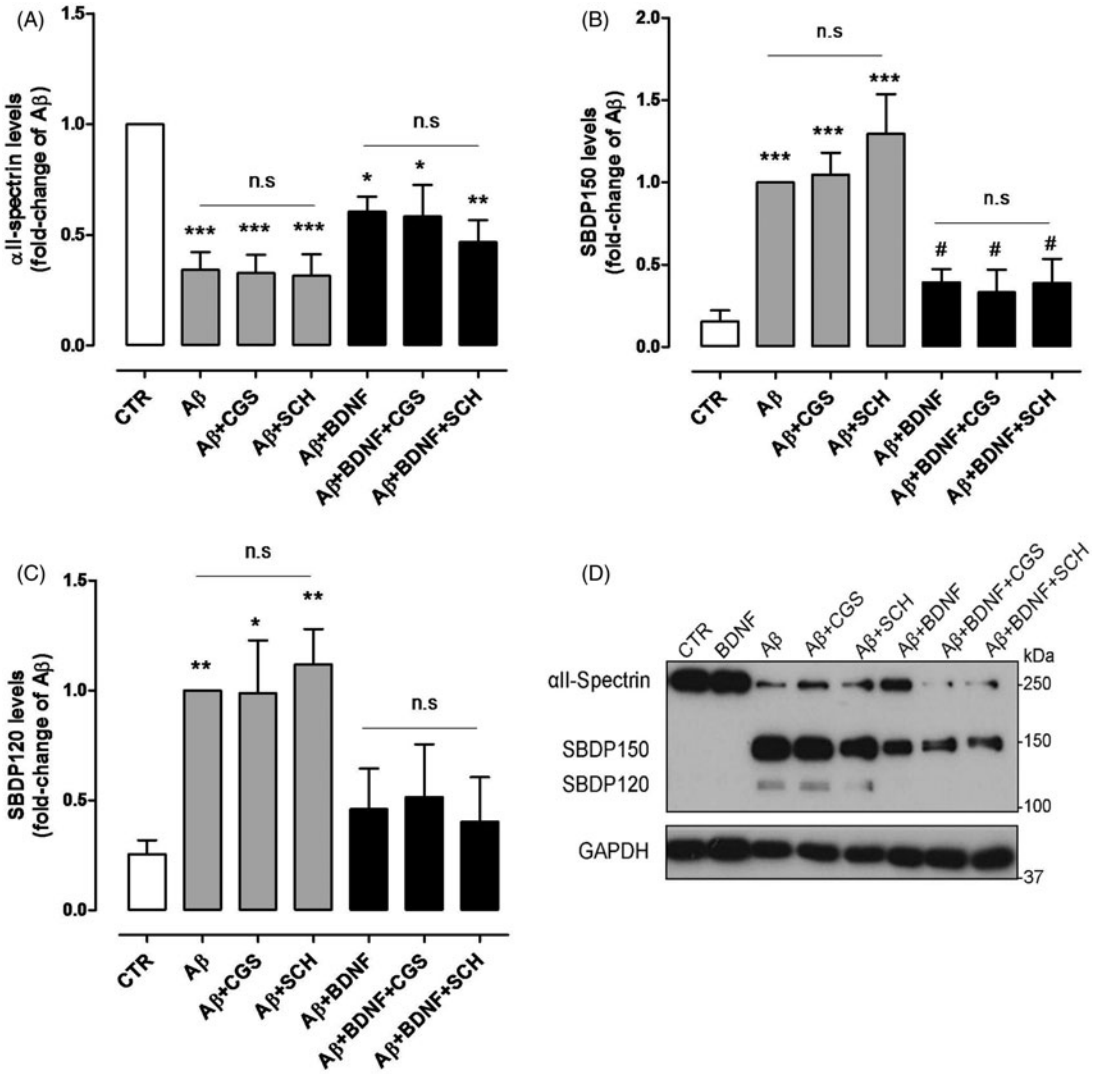

Figure 6. $A_{2A}R$ does not influence BDNF-mediated reduction in Aβ-induced spectrin breakdown. (A) Quantification of αII-spectrin breakdown with consequent formation of (B) SBDP150 and (C) SBDP120 breakdown products in 8 DIV cortical neurons non-treated or treated with $A\beta_{25–35}$ (25 μM) for 24 h in the absence or presence of BDNF (20 ng/mL) and SCH58261 (100 nM) or CGS21680 (10 nM). SBDP150 and SBDP120 levels are normalized to Aβ condition given their almost undetectable amount in control conditions. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, when comparing to control, n.s (not significant) when compared between the conditions indicated by the horizontal line and $\#p<0.05$ when comparing to Aβ ($n=6$, ANOVA followed by Bonferroni post-test) (D) Representative western-blot used in (A–C), showing the protein levels of αII-spectrin, SBDP150 and SBDP120. GAPDH was used as a loading control. Data represented are mean ± SEM of n independent experiments.

However, the results presented here indicate that $A_{2A}R$ activation is not required for BDNF neuroprotective actions upon Aβ insult to 7 DIV primary cortical cultures. One might speculate whether the lack of $A_{2A}R$ expression, in the cultures, could explain the absence of $A_{2A}R$ effects. However, this is not the case since although $A_{2A}R$ are not abundantly expressed in the hippocampus or neocortex (Ribeiro et al., 2002), these adenosine receptors have been detected in 6–9 DIV cortical cultures by immunocytochemistry (Rebola et al., 2005), and by western-blot (Ribeiro et al., 2015; Valadas et al., 2012). Furthermore, it has been shown, that similar concentrations of the $A_{2A}R$ agonist, CGS21680, can trigger $A_{2A}R$-mediated effects on hippocampal and cortical neuronal cultures (Jeon et al., 2011; Lee & Chao, 2001). Remarkably, using the same 3–7 DIV cortical cultures, data from our lab recently showed that $A_{2A}R$ are present and functional, promoting axonal elongation and dendritic branching (Ribeiro et al., 2015). Consequently, we might speculate that while most of $A_{2A}R$-dependent actions of BDNF are synaptic and fast-mediated actions, the slow and long-lasting events mediating neuroprotective actions by BDNF are not dependent on $A_{2A}R$ activation.

Controversial data have shown protective effects against insults either by blocking or activating $A_{2A}R$. Accordingly, it has been shown that $A_{2A}R$ activation, by CGS21680, reduces kainate-induced excitotoxicity by 40%, in 6 DIV cortical cultures (Rebola et al., 2005). In opposition, the blockade of $A_{2A}R$ by the selective antagonist SCH58261 improves cell viability against the glutamate (20–1000 μM, 24 h) insult, whereas $A_{2A}R$ activation does not protect neurons, in 9 DIV cortical cultures (Valadas et al., 2012). In addition, both caffeine (a non-selective adenosine receptor antagonist) and $A_{2A}R$ antagonists prevent the toxicity induced by 48 h of $A\beta_{25–35}$ (25 μM) incubation on cultured cerebellar granule neurons (Dall'Igna et al., 2003). Moreover, $A_{2A}R$ blockade, or genetic deletion of $A_{2A}R$, prevents synaptotoxicity and

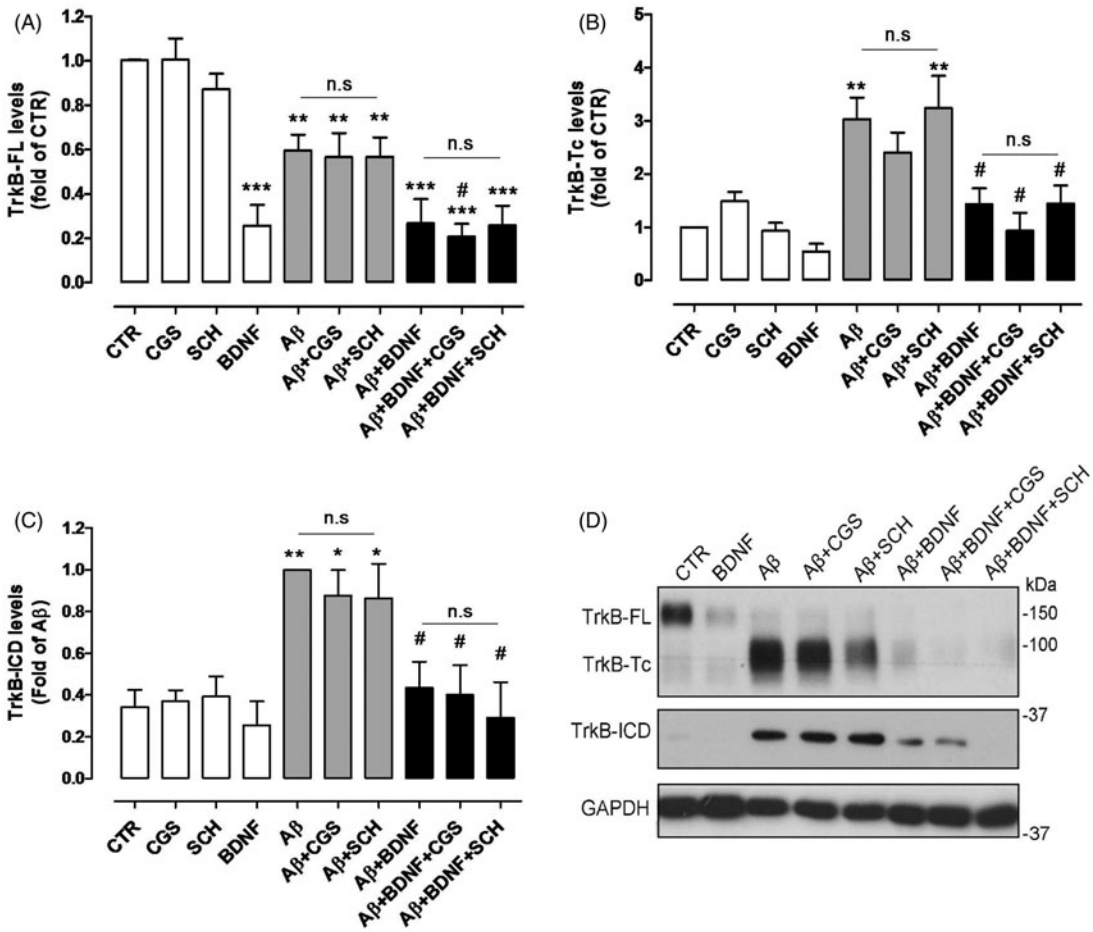

Figure 7. (A) Effect of $A_{2A}R$ and BDNF upon Aβ-induced TrkB truncation. Quantification of TrkB-FL cleavage with consequent formation of (B) truncated TrkB and (C) TrkB-ICD fragment, in 8 DIV cortical neurons non-treated and treated with $A\beta_{25–35}$ (25 μM) for 24 h in the absence or presence of BDNF (20 ng/mL) and SCH58261 (100 nM) or CGS21680 (10 nM). TrkB-ICD levels are normalized to Aβ condition given their almost undetectable amount in control conditions. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, when comparing to control, n.s (not significant) when compared between the conditions indicated by the horizontal line and $\#p<0.05$ when comparing to Aβ ($n=6$, ANOVA followed by Bonferroni post-test) (D) Representative western-blot used in (A–C), showing the protein levels of TrkB-FL, TrkB-Tc and TrkB-ICD. GAPDH was used as a loading control. Data represented are mean ± SEM of n independent experiments.

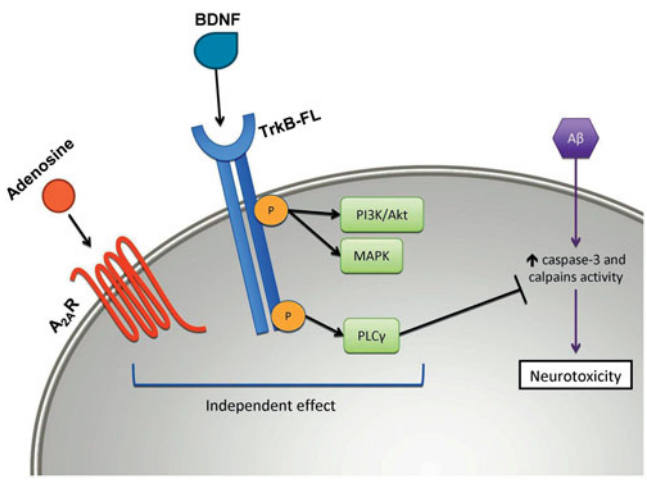

Figure 8. Schematic illustration outlining the conclusions supported by the results. BDNF exerts protective effects against Aβ toxicity leading to a reduction on caspase-3 and calpain activation, in a mechanism that might be mediated by PLCγ pathway and independent on $A_{2A}R$ activation.

memory dysfunction caused by intracerebroventricular administration of 2 nmol of $A\beta_{1–42}$ in mice (Canas et al., 2009; Cunha et al., 2008). Finally, blockade of $A_{2A}R$, by SCH58261, prevented the reduction in cell viability induced by the incubation of oligomeric-enriched $A\beta_{1–42}$ (500 nM), in hippocampal cultures (Canas et al., 2009). In this work, using cortical neurons, no significant protective effect of the $A_{2A}R$ agonist, or antagonist, against the toxicity induced by the incubation of fibrillary-enriched $A\beta_{25–35}$ (25 μM) (Kemppainen et al., 2012) was seen. These discrepancies might result from the degree of toxicity induced by the insulting agent. In line with this hypothesis is the work previously published by us demonstrating that neuroprotection afforded by $A_{2A}R$ blockade against glutamate insult is only seen for lower concentrations of glutamate (50 and 100 μM, 24 h), failing for higher concentrations (500and 1000 μM) (Rebola et al., 2005). In addition, while higher concentrations of glutamate only decreased cell viability by around 30%, incubations with $A\beta_{25–35}$ (25 μM, 24 h) in conditions similar to those used in the present study, had a stronger impact on cell viability decreasing it by around 45%

(Rebola et al., 2005). It is thus likely that $A_{2A}R$ inhibition might be not able to prevent the toxicity of a stronger insult such as $A\beta_{25–35}$ (25 μM). Therefore, drugs that modulate $A_{2A}R$ might have stronger benefits in stages where Aβ accumulation and its effects are not massive. Nevertheless, it is noteworthy to highlight that BDNF was able to reduce, very significantly, both caspase-3 activation and αII-spectrin breakdown, upon the $A\beta_{25–35}$ (25 μM) insult.

We recently showed that Aβ incubation on cortical neurons induces to a calpain-mediated cleavage on TrkB-FL receptor, generating a truncated TrkB receptor and an intracellular TrkB fragment (Jerónimo-Santos et al., 2015). Here, we evaluated whether BDNF was able to prevent the Aβ-induced calpain-mediated truncation of TrkB. We found that BDNF significantly reduces the levels of TrkB-FL cleavage fragments (TrkB-T′ and TrkB-ICD). Accordingly, to what was initially described (Sommerfeld et al., 2000), the present results confirm that BDNF strongly down-regulates TrkB-FL protein levels after 24 h of incubation. This BDNF-mediated down-regulation of TrkB-FL is a fast event, in which total TrkB-FL receptor levels decrease by 80% in just 3 h after the ligand binding, a value that remain almost constant at least up to 24 h (Sommerfeld et al., 2000). Given that the calpain-mediated cleavage of TrkB-FL has a much slower time course (Jerónimo-Santos et al., 2015), it is likely that when calpains start to cleave TrkB-FL receptors, the levels of the receptor itself are already reduced due to the fast BDNF-mediated down-regulation and therefore there are less TrkB-FL receptors available to be cleaved. Thus, the observation that BDNF prevents the formation of TrkB-FL cleavage fragments might be explained by both calpain inhibition and by BDNF-mediated down-regulation of TrkB-FL.

Conclusions

This work shows that BDNF exerts a robust neuroprotective effect upon Aβ-induced toxicity, by reducing calpain and caspase-3 activation. In opposition to most fast synaptic actions of BDNF, the neuroprotective effect of this neurotrophin does not depend on $A_{2A}R$ activation (Figure 8).

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Declaration of interest

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