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1–42 β-Amyloid peptide requires PDK1/nPKC/Rac 1 pathway to induce neuronal death

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1–42 β-Amyloid ($A\beta_{1-42}$) peptide is a key molecule involved in the development of Alzheimer's disease. Some of its effects are manifested at the neuronal morphological level. These morphological changes involve loss of neurites due to cytoskeleton alterations. However, the mechanism of $A\beta_{1-42}$ peptide activation of the neurodegenerative program is still poorly understood. Here, $A\beta_{1-42}$ peptide-induced transduction of cellular death signals through the phosphatidylinositol 3-kinase (PI3K)/phosphoinositol-dependent kinase (PDK)/novel protein kinase C (nPKC)/Rac 1 axis is described. Furthermore, pharmacological inhibition of PDK1 and nPKC activities blocks Rac 1 activation and neuronal cell death. Our results provide insights into an unsuspected connection between PDK1, nPKCs and Rac 1 in the same signal-transduction pathway and points out nPKCs and Rac 1 as potential therapeutic targets to block the toxic effects of $A\beta_{1-42}$ peptide in neurons.
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Introduction

Small GTPases of the Rho family, whose best-characterized members include Rho A, Rac 1 and Cdc42, are key players in complex signaling networks that control normal activity in most if not all cell types.[1] Like other small GTPases, Rho GTPases function as molecular switches to control cellular signaling pathways. They are present in two conformations, an inactive form loaded with GDP and an active form loaded with GTP. Transition from the inactive to the active state is regulated by guanine nucleotide exchange factors.[2] In their active configuration, GTPases interact with downstream effector molecules to promote a variety of biological responses.[3,4] GTPases of the Rho family are best known for controlling the appropriate actin cytoskeleton reorganization in response to extracellular signals, although their implication in additional biological processes, such as gene expression regulation, cell polarity and cell migration have also been reported.[1]

As these monomeric G proteins are implicated in almost all aspects of cell biology,[5] changes in their regulatory cycles can affect normal cell functionality in an irreversible manner. Signaling pathways that originated from deregulated GTPase activities can be involved in cell transformation and metastasis,[6,7] in Wiskott–Aldrich syndrome,[8] faciogenital dysplasia, Tangier disease[9] and other pathologies. Recently, it has been shown that they may also be implicated in Alzheimer's disease (AD).[10] It is well established that RhoGTPases control cytoskeleton dynamics in neurons, thereby modulating synaptic plasticity.[11] In fact, AD entails progressive dendritic spine loss, synaptic dysfunctions and morphological changes in dendrites.[10] In normal brain, neuronal Rho A attaches to synapses and dendritic microtubules; however, in AD Rho A the expression is decreased in synapses and increased in dystrophic neuritis.[10]

The molecular mechanisms leading to the development of AD are not fully characterized. This neuropathology is associated with massive accumulation of two types of protein aggregates: senile plaques that are constituted mostly by 1–42 β amyloid ($A\beta_{1-42}$) peptide and by neurofibrillary tangles containing hyperphosphorylated Tau protein.[12] There is mounting evidence that $A\beta_{1-42}$ peptide mediates many aspects of AD pathogenesis. *In vitro* this peptide is toxic to endothelial cells,[13,14] smooth muscle cells,[15] astrocytes,[16] neurons[17,18] and oligodendrocytes.[19] The mechanisms through which $A\beta_{1-42}$ peptide exerts its cytotoxic action are not fully understood. Currently, there are ongoing efforts to discover the signaling pathways that are mediated by the $A\beta_{1-42}$ peptide. Several signaling cascades may be involved in cell damage and they appear to be activated by the $A\beta_{1-42}$ peptide, including oxidative stress generation,[18,20] impaired

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Ca^{2+} homeostasis,[21] mitochondrial dysfunction,[22] generation of NO[23] and microglia activation.[24]

Here, $A\beta_{1-42}$ peptide use of cellular machinery for signal transduction leading to neuronal cell death in the cell line SN4741 theat originated from mouse substantia nigra was studied[25,26] in primary embryonic cortical neurons from rats as well as in neuronal organotypic cultures of the hippocampus and the entorhinal cortex. This signaling cascade involves specifically Rac 1 GTPase, which is regulated upstream by the phosphatidylinositol 3-kinase (PI3K)/phosphoinositol-dependent kinase (PDK)1/ novel protein kinase C (nPKC) pathway. This novel molecular characterization identifies nPKCs and Rac 1 as potential therapeutic targets to block neuronal death program induced by the β-amyloid peptide.

Materials and methods

Reagents. AKT inhibitor II, GF 109203X, Gö 6976 and Rottlerin were obtained from Calbiochem (Darmstadt, Germany). HFIP (1,1,1,3,3,3-hexafluoro-2-propanol), LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) hydrochloride, phorbol 12 myristate 13 acetate (PMA), 6-mercaptopurine (6-MP) and dimethylsulfoxide were obtained from Sigma (Dorset, UK). Rac 1 inhibitor NCS23766 (*N*-(*N*-(3,5-difluorophenacetyl-L-alanyl))-*S*-phenylglycine) was obtained from Calbiochem (Merck Millipore, Darmstadt, Germany). OSU03012 was obtained from Cayman (Ann Arbor, MI, USA) and $A\beta_{1-42}$ peptide was obtained from Bachem (Weil am Rheim, Germany). Mouse monoclonal anti-Cdc42 and anti-Rac 1 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal anti-Rho A antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-phospho-p21-activated kinase (PAK)1 and anti-PAK1 antibodies and mouse monoclonal anti-enhanced green fluorescent protein (EGFP) antibody were obtained from Cell Signaling Technology (Danvers, MA, USA), and mouse anti-HA was obtained from Covance (Berkeley, CA, USA).

Preparation of synthetic Aβ oligomers. $A\beta_{1-42}$ oligomers were prepared as previously described by Klein *et al.*[27] Briefly, $A\beta_{1-42}$ peptide was initially dissolved to 1 mM in HFIP and separated into aliquots in sterile microcentrifuge tubes. HFIP was totally removed under vacuum in a speedvac system and the peptide film was stored desiccated at $-80\,^{\circ}C$. For the aggregation protocol, the peptide was first resuspended in dry dimethylsulfoxide to a concentration of 5 mM and Hams F-12 cell culture medium (PromoCell, Labclinics, Barcelona, Spain) was added to bring the peptide to a final concentration of 100 μM and incubated at $4\,^{\circ}C$ for 24 h. Preparation was then centrifuged at 14 000 *g* for 10 min at $4\,^{\circ}C$ to remove insoluble aggregates and supernatant containing soluble $A\beta_{1-42}$ peptide was transferred to clean tubes and stored at $4\,^{\circ}C$.

Natural oligomers from double transgenic PS1Δ9xAPPswe (PS1APP) mice. Soluble protein extraction (soluble fractions) from 18-month-old PS1APP double transgenic and non-transgenic (wild-type (WT)) mice were obtained by ultracentrifugation of homogenates, as described previously.[28] Protein concentration present in soluble fractions was determined by the method of Lowry *et al.*[29] To diminish the potential inter-individual variability, soluble fractions from four different mice were pooled.[28] For *in vitro* experiments, soluble fractions were thawed immediately before use, diluted with Dulbecco's modified Eagle's medium (w/o fetal bovine serum (FBS)), sterilized and added to cultures.

Cell culture and DNA transfection. SN4741 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 4 mM L-glutamine, 3.7 g sodium bicarbonate and $4.5\,g\,l^{-1}$ glucose w/o sodium pyruvate, $100\,ng\,ml^{-1}$ streptomycin and $100\,U\,ml^{-1}$ of penicillin (all from Invitrogen, Alcobendas, Madrid, Spain). They were maintained at $37\,^{\circ}C$ and 5% CO_2. For transient transfection assays, cells were washed two times with PBS and trypsinized, and then 5×10^6 cells (per condition) were resuspended into 200 ml of serum-free medium containing either 10 μg pEGFP or pEGFP-PKCθ or pcDNA3-HA-Tiam-1 were electroporated at 260 V, 950 μF in a Gene Pulser Xcell Electroporator (Bio-Rad, Alcobendas, Madrid, Spain). Cells were collected into 10 ml of complete Dulbecco's modified Eagle's medium for 24 h, washed and then cultured in serum-free medium for additional 24 h. After this period, transfected cells were pretreated either with Rottlerin or not for 1 h followed by treatment with $A\beta_{1-42}$ peptide.

Primary neuron cultures were obtained from the cortical lobes of E18 Sprague–Dawley rat embryos, as described previously.[21] Cells were resuspended into 0.5% B27-Neurobasal medium plus 10% FBS and then seeded onto 6-well plates at 1.5×10^6 cells per well. After 1 day, the medium was replaced by serum-free 0.5% B27-supplemented neurobasal medium. Cell cultures were essentially free of astrocytes and microglia and they were maintained at $37\,^{\circ}C$ and 5% CO_2. Cultures were used 8–10 days after plating. For transient transfection assays, rat neurons obtained as described above were transfected with 3 μg pEGFP or pEGFP-RBD (Rac binding domain of PAK1)[30] using Amaxa Rat Neuron Nucleofector Kit (Lonza, Basel, Switzerland), according to the manufacturer's instructions. For microscopy analysis, transfected cells were plated onto glass-bottom μ-dishes (Ibidi GmbH, Munich, Germany), maintained as described above and used 8 days after plating.

Preparation of organotypic cultures and cell viability. Rat brains were removed and the two separated hemispheres were placed in Hank's balanced salt solution medium. Thalamus and midbrain were removed and each hemisphere was sliced using a tissue chopper (McIlwan Tissue Chopper; Campden Instruments, Lafayette, IN, USA) to obtain 400 μm coronal slices. The entorhinal cortex, connected to the hippocampus, was isolated using a dissection microscope, two slices were plated on each Millicell CM culture inserts (Millipore Ibérica, Madrid, Spain) and maintained in Neurobasal medium supplemented with 0.5% B27, 25% horse serum, 25% Hank's balanced salt solution and $25\,mg\,ml^{-1}$ gentamycin at $37\,^{\circ}C$ for 7 days.

Hippocampus–entorhinal cultures were exposed to $A\beta_{1-42}$ peptide oligomers at 100 nM for 4 additional days. Inhibitors were added to cultures 30 min before the $A\beta_{1-42}$ peptide oligomers. Slices were stained with 10 μM propidium iodide for 2 h at 37 °C, washed two times with PBS for 10 min and fixed with 4% paraformaldehyde in PBS for 40 min at room temperature.[21] Subsequently, slices were excited with 510–560 nm light and emitted fluorescence was acquired at 610 nm using a rhodamine filter with an inverted fluorescence microscope (Cell Observer Z1; Zeiss, Jena, Germany). Propidium iodide fluorescence images were captured with a Plan NeoFluar ×2.5 objective (Zeiss), using an EM CCD camera (Hamamatsu Photonics, Hamamatsu, Japan; C9100-13) and controlled by Axio Vision program (Zeiss). Images were analyzed with the ImageJ analysis program (NIH, Bethesda, MD, USA).

Rho A, Rac 1 and Cdc42 activation assays. Rho family GTPases were pulled down as described previously.[30] Briefly, cells deprived of serum for 24 h were pretreated with inhibitors or not for 1 h and subsequently treated with $A\beta_{1-42}$ peptide oligomers for the indicated times and lysed. Cell lysates were collected after centrifugation and incubated for 1 h at 4 °C with specific GST-fusion proteins coupled to glutathione-sepharose beads. Precipitated proteins were eluted, electrophoresed by sodium dodecyl sulfate-polyacryalmide gel electrophoresis and analyzed by western blotting with specific antibodies. Immunoreactive bands were visualized using ECL.

PAK1 phosphorylation assay. Primary neuron cultures were pretreated or not with Rac 1 inhibitor for 1 h. Subsequently, cells were treated with natural oligomers from transgenic mice for 30 min, as indicated. Cultures were washed with PBS, and lysed directly in Laemmli buffer. Lysates were electrophoresed by sodium dodecyl sulfate-polyacryalmide gel electrophoresis and analyzed by western blotting with anti-phospho-PAK1 and anti-PAK1 antibodies. Immunoreactive bands were visualized using ECL.

Immunoprecipitation assay. SN4741 cells transfected with pcDNA3-HA-Tiam-1 were treated or not with $A\beta_{1-42}$ peptide oligomers for 30 min, washed three times in ice-cold PBS and lysed in RIPA buffer. HA-Tiam-1 was immunoprecipitated at

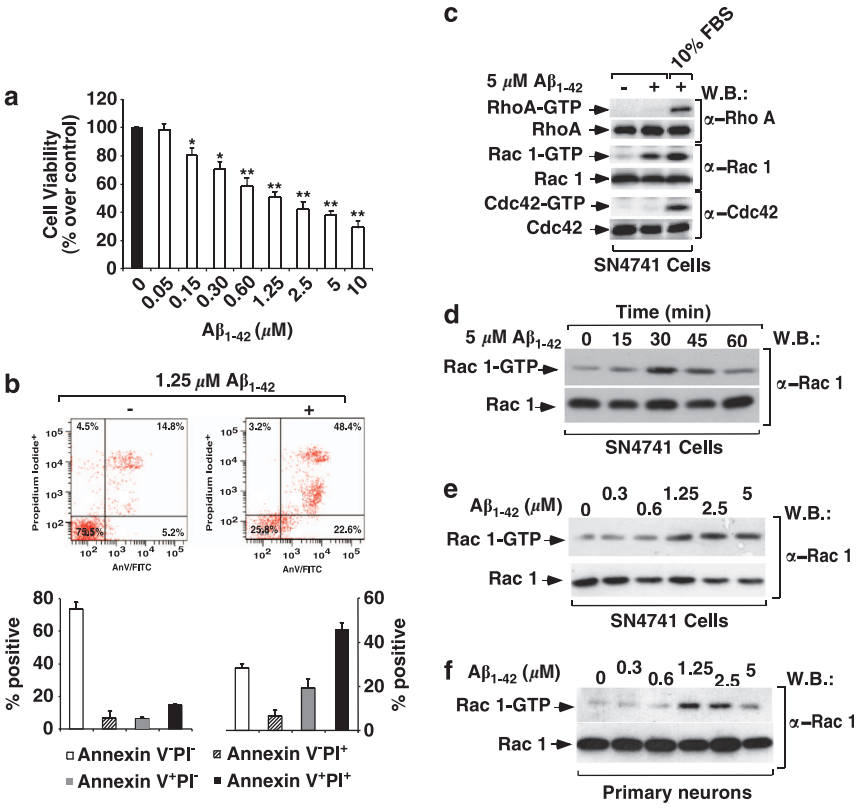

Figure 1 1–42 β-Amyloid ($A\beta_{1-42}$) peptide exposure leads to cytotoxicity and Rac 1 activation in neurons. (**a**) Histogram represents the mean of three independent experiments ± s.e.m. (*$P<0.05$, **$P<0.01$, comparing control cells vs $A\beta_{1-42}$ peptide-treated cells). (**b**) Dot plots represent the distribution of early apoptotic (lower right, annexin V^+PI^-), necrotic (upper left, annexin V^-PI^+), late apoptotic (upper right, annexin V^+PI^+) and viable (lower left, annexin V^-PI^-) cells. Dot plots are representative of three independent experiments. Histograms represent mean ± s.e.m. values of three independent experiments. (**c**) Small GTPases activation. SN4741 cells were treated (+) or not (−) with 5 μM $A\beta_{1-42}$ peptide for 1 h or 10 min with 10% fetal bovine serum (FBS) and cell extracts were used to measure Rho A, Rac 1 and Cdc42 activation (loaded with GTP) by affinity precipitation assays. Results are representative of three independent experiments. Panels 2, 4 and 6 show total small GTPases in cell lysates. (**d**) Kinetics of Rac 1 activation induced by $A\beta_{1-42}$ peptide. Results are representative of four independent experiments. (**e**) and (**f**) $A\beta_{1-42}$ peptide activates Rac 1 in a dose-dependent manner in SN4741 cells and primary neurons. Immunoblot depicts a representative experiment out of three. Lower panels in (**d–f**) show total Rac 1 in cell lysates. PI, propidium iodide; W.B., western blot.

4 °C for 2 h with the anti-HA antibodies. Immune complexes were recovered using Gamma Bind Plus Sepharose beads (GE Healthcare, Pittsburgh, PA, USA), washed and eluted from beads using 2 × Laemmli's buffer, electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by western blotting with anti-threonine or anti-HA antibodies. Immunoreactive bands were visualized using ECL.

Cell viability and toxicity assays. SN4741 cells were seeded at 10^4 cells per well in a 96-well plate, serum-starved for 24 h, pretreated with different inhibitors for 1 h, as indicated, and treated or not with $A\beta_{1-42}$ peptide oligomers for 24 h. Primary neuronal cultures were pretreated or not with Rac 1 inhibitor for 1 h. Subsequently, cells were treated with synthetic $A\beta_{1-42}$ peptide or natural oligomers from transgenic mice for 24 h, as indicated. Cell survival was measured with CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Madison, WI, USA), according to the manufacturer's instructions.

In SN4741 cells apoptosis was assessed staining cells with annexin V-FITC (FITC Annexin V Apoptosis detection kit; BD, Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. A total of 3×10^6 cells per 100 mm plates were serum-starved for 24 h, pretreated with different inhibitors for 1 h as indicated and treated with $A\beta_{1-42}$ peptide oligomers for 24 h. Cells were detached and 1×10^6 cells per ml were incubated with annexin V-FITC and propidium iodide and analyzed on a FACScan.

In neuronal primary cultures, apoptosis was estimated by staining cells with annexinV-Cy5 (BD), according to manufacturer's instructions. Primary cultures transfected with either pEGFP or pEGFP-RBD were treated or not with 5 μM $A\beta_{1-42}$ peptide for 24 h, and live-cell imaging was performed by laser-scanning confocal microscopy (Olympus Fluoview FV500, Hamburg, Germany).

Statistical analysis. Student's *t*-test for the mean of two-paired samples was used to determine the significance between data means ($^{\#,*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$).

Results

$A\beta_{1-42}$ peptide activates Rac 1 GTPase in a dose- and time-dependent manner. We examined whether neuronal cell line SN4741 was sensitive to the toxicity induced by the $A\beta_{1-42}$ peptide-derived diffusible ligands (ADDLs).[27] Cells were incubated with increasing concentrations of $A\beta_{1-42}$ peptide for 24 h and cell viability was determined by MTT assay. As shown in Figure 1a, cell viability, measured as mitochondrial metabolic activity, correlated inversely with the concentration of $A\beta_{1-42}$ peptide added. Nevertheless, MTT assay is a colorimetric method that cannot discriminate whether cells die by necrosis or apoptosis.[31,32]

Therefore, SN4741 cells were treated (+) or not (−) with 1.25 μM $A\beta_{1-42}$ peptide (a concentration that reduced cellular viability by 50%, as shown in Figure 1a) for 24 h and apoptosis was determined by staining plasma membrane with annexin V combined with propidium iodide followed by flow cytometry analysis. As shown in Figure 1b (histogram graphs and dot plots), $A\beta_{1-42}$ peptide reduced SN471 cells viability from 73 to

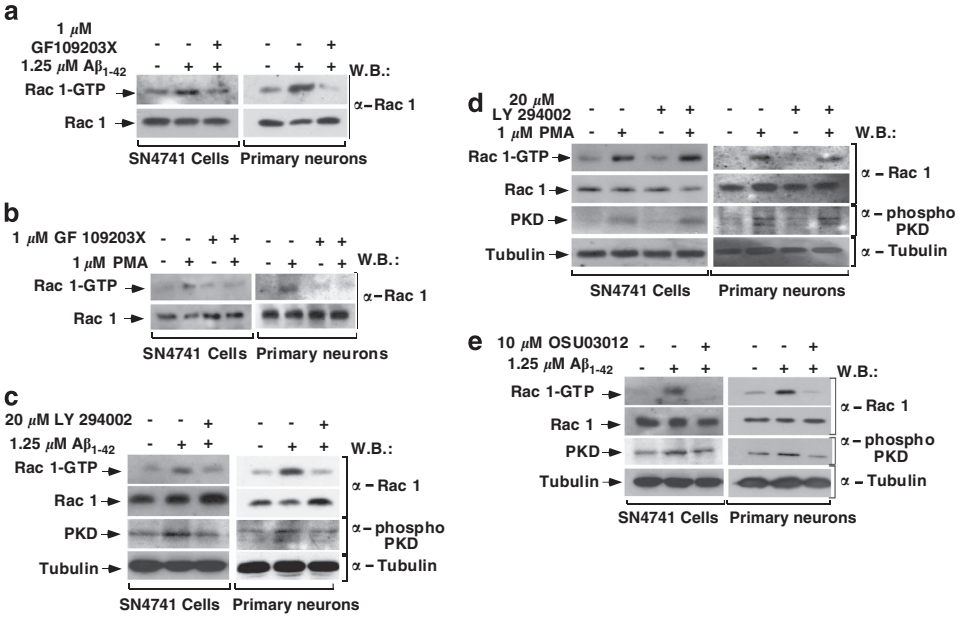

Figure 2 Rac 1 activation induced by 1–42 β-amyloid $A\beta_{1-42}$ peptide requires the involvement of protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and phosphoinositol-dependent kinase (PDK)1 in neurons. (**a**–**f**) SN4741 cells (left panels) and primary neurons (right panels) were pretreated with the indicated inhibitors for 1 h and then treated or not with 1.25 μM $A\beta_{1-42}$ peptide for 30 min or 1 μM phorbol 12 myristate 13 acetate (PMA) for 15 min and lysed. Cell extracts were used to measure Rac 1 activation by affinity precipitation assays (Rac 1-GTP); a sample of total cell lysate was used to determine the PKD phosphorylation treatment, Rac 1 shows total Rac 1 in cell lysates and tubulin immunoblot represents the loading protein control. Results are representative of five (**a**, **d** and **e**) and three (**b** and **c**) independent experiments, respectively.

28% (Figure 1b, empty bars, left histogram vs right histogram) and increased apoptosis in the same proportion, that is, early apoptosis from 6 to 19% (Figure 1b, gray bars, left histogram vs right histogram) and late apoptosis from 14 to 46% (Figure 1b, solid bars, left histogram vs right histogram). Percentage of annexinV$^-$/propidium iodide$^+$ cells in both untreated and treated cells was maintained at around 6% (Figure 1b, hached bars, left histogram vs right histogram). Taken together, these observations indicate that SN4741 cell line is suitable for studying $A\beta_{1-42}$ peptide-induced apoptosis.

As small GTPases of the Rho family are key regulators of cell signaling, the hypothesis that they can be activated by $A\beta_{1-42}$ peptide treatment was tested. SN4741 cells were treated with 5 μM $A\beta_{1-42}$ peptide for 1 h; lysed and small GTPases were pulled down and visualized, as described in the Materials and methods section. As shown in Figure 1c, $A\beta_{1-42}$ peptide stimulated Rac 1 activation (Rac 1-GTP); however, this peptide was unable to activate either Rho A or Cdc42. To verify whether these GTPases were functional in this cellular system, cells were stimulated with 10% FBS for 10 min and processed in the same manner as cells treated with $A\beta_{1-42}$ peptide. All three GTPases were effectively activated under this stimulatory condition (Figure 1c, line 3).

The effect of $A\beta_{1-42}$ on Rac 1 activation was characterized further by examining the kinetics of activation of this GTPase. SN4741 cells were treated with 5 μM $A\beta_{1-42}$ peptide at different times, as indicated in Figure 1d. Maximal activation of Rac1 was observed 30 min after peptide addition and decreased progressively reaching basal levels 60 min thereafter (Figure 1d). The optimum time to show Rac 1 activation was at 30 min after stimulation, which was taken into consideration when performing the dose–response curve with SN4741 cells. Minimal concentration of $A\beta_{1-42}$ peptide required to produce maximal activation of Rac 1 was 1.25 μM, as shown in Figure 1e. This result was confirmed using primary neuron cultures, where again, the minimal $A\beta_{1-42}$ peptide concentration to induce maximal Rac 1 activation was 1.25 μM (Figure 1f). These results prompted us to characterize the signaling cascades used by the $A\beta_{1-42}$ peptide to activate Rac 1 and to determine whether they are involved in neuronal cell death.

Signaling cascade activated by $A\beta_{1-42}$ peptide: the PI3K and Rac 1 connection. PKC family is one of the most important serine–threonine kinase families implicated in early intracellular signal transduction. We initiated the study examining whether Rac 1 activation mediated by $A\beta_{1-42}$

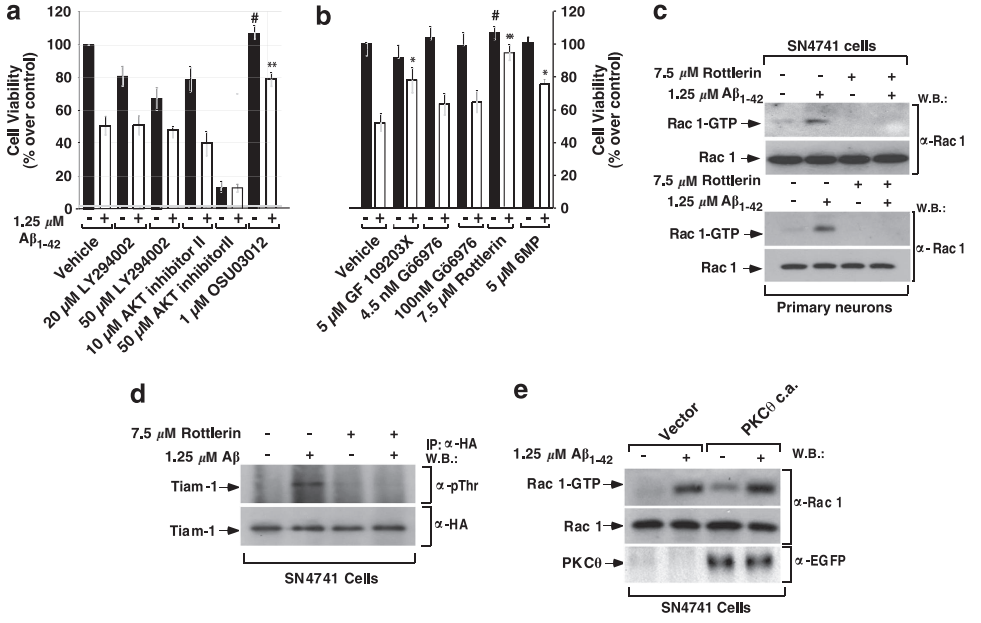

Figure 3 Phosphatidylinositol 3-kinase (PI3K)/ phosphoinositol-dependent kinase (PDK)1/novel protein kinase C (nPKC)/Rac 1 pathway is a target to block SN4741 apoptosis induced by 1–42 β-amyloid ($A\beta_{1-42}$) peptide *in vitro*. (**a**, **b**) SN4741 cells were pretreated or not with pharmacological inhibitors for PI3K, AKT, PDK1, PKC or Rac 1 for 1 h and treated with $A\beta_{1-42}$ peptide oligomers (+) or not (−) for additional 24 h. Results show the mean of: (**a**) six independent experiments ± s.e.m. (**$P<0.01$, comparing $A\beta_{1-42}$ peptide vs OSU03012 treatment, #$P<0.05$, comparing untreated vs OSU03012 treatment) and (**b**) four independent experiments ± s.e.m. (*$P<0.05$, **$P<0.01$, comparing $A\beta_{1-42}$ peptide vs inhibitors treatments, #$P<0.05$, comparing untreated vs Rottlerin treatment). (**c**) SN4741 cells (upper panels) and primary neurons (lower panels) were pretreated with 7.5 μM Rottlerin (+) or not (−) for 1 h and then treated (+) or untreated (−) with 1.25 μM $A\beta_{1-42}$ peptide for 30 min and lysed. Cell extracts were used to measure Rac 1 activation, and upper and lower second panels show total Rac 1 in cell lysates. Results are representative of four independent experiments. (**d**) Tiam-1 threonine phosphorylation induced by $A\beta_{1-42}$ peptide requires the involvement of PKCs. Transfected cells were pretreated with 7.5 μM Rottlerin (+) or not (−) for 1 h and then treated (+) or left untreated (−) with 1.25 μM $A\beta_{1-42}$ peptide for 30 min and lysed, cell extracts were subjected to immunoprecipitation with anti-hemagglutinin (HA) antibodies and immunoreactive bands were visualized using anti-phosphothreonine and anti-HA antibodies. Results are representative of three independent experiments. (**e**) SN4741 cells were transfected with an empty vector or a plasmid encoding the constitutively active form of PKCθ and the effects on Rac 1 activation induced by 1.25 μM $A\beta_{1-42}$ peptide were studied. Immunoreactive bands were visualized using anti-Rac 1 antibodies. Results are representative of three independent experiments. EGFP, enhanced green fluorescent protein; IP, immunoprecipitation; LY294002, (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one); 6-MP, 6-mercaptopurine; W.B., western blot.

peptide involved PKCs' activation. To this end, cell cultures were pretreated for 1 h with 1 μM GF 109203X, a broad-spectrum inhibitor for PKC family members,[33] followed by treatment with $A\beta_{1-42}$ peptide for 30 min. Active Rac 1 was pulled down and visualized as described above. Here, inhibition of PKC activity inhibited Rac 1 activation stimulated by $A\beta_{1-42}$ peptide. Results were obtained with both: SN4741 cells and primary neurons (Figure 2a, upper left and right panels, respectively). To verify a putative connection between PKC and Rac 1 activation in these cellular systems, PMA, a natural analog of DAG, was used to activate directly PKC. PMA was able to induce Rac 1 activation in both cellular models (Figure 2b upper left and right panels, lanes 2 and 6). Accordingly, PMA-stimulated Rac 1 activation was blocked when cells were pretreated with GF 109203X (Figure 2b, upper left and right panels, lanes 4 and 8). It is well established that protein kinase D (PKD) requires PKCs to be phosphorylated and become active.[34] Thus, to further understand the involvement of PKC in Rac 1 activation stimulated by $A\beta_{1-42}$ peptide, the phosphorylation state of PKD was examined in the same samples used to check the involvement of PKC in Rac 1 activation stimulated by $A\beta_{1-42}$ peptide. Results show that $A\beta_{1-42}$ peptide treatment correlated with an increase in PKD phosphorylation (Figure 2c, third panels, lanes 2 and 5). These results suggest an active role of PKCs in the $A\beta_{1-42}$ peptide-stimulated Rac 1 activation.

To identify additional components of this novel signal-transduction pathway, a potential implication of PI3K was examined. SN4741 cells were pretreated for 1 h with 20 μM LY294002, a specific inhibitor of PI3Ks, followed by incubation with 1.25 μM $A\beta_{1-42}$ peptide for 30 min. Results show that LY294002 blocked Rac 1 activation induced by $A\beta_{1-42}$ peptide (Figure 2c, first panel on the left, lane 3). Similar results were obtained using primary neurons (Figure 2c, first panel on the right, lane 6). This result suggests that PI3K mediates Rac 1 activation stimulated by $A\beta_{1-42}$ peptide treatment in SN4741 cells. Furthermore, pretreatment of SN4741 and primary neuron monolayers with LY294002 resulted in a decrease in PKD phosphorylation (Figure 2c, third panels, left and right, lanes 3 and 6, respectively).

The relationship between PI3K, PKC and Rac 1 activation in SN4741 and primary neurons was further probed using PMA, as described before. PMA was able to induce Rac 1 activation in both cellular models (Figure 2d, upper left and right panels, lanes 2 and 6). Moreover, Rac 1 activation was not blocked when cells were treated with LY294002 (Figure 2d, upper left and right panels, lanes 4 and 8). Finally, PI3K inhibitor did not

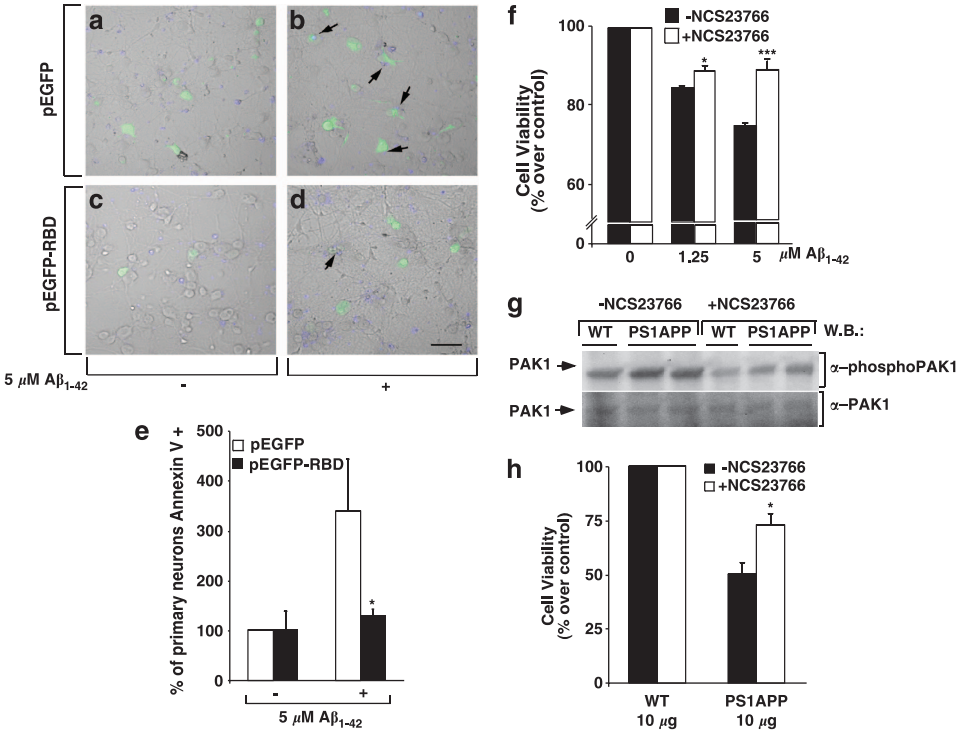

Figure 4 1–42 β-Amyloid ($A\beta_{1-42}$) peptide-induced apoptosis in primary cultures of neurons requires Rac 1 pathway involvement. Confocal images of primary neurons, showing apoptotic cells. Arrows identify annexin V^+ (blue) cells in (**a**) enhanced green fluorescent protein (EGFP)$^+$ and untreated cells, (**b**) EGFP$^+$ and $A\beta_{1-42}$ peptide-treated cells, (**c**) EGFP-RBD$^+$ and untreated cells and (**d**) EGFP-RBD$^+$ and $A\beta_{1-42}$ peptide-treated cells. Scale bar: 50 μm. Histogram bars (**e**) represents normalized mean ± s.e.m.% of annexin V^+ primary neurons cultures ($n = 3$), $^*P < 0.05$ comparing EGFP$^+$- vs EGFP-RBD$^+$-treated cells. Scale bar: 50 μm. (**f**) Synthetic $A\beta_{1-42}$ oligomers reduce cortical neuronal cell viability through Rac1. Results represent the mean ± s.d. of three independent experiments, $^*P < 0.05$ and $^{***}P < 0.001$ comparing 1.25 and 5 μM $A\beta_{1-42}$ peptide vs NCS23766 (*N*-(*N*-(3,5-difluorophenacetyl-L-alanyl))-*S*-phenylglycine) treatment, respectively. (**g**) Soluble brain fractions, derived from 18-month-old PS1APP mice induce p21-activated kinase (PAK)1 phosphorylation. PAK1 phosphorylation levels (Ser199/204) were determined by western blot (W.B.). Membrane was stripped and probed with anti-PAK1 antibody (lower panel). Results are representative of two independent experiments. (**h**) Soluble brain fractions, derived from 18-month-old PS1APP mice, reduce cortical neuronal cell viability through Rac 1. Results represent the mean ± s.d. of three independent experiments $^*P < 0.05$ comparing 10 μg PS1APP vs NCS23766 treatment.

affect PKD phosphorylation (Figure 2d, third panels, lanes 4 and 8). Taken together, these results suggested that PI3K is upstream of PKC-mediated Rac 1 activation.

These results prompted us to investigate further the link between PI3K and PKC initiated by $A\beta_{1-42}$ peptide. A well-described enzyme with the ability to connect these two kinases is the phosphoinositol-dependent kinase 1.[35,36] At this point, we hypothesized that PDK1 could be our candidate connecting PI3K and PKC. To test this hypothesis, we used the pharmacological inhibitor for PDK1, OSU-03012. SN4741 cells were pretreated for 1 h with 10 μM OSU-03012 followed by 1.25 μM $A\beta_{1-42}$ peptide treatment for 30 min. Results showed that OSU-03012 blocked Rac 1 activation stimulated by the $A\beta_{1-42}$ peptide (Figure 2e, first panel left, lane 2 vs lane 3). Similar results were obtained with primary neurons (Figure 2e, first panel right, lane 5 vs lane 6). Furthermore, $A\beta_{1-42}$ peptide stimulated PKD phosphorylation (Figure 2e, third panels, lanes 2 and 5) was blocked by OSU-03012, both in SN4741 cells (Figure 2e, third panel left, lane 3) and in primary neurons (Figure 2e, third panel right, lane 6). These results suggest that PDK1 may have a role in the signaling pathway that leads to Rac 1 activation in response to $A\beta_{1-42}$ peptide stimulation.

$A\beta_{1-42}$ peptide activates PI3K/PDK1/PKC/Rac 1 pathway leading to neuronal death: potential therapeutic targets. A detailed study of the PI3K/PDK1/PKC/Rac 1 signaling pathway controlled by the $A\beta_{1-42}$ peptide and its putative role on neuronal death was carried out. First, examination of signaling pathway regulated by PI3K and its downstream targets, PDK1 and AKT, was performed. To this end, SN4741 cells were pretreated with inhibitors for 1 h before $A\beta_{1-42}$ peptide addition and maintained in culture for further 24 h. Finally, cell viability was determined by an MTT assay. As shown in Figure 3a, LY294002 alone decreased SN4741 cell viability in a dose-dependent manner. Consequently, the inhibitor did not prevent cell death induced by the $A\beta_{1-42}$ peptide. The observed effect was more pronounced when using AKT inhibitor II than the effect of the $A\beta_{1-42}$ peptide alone. At 50 μM AKT inhibitor II cell viability was reduced to $<20\%$. This indicated that both LY294002 and AKT inhibitor II were toxic to cells on their own. On the other hand, 1 μM OSU03012 (PDK1 inhibitor) alone increased SN4741 cell viability compared to untreated cells, but more importantly, pretreament of cells with 1 μM OSU03012 prevented the toxic effect induced by 1.25 μM $A\beta_{1-42}$ peptide, increasing cell survival from 50.28 to 78.83% (Figure 3a).

Next, we examined whether PKCs' were involved in this neurotoxic pathway initiated by the $A\beta_{1-42}$ peptide. We showed that GF 109203X was able to inhibit activation of Rac 1 upon $A\beta_{1-42}$ peptide stimulation. GF 109203X inhibits both classical and novel PKCs.[33,37–39] To differentiate between classic and novel PKCs, Gö 6967, an inhibitor of classic PKCs (mainly α and β), was chosen, and Rottlerin, which was initially described as a selective inhibitor of the novel PKC isoform δ was[40] subsequently described to inhibit PKCθ also.[41,42] As shown in Figure 3b, pretreatment of SN4741 cells with GF 109203X was partially able to protect cells against toxicity caused by the $A\beta_{1-42}$ peptide. Cell cultures treated with 1.25 μM $A\beta_{1-42}$ peptide showed 50.28% viability; however, GF 109203X protected cell cultures increasing their viability to 77.96%. In contrast, Gö 6967 did not prevent the toxic effect induced by the peptide, neither at 4.5 nM nor at 100 nM (Figure 3b). Strikingly, 7.5 μM Rottlerin alone not only increased SN4741 cells viability compared with untreated cells but also under the experimental conditions described. Rottlerin also completely blocked the toxic effect produced by the $A\beta_{1-42}$ peptide while maintaining cell viability close to 100% (Figure 3b). Finally, we determined the role of Rac 1 in $A\beta_{1-42}$ peptide-induced toxicity using 6-MP, a compound described to be a Rac 1 inhibitor.[43] Cells were pretreated for 1 h with 5 μM 6-MP before adding the $A\beta_{1-42}$ peptide and cells viability was analyzed after 24 h. As shown in Figure 3b, blocking Rac 1 activity by 6-MP partially prevented

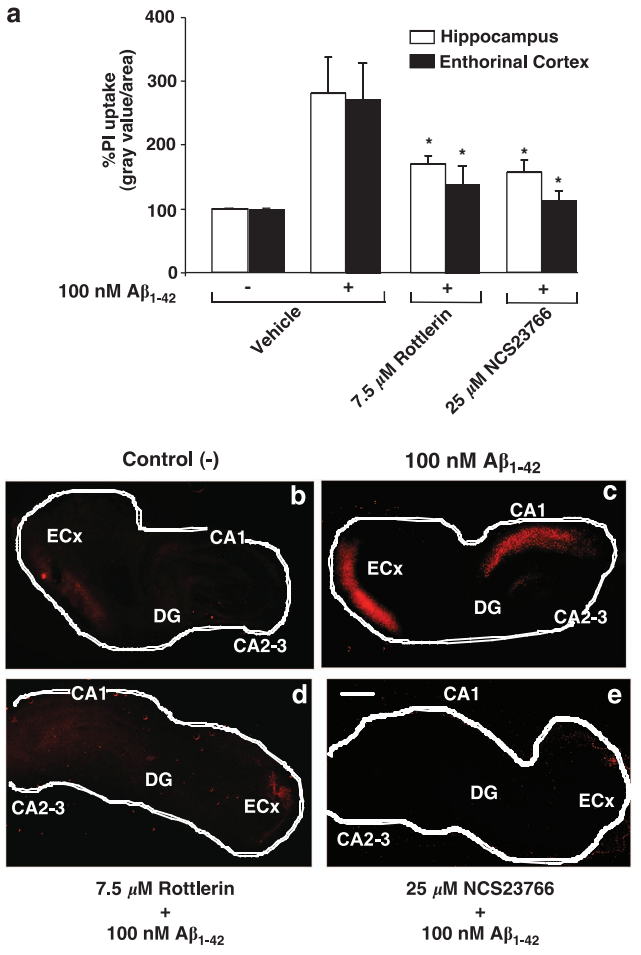

Figure 5 nPKCs and Rac 1 are molecular targets that can block apoptosis of neurons induced by 1–42 β-amyloid ($A\beta_{1-42}$) peptide. (**a**) Histogram and representative fields showing (**b**) untreated, (**c**) $A\beta_{1-42}$ peptide (100 nM, 4 days) toxicity in cultures treated after seven divisions and (**d**, **e**) protection when oligomers are applied in combination with 7.5 μM Rottlerin and 25 μM NCS23766 (*N*-(*N*-(3,5-difluorophenacetyl-L-alanyl))-*S*-phenylglycine), respectively; scale bar in (**e**) represents 500 μm. (**a**) Bars (gray value per area) represent neuronal apoptosis occurring in $A\beta_{1-42}$ peptide-treated hippocampus and entorhinal cortex. Bars in (**a**) represent the mean ± s.e.m. out of five independent experiments for Rottlerin and three independent experiments for NCS23766. $^*P<0.05$ comparing $A\beta_{1-42}$ peptide vs Rottlerin or NCS23766 treatment. ECx, entorhinal cortex; CA1, cornu ammonis 1; CA2-3, cornu ammonis 2-3 of the hippocampus; DG, dentate gyrus; PI, propidium iodide.

the toxicity induced by the $A\beta_{1-42}$ peptide. Typically, this peptide reduced cell culture viability to 50.80%; however, Rac 1 inhibition of the $A\beta_{1-42}$ peptide-stimulated pathway increased cell culture viability by 25% from 50.80 to 75.52%.

To examine whether nPKCs mediated $A\beta_{1-42}$ peptide-stimulated Rac 1 activation, the effect of Rottlerin was tested. Treatment of SN4741 cells with this inhibitor prevented Rac 1 activation induced by the $A\beta_{1-42}$ peptide (Figure 3c, upper first panel, lane 4 vs lane2). $A\beta_{1-42}$ peptide also stimulated Rac 1 activation in primary cultures of neurons (Figure 3c, lower first panel, lane 2). The stimulatory effect of $A\beta_{1-42}$ peptide on Rac 1 activation in these cell cutures was also abrogated by treatment with Rottlerin (Figure 3c, lower first panel, lane 4). Small GTPases activation, including Rac 1, are regulated by guanine nucleotide exchange factors.[2] Some guanine nucleotide exchange factors for Rac 1 have been described to be activated by tyrosine phosphorylation mediated by tyrosine kinases, such as Vav proteins,[44] by threonine phosphorylation mediated by PKCs, such as Tiam-1,[45] or by serine/threonine phosphorylation mediated by PKA, such as STEF/Tiam-2.[46] Our results indicate that Rac 1 activation was mediated by PI3K/PDK1/PKCs. This scenery prompted us to test if Tiam-1 could be the link between PKCs and Rac 1 in the $A\beta_{1-42}$ peptide neurotoxic pathway. To this end, SN4741 cells overexpressing HA-Tiam-1 were pretreated for 1 h with 7.5 μM Rottlerin, followed by 1.25 μM $A\beta_{1-42}$ peptide for 30 min. Cells were lysed and cell lysates were immunoprecipitated as described in the Materials and methods section. As shown in Figure 3d (line 2), $A\beta_{1-42}$ peptide induced Tiam-1 threonine phosphorylation, whereas Rottlerin prevented Tiam-1 threonine phosphorylation induced by $A\beta_{1-42}$ peptide (Figure 3d, lane 4).

Results presented in Figure 3 suggest that $A\beta_{1-42}$ peptide stimulates Tiam-1/Rac 1 pathway activation through novel PKCs. To examine this possibility further, SN4741 cells were transiently transfected with empty vector as control and with cDNA encoding the PKCθ isoform, which had been rendered constitutively active.[47] Equal Rac 1 expression levels in SN4741-transfected cells were verified by immunoblotting. As shown in Figure 3e, overexpression of the constituvely active isoform of PKCθ was enough to induce Rac 1 activation in SN4741 cells (Figure 3e, lane 3). PKCθ overexpression potentiated $A\beta_{1-42}$ peptide-stimulated Rac 1 activation (Figure 3e, lane 2 compared with lane 4). However, it was not possible to detect any effects when cells were transfected with cDNA encoding for PKCα and PKCε (data not shown). Equal levels of Rac 1 expression in SN4741-transfected cells was verified by immunoblotting.

Taken together, these results suggest that the novel PKCs mediate Tiam-1/Rac 1 activation induced by the $A\beta_{1-42}$ peptide, and point to Rac 1 as a key player in transducing the toxic message initiated by the $A\beta_{1-42}$ peptide.

Role of Rac 1 in neuronal cell death induced by the $A\beta_{1-42}$ peptide. Effector molecules for small GTPases contain a region which binds specifically to the active conformation of the GTPases.[30] With this in mind, it was hypothesized that overexpression of RBD of PAK1 in primary cultures of neurons could block death signaling induced by $A\beta_{1-42}$ peptide, as this RBD would bind to the active configuration of Rac 1 blocking the signal-transduction pathway through Rac 1 emanating from exposure to the $A\beta_{1-42}$ peptide.

As shown in Figure 4, $A\beta_{1-42}$ peptide significantly increased neuronal apoptosis from basal level (100%) ($EGFP^+$-untreated cells) to 340 ± 119% (Figures 4 a, b and e, empty bars). However, when we examined the effect of $A\beta_{1-42}$ peptide on neurons overexpressing RBD of PAK, we observed that the construct blocked the toxic effect of $A\beta_{1-42}$ peptide, as there was no significant difference beween EGFP-RBD^+-untreated (100 ± 46%) and -treated cells (136 ± 26%) (Figures 4 c, d and e, solid bars). Prompted by these findings, the impact of the Rac 1 inhibitor NCS23766[48] on the Rac 1-mediated neuronal death induced by $A\beta_{1-42}$ peptide was examined. To this end, primary neurons were pretreated or not with 25 μM NCS23766 for 1 h followed by $A\beta_{1-42}$ peptide treatment for 24 h and cell viability was determined by an MTT assay. As shown in Figure 4f (solid bars), cell viability correlated inversely with the concentration of $A\beta_{1-42}$ peptide added. However, NCS23766 efficiently blocked the toxic effect of the $A\beta_{1-42}$ peptide (Figure 4f, empty bars).

To strengthen the results obtained with $A\beta_{1-42}$ synthetic peptide, the ability of soluble brain extracts derived from 18-month-old PS1APP transgenic mice (natural oligomers) to stimulate Rac 1 activation was also investigated. To this end, the effects of 10 μg of soluble brain extracts from 18-month-old WT and PS1APP mice on PAK1 phosphorylation were determined. As shown in Figure 4g, incubation of cell cultures with 10 μg of soluble brain fractions from PS1APP mice for 30 min induced a robust phosphorylation of PAK1 compared with soluble extract from WT mice. This phosphorylation was mediated by the activation of Rac 1, as NCS22766 prevented PAK1 phosphorylation induced by the soluble brain extracts from PS1APP mice (Figure 4g, compare lines 2 and 3 with 5 and 6).

In addition, we also determined the effect of 18-month-old mice-derived soluble fractions on neuronal cell death. Primary neurons were pretreated or not with 25 μM NCS23766 for 1 h followed by exposition to soluble brain extracts from 18-month-old WT and PS1APP mice for 24 h. Cell viability was determined by an MTT assay. As expected, soluble fractions of PS1APP mice reduced to 50% the cell viability (Figure 4h, solid bars 100 ± 0.1% vs 50 ± 2.5%). However, NCS23766 blocked the toxic effect induced by these soluble brain extracts (Figure 4f, solid bar 50 ± 2.5 vs 75 ± 2.4). WT-derived soluble fractions did not have any measurable effect on neuronal cell death.

Taken together, these results show that both the synthetic $A\beta_{1-42}$ peptide, as well as brain-derived soluble fractions from PS1APP mice induced apoptosis in primary cultures of neurons and cell death in this context is actively dependent on Rac 1 activity.

Rottlerin (nPKC inhibitor) and NCS23766 (Rac 1 inhibitor) block $A\beta_{1-42}$ peptide induced neuronal cell death in organotypic cultures of the hippocampus and the entorhinal cortex. To determine whether these results could be reproduced under nearest to the neuronal physiological conditions as possible, we investigated the effects of nPKCs and Rac 1 inhibition in organotypic cultures of the

hippocampus and the entorhinal cortex treated with $A\beta_{1-42}$ peptide. Both cerebral structures are vulnerable in the initial stages of AD. Organotypic cultures were untreated or treated with 100 nM $A\beta_{1-42}$ peptide alone or together with 7.5 μM Rottlerin or 25 μM NCS23766 for 72 h. After which brain sections were stained with propidium iodide. As shown in Figure 5a (bars 3 and 4) and c, $A\beta_{1-42}$ peptide induced cell death in the hippocampus and in the entorhinal cortex. However, treatment with Rottlerin or NCS23766 efficiently prevented $A\beta_{1-42}$ peptide-induced cell death in both cerebral structures: in the hippocampus and in the entorhinal cortex (Figure 5a (bars 5–8) and d,e). Our results describe for the first time how $A\beta_{1-42}$ peptide transduces cell death signals through PI3K/PKD1/nPKC/Rac 1 to induce neuronal cell death. This characterization also allows us to point out nPKC/Rac 1 as potential targets to revert the programmed cell death induced by the $A\beta_{1-42}$ peptide.

Discussion

Functional specificity of small GTPases of the Rho family in intracellular signaling pathways depends mainly on the cellular system, type of stimuli and their intracellular localization. Regarding the nervous system, GTPases of the Rho family participate actively controlling cytoskeleton dynamics in neurons, thereby modulating synaptic plasticity;[11] however, their molecular partners in $A\beta_{1-42}$ peptide-mediated neuronal toxicity are not known.

$A\beta_{1-42}$ peptide is the primary neurotoxic factor in the pathogenesis of AD and it can be present in different molecular structures. To date, the prevailing hypothesis has been the amyloid cascade,[49] where long insoluble polymers generated by aggregates of the β-amyloid peptide (40–42 amino acids), localized in senile plaques lead to neuronal death and thus are involved in the development of AD and other forms of dementia. However, presently there is additional evidence suggesting that amyloid insoluble fibrils are not the only toxic forms of the $A\beta_{1-42}$ peptide. It has been reported that ADDLs cause loss of short-term synaptic plasticity and selective neuronal death in the long term and this toxic form of the peptide has become increasingly more important in the understanding of AD neuropathogenesis.[27,50–52]

In this study, we show that ADDLs' engagement and natural oligomers from transgenic mice induce a robust Rac 1 activation, triggering a signaling cascade that results in cellular death. Importantly, we identified PI3K/PDK1/nPKCs as a specific upstream regulatory pathway for Rac 1 in $A\beta_{1-42}$ peptide-treated cells, and provide new evidence supporting, on the one hand, the relevance of this cellular death cascade controlled by ADDLs and on the other hand, the possibility of using pharmacological inhibition of these signaling molecules to prevent peptide-induced toxicity in neurons.

The best-characterized neuronal survival pathway described is the PI3K/AKT cascade[53] and it has been suggested that some neurodegenerative diseases such as schizophrenia and/or AD this signaling pathway is altered.[54–56] The expression of intracellular $A\beta_{1-42}$ peptide causes a decrease in the AKT phosphorylation and in the induction of apoptosis in neurons.[57] Recently, it has also been reported that ADDLs block PDK1-mediated AKT phosphorylation by preventing a direct interaction between them. The effect of rendering this antiapoptotic pathway non-functional leads to an increase in neuronal cell death.[58] Accordingly, we observed that when PI3K and AKT basal activities were blocked with specific inhibitors, cell viability was reduced and these pretreatments were unable to prevent $A\beta_{1-42}$ peptide-stimulated toxicity in neurons. Nevertheless, pretreatment of SN4741 cells and primary neurons with OSU03012, a PDK1 inhibitor, prevented not only Rac 1 activation but also cell death induced by the $A\beta_{1-42}$ peptide. These results suggest that PDK1 participates in other AKT-independent signaling pathways.

Indeed, PDK1 can also phosphorylate and activate other key intracellular signaling molecules, such as PKA,[59] p70-S6K[59] and some PKC family members: including the novel PKCs (δ and θ).[35,36] PKCs are critical components in the signal-transduction pathways that control not only cell survival[42,60] but also proapoptotic pathways in neurons.[61,62] It is still unknown how PKCs maintain proper balance between cell survival and apoptosis in neurons. Probably this equilibrium depends on the different stimuli reaching cells. As several studies have demonstrated, the novel PKCs are involved in programmed cell death signaling acting through proapoptotic signals, and the PKC inhibitor Rottlerin is able to protect cells from apoptosis induced by H_2O_2, ultraviolet radiation, taxol and other toxins.[63–67] In addition, it has been reported that the novel PKCδ is activated in response to oxidative stress stimulated by glutamate[61,62] and that PKCδ activation in retinal neurons contributes to apoptosis.[68,69] In agreement with this, we have found that inhibition of novel PKC (δ and θ) activities by Rottlerin prevent Rac 1 activation and apoptosis induced by the $A\beta_{1-42}$ peptide in SN4741 cells, neuronal primary cultures and organotypic cultures of the hippocampus and the entorhinal cortex. Therefore, we point out that nPKCs would be key molecules in cell signaling mediated by the $A\beta_{1-42}$ peptide and could be actively involved in neuronal degeneration in AD.

$A\beta_{1-42}$ peptide increases the intracellular concentration of reactive oxidative species, which cause permanent damage to brain structures.[70,71] Some mechanisms have been suggested by which $A\beta_{1-42}$ peptide could increase the concentration of reactive oxidative species. One of them proposed that Aβ would be activating the NADPH oxidase complex via the canonical pathway involving Rac 1, in both microglia and other cell types, including monocytes and neutrophils.[72,73] However, another mechanism would involve PI3K and PKC in the activation of NADPH oxidase by a non-canonical pathway.[74–76] It is also possible that the key to this process lies in another effector molecule for Rac 1, such as PAK. In this regard, Ma and co-workers[77] have reported that Rac 1 activation by soluble oligomeric $A\beta_{1-42}$ peptide leads to aberrant activation and membrane translocation of PAK. These authors demonstrate an aberrant activation of PAK in neurons from the hippocampus, inducing an alteration of dendritic spines that leads to a loss of synaptic contacts between cells. Related to this, we have found that inhibition of Rac 1 activity by 6-MP or NCS23766 prevent apoptosis induced by the $A\beta_{1-42}$ peptide and natural oligomers in SN4741 cells, neuronal primary cultures and organotypic

cultures of the hippocampus and the entorhinal cortex. Therefore, we postulate that Rac 1 could be another key molecule downstream of nPKCs in cell signaling mediated by the $A\beta_{1-42}$ peptide and it could be actively involved in neuronal degeneration in AD.

In conclusion, we have characterized the signaling pathway PI3K/PDK1/nPKCs/Rac 1 involved in cellular toxicity induced by the $A\beta_{1-42}$ peptide. Furthermore, this signaling pathway has allowed us to identify PDK1, nPKC and Rac 1, as putative therapeutic targets given that their inhibition is able to protect neurons from cell death induced by the $A\beta_{1-42}$ peptide or natural oligomers, albeit nPKCs inhibition do so more efficiently. However, the receptor system through which $A\beta_{1-42}$ peptide delivers death signals through PI3K/PDK1/nPKC/Rac 1 pathway is still unknown. There is mounting evidence indicating a direct interaction between the $A\beta_{1-42}$ peptide with the post-synaptic receptors, such as N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA0 and/or α-7-nicotine acetylcholine receptors.[78] Future studies will allow us to characterize the receptors and signaling molecules that participate upstream of this signal-transduction pathway.

Conflict of interest

The authors declare no conflict of interest.

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