Activation of the L Voltage-sensitive Calcium Channel by Mitogen-activated Protein (MAP) Kinase following Exposure of Neuronal Cells to β-Amyloid

MAP KINASE MEDIATES β-AMYLOID-INDUCED NEURODEGENERATION*

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Neuronal degeneration in Alzheimer’s disease (AD) has been variously attributed to increases in cytosolic calcium, reactive oxygen species, and phosphorylated forms of the microtubule-associated protein tau. β-Amyloid (βA), which accumulates extracellularly in AD brain, induces calcium influx in culture via the L voltage-sensitive calcium channel. Since this channel is normally activated by protein kinase A-mediated phosphorylation, we examined kinase activities recruited following βA treatment of cortical neurons and SH-SY-5Y neuroblastoma. βA increased channel phosphorylation; this increase was unaffected by the protein kinase A inhibitor H89 but was reduced by the mitogen-activated protein (MAP) kinase inhibitor PD98059. Pharmacological and antisense oligonucleotide-mediated reduction of MAP kinase activity also reduced βA-induced accumulation of calcium, reactive oxygen species, phospho-tau immunoreactivity, and apoptosis. These findings indicate that MAP kinase mediates multiple aspects of βA-induced neurotoxicity and indicate that calcium influx initiates neurodegeneration in AD. βA increased MAP kinase-mediated phosphorylation of membrane-associated proteins and reduced phosphorylation of cytosolic proteins without increasing overall MAP kinase activity. Increasing MAP kinase activity with epidermal growth factor did not increase channel phosphorylation. These findings indicate that redirection, rather than increased activation, of MAP kinase activity mediates βA-induced neurotoxicity.

Alzheimer’s disease (AD) is a neurodegenerative disorder that affects the cognitive function of the brain. Pathological changes in AD are punctuated by the formation of amyloid plaques and neurofibrillary tangles as well as extensive neuronal loss (1). Amyloid plaques, which accumulate extracellularly, are composed of aggregated β-amyloid (βA; Refs. 2 and 3).

This 42-amino acid peptide is generated by alternate cleavage from the larger “amyloid precursor protein” (4, 5). βA is either directly toxic to neurons in culture or potentiates neuronal vulnerability to excitatory neurotoxins (e.g. Refs. 6 and 7). These effects may derive from the formation of ion channels within the cell membrane by βA, fostering direct leakage of calcium into cells (8). Additional studies indicate that, rather than forming de novo calcium channels, βA induces calcium influx via existing channels (9), including the L voltage-sensitive calcium channel (10). Increased calcium influx, with resultant disruption of calcium homeostasis, may be the underlying cause of βA neurotoxicity and ultimate neurodegeneration (7, 11–15).

A consequence of disruption in calcium homeostasis is the induction of oxidative stress and accumulation of free radicals, collectively referred to as reactive oxygen species (ROS; Ref. 15). One target of ROS is the phospholipid membrane (16–18). Cumulative ROS-induced membrane damage compromises membrane integrity and increases the permeability of several ions, including calcium; resultant calcium influx is a crucial factor in neurodegeneration (19). Calcium influx, moreover, promotes recruitment of other ion channels and generation of additional ROS, resulting in neuronal excitotoxicity (20–22). Importantly, ROS accumulation fosters calcium influx, which, in turn, fosters yet further ROS accumulation (23). βA-aberrant calcium influx may therefore initiate a cascade of cytosolic calcium and ROS accumulation. In this regard, additional studies have attributed βA toxicity to ROS accumulation (24–26). Since the L voltage-sensitive calcium channel (activated by βA; Ref. 10) is activated by phosphorylation (27), elucidation of kinase pathways activated by βA represents one potential approach toward understanding βA-induced neurodegeneration.

In the present study, we demonstrate that βA induces calcium influx by MAP kinase-mediated phosphorylation of the L voltage-sensitive calcium channel and that inhibition of MAP kinase activity prevents multiple known aspects of βA toxicity.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—SH-SY-5Y human neuroblastoma cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro) containing 10% fetal bovine serum in 5% CO₂. Cultures were differentiated for 7 days with 10 μM retinoic acid, during which time they elaborate extensive neurites that exhibit characteristics of axons (28). Cortical neurons were harvested from day 16 embryonic mice and cultured on poly-L-lysine in Dulbecco’s modified Eagle’s medium/F-12 containing B27 supplements (Life Technologies, Inc.) in the absence of serum according to Ref. 29. Cells were utilized 3 days after plating, during which time they had established polarity and elaborated axons (e.g. Fig. 1). SH-SY-5Y cells and cortical neurons were deprived of serum and treated for 2 h with one or more of the following: βA1–40, βA25–35, and βA1–25.

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‡ These abbreviations used are: AD, Alzheimer’s disease; βA, β-amyloid; ROS, reactive oxygen species; MAP, mitogen-activated protein; BAPTA, bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid.
(Sigma; 10–25 μM for cortical neurons and 22–40 μM for SH-SY-5Y cells (9, 28)), the antioxidant vitamin E (0.15 mg/ml; Ref. 24), the calcium chelator BAPTA (1 mM; Molecular Probes, Inc., Eugene, OR), the MAP kinase inhibitor PD98059 (10–20 μM; RBI, Natick, MA; Ref. 30), the PKA inhibitor H89 (200 μM; Calbiochem; Ref. 31), or 10–12 μM epidermal growth factor to activate MAP kinase (32). βA25–35 and βA39–40 were initially dissolved in a small volume of Me2SO, and diluted to a final stock concentration of 200 μM in serum-free medium. βA25–35 and βA39–40 were then aggregated overnight at 37 °C before use. Cultures of SH-SY-5Y cells were radiolabeled by incubation of 100 μCi of [32P]orthophosphate in culture medium during these incubations as described previously (33). Additional cultures were incubated with oligonucleotides corresponding to the sequence of MAP kinase in sense (ATG GCG GCG GCG GCG GCG GCT) or antisense (AGC CGC CGC CGC CGC CGC CAT) orientation; these antisense oligonucleotides have previously been demonstrated effective at down-regulation of MAP kinase steady-state levels (34). Oligonucleotides (32 μg) were incubated for 15 min at room temperature with 32 μl of Superfect (Qiagen) and stored in a Macintosh Power PC 7100AV (e.g. Refs. 35 and 36). Analysis was carried out with NIH Image software (28).

**Monitoring of Intracellular Calcium Concentrations**—Intracellular calcium concentration was monitored as described previously (28) by incubation with Fluo-3 (acetoxymethyl ester; Molecular Probes) for 30 min. Images were captured using a DAGE CCL-72 cooled CCD camera via a Scion LG-3 frame grabber operated by NIH Image analysis software and stored in a Macintosh Power PC 7100AV (e.g. Refs. 35 and 36). Analysis was carried out with NIH Image software (28).

**Monitoring of Intracellular Peroxide Concentrations**—Cultures were treated with 2,7’-dichlorofluorescein diacetate (Eastman Kodak Co.) for 20 min. The cultures were rinsed with serum-free medium. Intracellular peroxide levels were measured in individual cells in multiple cultures under a fluorescence optic (24). The antioxidant vitamin E (0.15 mg/ml) was included in some cultures to curtail intracellular ROS accumulation, and additional cultures received 10 mM H2O2 to induce ROS (24).

**Immunofluorescence**—Cells fixed for 15 min with 4% paraformaldehyde in 0.1 M phosphate buffer and immunostained by sequential reaction with a mouse monoclonal antibody (PHF-1; generous gift of Dr. Peter Davies) raised against tau from paired helical filaments from AD brains, followed by rhodamine-conjugated goat anti-mouse IgG and visualization by standard methods (37). Identical results were obtained following substitution of methanol for paraformaldehyde. Additional controls, which yielded only background fluorescence, included substitution of nonimmune murine IgG for PHF-1 or omission of primary antibody.

**Analysis of Membrane and Cytosolic Protein Phosphorylation**—Cells were harvested by scraping with a rubber policeman, and membrane preparations were generated by centrifugation of the homogenate for 15 min at 13,000 × g (38). Aliquots of resultant membranes (pellet) and cytosolic (supernatant) proteins were subjected to SDS-gel electrophoresis and autoradiography. The L voltage calcium channel was immunoprecipitated from membrane preparations using a mouse antidiarylsphingosine antibody specific for this channel (Upstate Biotechnology Inc., Lake Placid, NY; Ref. 38) followed by protein G-Sepharose via standard procedures (36, 38). Immunoprecipitated material was subjected to SDS-gel electrophoresis. Dried gels were placed against X-Omat film (Kodak) to generate autoradiographs.

**Analysis of MAP Kinase Activity**—βA-treated and untreated SH-SY-5Y cultures were rinsed, scraped from the plate, and homogenized in 1% Triton X-100 in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 0.5 mM Na3VO4, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerol phosphate, and 0.1% 2-mercaptoethanol. Aliquots (200 μg) were subjected to immunoblot analysis with antibodies specific for phosphorylated p42 and p44 MAP kinase (NE BioLabs, Beverly, MA) which provided an index of relative levels of phosphorylated (active) kinase. MAP kinase activity was assayed via the "MAP kinase immunoprecipitation kinase cascade assay kit" according to the manufacturer’s instructions (catalog no. 17-184; Upstate Biotechnology). Briefly, MAP kinase was immunoprecipitated from additional aliquots (500 μg) by incubation for 2 h with anti-RAV MAP kinase. βA treatment altered intracellular kinase activities in cultured neurons and neuroblastoma. 

**RESULTS**

**βA Treatment Alters Intracellular Kinase Activities**—Our prior studies demonstrated that treatment of SH-SY-5Y human neuroblastoma cells and mouse cortical neurons with 40 and 25 μM βA, respectively, induced widespread neurodegeneration within 2 h (9). However, treatment of SH-SY-5Y cells and cortical neurons for 2 h with lower βA concentrations (22 and 10 μM, respectively, did not invoke significant overt cell loss for 24 h, which provided an experimental window to monitor upstream events prior to degeneration. Treatment with these lower concentrations for 30 min to 2 h induced accumulation of phosphorylated tau epitopes common to those that accumulate in affected neurons in AD (Fig. 1). In addition, a 2-h treatment of SH-SY-5Y human neuroblastoma cells with βA induced a >6-fold increase in phosphorylation of membrane-associated proteins (Fig. 2). These findings indicated these culture systems could serve as models for probing the intracellular consequences of βA toxicity.

**Phosphorylation of the L Voltage-sensitive Calcium Channel by βA**—Since calcium influx following βA exposure has been attributed to activation of the L voltage-sensitive calcium channel (10), we examined potential kinases affected by βA treatment. Immunoprecipitation of the channel followed by autoradiography confirmed that βA increased phosphorylation of the channel by >2-fold (Fig. 3). However, the channel is normally regulated during synaptic transmission by PKA, inclusion of the PKA inhibitor, H89, at up to twice the concentration reported to inhibit PKA (31) did not diminish βA-induced channel phosphorylation (Fig. 3; Table 1), suggesting that βA invoked an alternate kinase to phosphorylate this channel.
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**Fig. 2.** βA induces an increase in phosphorylation of membrane proteins. Representative autoradiograph of membrane preparations derived from SH-SY-5Y cells incubated with [32P]orthophosphate for 2 h in the presence and absence of 22 μM βA, 10 μM PD98059, or both. The accompanying graph presents densitometric analysis of these samples. Note that βA induces an approximately 2-fold increase in phosphorylation of membrane proteins and that co-treatment with the MAP kinase inhibitor P998059 attenuated this increase.

**Fig. 3.** βA induces phosphorylation of the L voltage calcium channel. Representative autoradiograph of material immunoprecipitated by an antibody directed against the L voltage-sensitive calcium channel from SH-SY-5Y cells incubated with [32P]orthophosphate for 2 h in the presence and absence of 22 μM βA, 10 μM PD98059, or both. The accompanying graph presents densitometric analysis of 3–5 such samples. Note that βA induces an approximately 2-fold increase in phosphorylation of the L voltage-sensitive calcium channel. The MAP kinase inhibitor PD98059 attenuated this increase, but the PKA inhibitor H89 did not. Treatment of cells for 2 h with 10 μM epidermal growth factor to activate MAP kinase did not result in increased channel phosphorylation.

**Table I**

Phosphorylation of the L voltage-sensitive calcium channel by βA

| Culture conditions | Incorporation of [32P] into channel | Fold change vs. control | p value vs. control | p value vs. βA |
|--------------------|-------------------------------------|-------------------------|---------------------|---------------|
| Control            | 137,317 ± 16,705                    | 2.2 ± 0.08              | 0.002               |               |
| βA                 | 302,312 ± 23,266                    | 1.46 ± 0.11             | 0.006               |               |
| PD98059            | 156,849 ± 22,559                    | 1.14 ± 0.14             | 0.512               | 0.008         |
| H89                | 200,448 ± 21,862                    | 1.46 ± 0.11             | 0.008               | 0.050         |
| PD98059 + βA       | 142,203 ± 8178                      | 1.04 ± 0.06             | 0.827               | 0.004         |

*Values present density of radiolabel associated with the L voltage-sensitive calcium channel immunoprecipitated from cultures treated with [32P]orthophosphate. Values represent the mean ± S.E., expressed in arbitrary units, derived from at least three experiments.

*Calculated by dividing the autoradiographic density of treated samples by that of control samples.

Student’s t test.

**Inhibition of MAP Kinase Prevented βA-induced Phosphorylation of the L Voltage-sensitive Calcium Channel and of Membrane Proteins—**Unlike the PKA inhibitor H89, PD98059, which inhibits MAP kinase (the immediate upstream activator of MAP kinase; Fig. 4; see also Ref. 30), diminished βA-induced channel phosphorylation (Fig. 3, Table I). Moreover, PD98059 reduced overall phosphorylation of membrane-associated proteins following βA treatment (Fig. 2). As expected, cell-free assays of MAP kinase activity revealed considerable constitutive MAP kinase activity in non-βA-treated SH-SY-5Y cells; no further increase was observed in cells treated for 2 h with βA (Fig. 5; but see also Ref. 39). These findings suggested, at least under conditions of prior activation of MAP kinase, that βA can redirect MAP kinase to phosphorylate the L voltage-sensitive calcium channel without necessarily invoking a significant increase in overall MAP kinase activity. In support of this conclusion, we observed that, along with increasing phosphorylation of membrane-associated proteins, βA induced a compensatory decrease in phosphorylation of cytosolic proteins (Fig. 6). Moreover, PD98059 diminished this decrease in cytosolic protein phosphorylation along with inhibiting the increase in membrane-associated protein phosphorylation.

**Inhibition of MAP Kinase Prevented βA-induced Degenerative Changes—**We next examined whether or not MAP kinase mediated additional aspects of βA toxicity by pharmacological and antisense oligonucleotide-mediated inhibition of MAP kinase activity and steady-state levels. SH-SY-5Y human neuroblastoma cells and mouse cortical neurons were treated for 30 min to 2 h with and without 22 μM (for neuroblastoma) or 10 μM (for neurons) βA25–35 or βA1–40 in the presence and absence of vitamin E, BAPTA, and 10 μM PD98059 and assayed for calcium influx, ROS and PHF-1 immunoreactivity (Figs. 7 and 8). Both βA peptides induced an increase in intracellular calcium, ROS, and phospho-tau immunoreactivity in both culture types (Figs. 7 and 8). Maximal cytosolic calcium levels were obtained prior to maximal levels of ROS and PHF-1 in both cell types (Fig. 7). The intracellular calcium chelator, BAPTA, inhibited the βA-induced increase in intracellular calcium, ROS, and PHF-1 (p < 0.001; Fig. 8). The antioxidant, vitamin E, blocked accumulation of ROS and PHF-1 (p < 0.001) but did not significantly lower the level of the βA-induced increase in intracellular calcium (Fig. 8). These latter findings suggested that accumulation of ROS and phospho-tau immunoreactivity may be secondary effects of the βA-induced increase in intracellular calcium. Antisense oligonucleotide-mediated reduction in SH-SY-5Y MAP kinase levels (Fig. 9) also diminished βA-induced cytosolic accumulation of calcium, ROS, and PHF-1 (Fig. 10). While short term (i.e., 2-h) incubation with βA at this relatively low concentration does not induce overt neuronal degeneration (10), continued treatment for 24 h results in approxi-
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Fig. 5. MAP kinase is constitutively active in differentiated SH-SY-5Y cells and its activity is not increased by short term βA treatment. Homogenates of untreated and βA-treated SH-SY-5Y cells were subjected to immunoblot analysis with an antibody that recognizes only phosphorylated MAP kinase (top panel) and a MAP activity assay using myelin basic protein as a substrate (lower panel). Note that phosphorylated MAP kinase is present in untreated SH-SY-5Y cells as evidenced by immunoblot analysis; the activity of this kinase was further confirmed by its ability to phosphorylate myelin basic protein (see also Fig. 4). Note further that no additional increase in MAP kinase activity (as indicated by lack of increase in phosphoisoforms and activity) was induced following βA treatment.

Fig. 6. βA reduces MAP kinase-mediated phosphorylation of cytosolic proteins. Representative autoradiograph of cytosolic proteins derived from SH-SY-5Y cells incubated with [32P]orthophosphate for 2 h in the presence and absence of 22 μM βA, 10 μM PD98059, or both. The accompanying graph presents densitometric analysis of these samples. Note that βA induces a decrease in phosphorylation of cytosolic proteins and that co-treatment with the MAP kinase inhibitor PD98059 attenuated this decrease.

Fig. 7. Pharmacological inhibition of MAP kinase diminishes βA-induced increases cytosolic calcium, ROS, and phospho-tau immunoreactivity. Densitometric data derived from multiple fields of SH-SY-5Y cells and cortical neurons following treatment for 30 min to 2 h with βA (10 μM for cortical cultures, 22 μM for SH-SY-5Y cells) in the presence and absence of 10 μM PD98059. Cells were processed to visualize cytosolic calcium, ROS, and PHF-1, and the relative fluorescence intensity of 50–100 cells in multiple fields was quantified via NIH Image software as described under “Experimental Procedures.” Values are presented as the mean ± S.E. of the mean. Note the increase in cytosolic calcium, ROS, and phospho-tau immunoreactivity following βA treatment and the attenuation of these increases by PD98059. Note further that maximum levels of cytosolic calcium were attained before those of ROS and PHF-1 following βA treatment of both cell types.

DISCUSSION

The findings of the present study confirm and extend the demonstration by Ueda and co-workers (10) that βA induces calcium influx via the L voltage-sensitive calcium channel. Our demonstration that βA-induced channel phosphorylation does not involve PKA, which is involved in synaptic channel activation under normal conditions (27), but rather is dependent upon MAP kinase, underscores that βA alters signal transduction pathways.

Altered signal transduction and aberrant kinase activities have been extensively studied with regard to tau phosphorylation. Several candidate intracellular tau kinases have been identified, including glycogen synthase kinase 3β (40–44) and cyclin-dependent kinase 5 (45, 44), calcium-calmodulin kinase (47), and protein kinase C (35, 36, 48, 49). Although MAP kinase clearly phosphorylates tau in cell-free analyses and, in doing so, increases phospho-tau immunoreactivity (49–51), the role of MAP kinase in intracellular tau phosphorylation remains controversial, since it has been reported to phosphorylate tau within cells in some (52–55) but not all studies (43, 44). The findings of the present study potentially shed light upon this controversy in that MAP kinase may not directly phosphorylate tau but instead may, by activating the L voltage-sensitive calcium channel, foster tau phosphorylation by other calcium-dependent kinases. Notably, the intracellular tau kinases, calcium-calmodulin kinase II and protein kinase C, are both calcium-dependent. In addition, prior phosphorylation of tau by CaM or protein kinase C facilitates subsequent phosphorylation by glycogen synthase kinase 3β (57, 58). MAP kinase-mediated calcium influx following βA treatment may therefore induce downstream activation of a succession of tau kinases. Similarly, βA-induced phosphorylation of the L voltage-sensitive calcium channel may involve additional kinases upstream and/or downstream of MAP kinase. In support of the
potential involvement of MAP kinase in AD, MAP kinase activity is detected within affected regions susceptible to AD neurodegeneration (59) and has been reported to be activated by 

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in AD (60), and at least one novel MAP kinase is detected within neurons (61). While 

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is capable of increasing MAP kinase activity (39), it is of interest that, under conditions of constitutive MAP kinase activity, 

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is capable of inducing this kinase to phosphorylate a distinct set of substrates without necessarily invoking a significant increase in kinase activity. 

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also did not increase MAP kinase activity in a prior study (62). These findings suggest that 

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can induce neurodegenerative effects by relatively subtle alteration of signal transduction pathways. Subtle alteration of signal transduction is consistent with the protracted degeneration of neurons in situ in AD. Moreover, depending upon the mode of analysis (e.g. canvassing exclusively for increased activity of one or more kinases), such subtle consequences may escape detection.

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-induced neurotoxicity has been separately attributed to
calcium influx, ROS accumulation, and increased phosphorylation of tau (7–9, 11–15, 19, 24–28). In addition to inhibition of tau phosphorylation, inhibition of MAP kinase prevented multiple consequences of βA toxicity, including accumulation of cytosolic calcium ROS, and ultimate neuronal death. These findings indicate that MAP kinase plays a pivotal role in βA neurotoxicity and highlight MAP kinase-mediated pathways as potential therapeutic targets to alleviate the progression of AD (63). In this regard, calcium chelation by BAPTA suppressed both ROS-phospho-tau accumulation following βA treatment, demonstrating that calcium influx alone can provoke the additional aspects of βA neurotoxicity. This interpretation is further supported by the observation of maximal calcium accumulation prior to that of ROS or PHF-1 during βA treatment of both cortical neurons and SH-SY-5Y cells. Our data also demonstrate that prevention of accumulation of ROS attenuates βA neurotoxicity despite cytosolic calcium accumulation, further suggesting that ROS accumulation is downstream of calcium accumulation in βA toxicity. These findings collectively suggest that aberrant activation of the L voltage-sensitive calcium channel by MAP kinase may represent the initial toxic event resulting from βA treatment. This latter conclusion should be viewed with caution, however, since βA induced MAP kinase-mediated phosphorylation of many membrane-associated proteins, one or more of which may also contribute to neurodegeneration.

An important unexpected aspect of the present study is that redirection, rather than increased activity, of MAP kinase by βA is what promotes the above intracellular cascade leading to neurodegeneration. Epidermal growth factor, which increases τ phosphorylation, inhibition of MAP kinase prevented multiple consequences of βA toxicity, suggesting that ROS accumulation is downstream of calcium influx. Inhibition of MAP kinase prevented multiple consequences of βA toxicity, indicating accumulation of cytosolic calcium ROS, and ultimate neuronal death. These findings indicate that MAP kinase plays a pivotal role in βA neurotoxicity and highlight MAP kinase-mediated pathways as potential therapeutic targets to alleviate the progression of AD (63). In this regard, calcium chelation by BAPTA suppressed both ROS-phospho-tau accumulation following βA treatment, demonstrating that calcium influx alone can provoke the additional aspects of βA neurotoxicity. This interpretation is further supported by the observation of maximal calcium accumulation prior to that of ROS or PHF-1 during βA treatment of both cortical neurons and SH-SY-5Y cells. Our data also demonstrate that prevention of accumulation of ROS attenuates βA neurotoxicity despite cytosolic calcium accumulation, further suggesting that ROS accumulation is downstream of calcium accumulation in βA toxicity. These findings collectively suggest that aberrant activation of the L voltage-sensitive calcium channel by MAP kinase may represent the initial toxic event resulting from βA treatment. This latter conclusion should be viewed with caution, however, since βA induced MAP kinase-mediated phosphorylation of many membrane-associated proteins, one or more of which may also contribute to neurodegeneration.

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