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Lithium protects cultured neurons against β-amyloid-induced neurodegeneration

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Abstract The deposition of β-amyloid peptide (Aβ), the hyperphosphorylation of tau protein and the death of neurons in certain brain regions are characteristic features of Alzheimer's disease. It has been proposed that the accumulation of aggregates of Aβ is the trigger of neurodegeneration in this disease. In support of this view, several studies have demonstrated that the treatment of cultured neurons with Aβ leads to the hyperphosphorylation of tau protein and neuronal cell death. Here we report that lithium prevents the enhanced phosphorylation of tau protein at the sites recognized by antibodies Tau-1 and PHF-1 which occurs when cultured rat cortical neurons are incubated with Aβ. Interestingly, lithium also significantly protects cultured neurons from Aβ-induced cell death. These results raise the possibility of using chronic lithium treatment for the therapy of Alzheimer's disease.

Key words: Alzheimer's disease; Amyloid-β; Cell death; Glycogen synthase kinase-3; Lithium; Rat cortical neuron

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder in which the loss of neurons in certain brain regions including the cortex and hippocampus is associated with the presence of two distinct types of fibrillar deposits, amyloid plaques and neurofibrillary tangles [1,2]. Amyloid deposits are mainly constituted of the β-amyloid peptide (Aβ), a 39–43-amino acid peptide which is a fragment of an integral membrane protein referred to as the amyloid precursor protein (APP) [3]. Neurofibrillary tangles contain hyperphosphorylated tau protein as a major component and are also found in other neurodegenerative diseases [4].

A crucial role for Aβ in AD is strongly suggested as certain mutations in the gene encoding APP which are causally linked to some early-onset familial forms of the disease give rise to increased Aβ levels [3]. Animal and cell culture studies also support the view that accumulation of aggregated Aβ may lead to neurodegeneration. The overexpression of a mutant APP in a transgenic mouse leads to a massive amyloid deposition which is accompanied by increased tau protein phosphorylation and neuronal cell death [5,6]. The microinjection of fibrillar aggregates of synthetic Aβ into the aged rhesus monkey cerebral cortex also results in tau protein hyperphosphorylation and neuronal loss [7]. Furthermore, synthetic Aβ induces cell death when added to primary cultures of rodent cortical and hippocampal neurons [8–13]. Interestingly, the phosphorylation of tau protein is also enhanced by the treatment of cultured neurons with fibrillar aggregates of Aβ [9,14,15].

Tau is a microtubule-associated protein presumably involved in the stabilization of axonal microtubules [16]. Hyperphosphorylation of tau protein appears to be an early event preceding the formation of neurofibrillary tangles in the brains of AD patients [17]. As mentioned above, increased tau phosphorylation has also been observed both in animal models of AD [5,7] and in cultured neurons treated with Aβ [9,14,15]. It has been hypothesized that tau hyperphosphorylation might lead to microtubule destabilization and tau protein aggregation, thus causing a deficit of axonal transport which could eventually result in neuronal cell death [4,16,18]. According to this view, the protein kinases responsible for Aβ-induced tau protein hyperphosphorylation should be considered potentially interesting targets for therapeutic intervention in AD.

We have recently reported that lithium blocks the phosphorylation of a number of sites on the tau molecule under physiological conditions both in cultured neurons and in vivo in rat brain [19]. Assuming that lithium behaves as a specific inhibitor of glycogen synthase kinase-3 (GSK-3) under the conditions used in these assays [19–21], GSK-3 appears to be a tau kinase in normal brain. Here we have investigated the effects of lithium on the Aβ-induced tau protein phosphorylation and cell death in primary cultures of rat cortical neurons.

2. Materials and methods

2.1. Materials

Tau monoclonal antibodies 7.51 [22] (a kind gift of Dr. C. Wischik, MRC, Cambridge, UK), Tau-1 [23] (a kind gift of Dr. L. Binder, MGC, Illinois, USA) and PHF-1 [24] (a kind gift of Dr. P. Davies, Albert Einstein College, Bronx, NY, USA) were used. All these antibodies react with both human and rat tau proteins [22–24]. According to the residue numbering of the longest human tau isoform of 441 amino acids [25], antibody Tau-1 recognizes tau protein only when serines 195, 198, 199 and 202 are dephosphorylated [26] and antibody PHF-1 recognizes tau when serines 396 and 404 are phosphorylated [27].

Synthetic human 40-amino acid β-amyloid peptide ($A\beta_{1-40}$) was

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Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid peptide; APP, amyloid precursor protein; GSK-3, glycogen synthase kinase-3; IGF-1, insulin-like growth factor-1; MEM, minimal essential medium; PBS, phosphate-buffered saline

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purchased from QCB (Quality Controlled Biochemicals, Inc.). The $A\beta_{1-40}$ peptide was dissolved in double distilled water at 1 mg/ml and incubated at 37°C for 7 days to preaggregate the peptide into a largely fibrillar form. Calcein acetoxymethyl ester (calcein AM) was purchased from Molecular Probes (Eugene, OR, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA)

2.2. Neuronal culture

Cortical neuronal cultures were prepared from 18-day-old Wistar rat embryos essentially as previously described [28,29]. Cerebral cortices were enzymatically dissociated in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 0.4 mg/ml papain, and 6 mM glucose. Dissociated cells were collected by centrifugation ($800\times g$, 5 min) and resuspended in medium supplemented with 20% horse serum. Culture medium consists of minimal essential medium (MEM), 1% glutamax-I (Gibco, Grand Island, NY, USA) and the components of B18 supplement [30] omitting glutamate, glutamine, retinol, retinyl acetate, catalase, superoxide dismutase, and triiodothyronine. The cells were then plated at 1×10^5 cells/cm^2 on plastic or glass coverslips pretreated for 2–3 days with 10 μM poly-L-lysine and for 2 h with laminin (1.38 μg/ml). The 20% horse serum-containing medium was replaced after 3 h by 5% horse serum-containing medium. After 2 days in vitro, cultures were switched to serum-free defined medium, two thirds of which was replaced every second day. The experiments were performed after 7 or 9 days in vitro. Under our culture conditions, neurons represented 86% of the cell population, astrocytes 7.5% and microglial cells were hardly detectable [29].

2.3. Neuronal viability assays

For Aβ toxicity experiments, cultures were shifted to serum-free MEM supplemented with N2 components [31], and neurons were exposed to Aβ for different times. Cell viability was assessed by calcein/propidium iodide uptake [32]. Calcein AM is taken up and cleaved by esterases present within living cells yielding yellowish-green fluorescence, whereas propidium iodide (PI) is only taken up by dead cells which become orange-red fluorescent. In brief, neurons were incubated for 30 min with 2 μM PI (Sigma) and 1 μM calcein-AM (Molecular Probes). Then, the cultures were rinsed three times with HBSS containing 2 mM $CaCl_2$ and the cells were visualized by fluorescence microscopy using a Zeiss Axiovert 135 microscope. Three fields (selected at random) were analyzed per well (approximately 300 cells/field) in at least three different experiments. Cell death was expressed as percentage of PI-positive cells from the total number of cells. In every experiment, specific Aβ-induced cell death was obtained after subtracting the number of dead cells present in vehicle-treated cultures.

2.4. Protein preparation, gel electrophoresis and immunoblot analyses

Cells (10^6 cells for each experimental point) were harvested by scraping, washed in chilled PBS, resuspended and homogenized in a buffer consisting of 20 mM HEPES, pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). Lysates were centrifuged at $10\,000\times g$ for 10 min at 4°C. The resulting supernatants were collected and their protein contents determined by the bicinchoninic acid (BCA) assay.

Samples were mixed with Laemmli buffer, boiled for 3 min, separated by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) on 12% resolving gels and transferred to nitrocellulose according to standard procedures. A blocking buffer of 5% non-fat powdered milk in PBS with 0.1% Tween-20 was used for all incubations. Monoclonal antibodies to tau protein were obtained as hybridoma-conditioned culture media and used at a dilution of 1/10. Immunoreactive proteins were visualized by the use of a peroxidase-conjugated anti-mouse antibody and enhanced chemiluminescence detection (Amersham). Quantification of immunoreactivity was performed by densitometric scanning. Individual tau bands may include multiple distinct tau proteins (arising from alternative splicing and differential phosphorylation) that comigrate and were therefore not quantitated separately. Thus, total immunoreactivity for all tau bands was quantitated for each lane of immunoblots. The densitometry values obtained for blots corresponding to the antibodies recognized phosphorylation-sensitive epitopes (Tau-1 and PHF-1) were normalized with respect to the values obtained for antibody 7.51 which reacts with a phosphorylation-independent epitope.

3. Results

3.1. β-Amyloid peptide induces an early increase in tau protein phosphorylation which is followed by neuronal cell death

The effect of fibrillar $A\beta_{1-40}$ on the in situ phosphorylation state of tau protein was analyzed by immunoblotting of cell extracts with antibodies which react with phosphorylation-sensitive epitopes as previously described [19]. Fig. 1A shows that the treatment of cultured rat cortical neurons with 25 μM $A\beta_{1-40}$ augments the immunoreactivity of tau protein to antibody PHF-1, which recognizes a phosphorylated epitope, with respect to the immunoreactivity to antibody 7.51, which reacts with a phosphorylation-independent epitope. This indicates that Aβ enhances the phosphorylation of tau protein at the epitope recognized by antibody PHF-1. The augmented phosphorylation of tau is observed as early as 1 h after the addition of Aβ and attains its maximal level after 3 h of treatment. In addition to this enhancement in tau phosphorylation, a less important but reproducible increase in the immunoreactivity of tau protein to antibody 7.51 is also seen after 6 h of Aβ treatment (see Fig. 1A, upper panel). This suggests that not only the phosphorylation but also the total level of tau protein is increased within neurons incubated with Aβ, which agrees well with previous observations [9,33].

The neurotoxicity of the treatment of cultured rat cortical neurons with 25 μM $A\beta_{1-40}$ was also studied. Fig. 1B shows that $A\beta_{1-40}$ induces a specific death of cultured neurons which is clearly observed after 24 h of treatment. Neurotoxicity was also observed with a shorter fragment of Aβ, referred to as $A\beta_{25-35}$, whereas scrambled peptides lack any neurotoxic effect [34]. Aβ-induced neuronal cell death was not attenuated

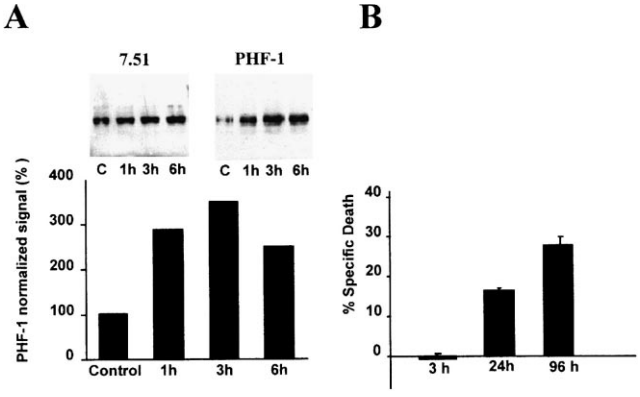

Fig. 1. β-Amyloid peptide enhances tau protein phosphorylation and induces cell death in cultured rat cortical neurons. A: Neurons were cultured for 9 days in vitro and then treated with 25 μM $A\beta_{1-40}$ at the times indicated (1, 3 and 6 h). Cell extracts were prepared, electrophoresed, blotted and probed with antibodies 7.51 and PHF-1. Upper panels show the corresponding blots. 'C' refers to untreated (control) neurons. Lower diagram represents the immunoreactivity of tau protein to antibody PHF-1, which reacts with a phosphorylated epitope, as densitometry values normalized with respect to the immunoreactivities to antibody 7.51 which recognizes a phosphorylation-independent epitope. The PHF-1 normalized signal is a measure of the state of phosphorylation of tau protein at the PHF-1 epitope. B: Neurons were cultured for 7–9 days in vitro and then treated with either 25 μM $A\beta_{1-40}$ or vehicle at the times indicated (3, 24 and 96 h). Percentages of specific Aβ-induced cell death were determined by calcein/propidium iodide uptake as described in Section 2. The data represent mean values ± S.E.M. of $n=4$ different experiments performed in triplicate.

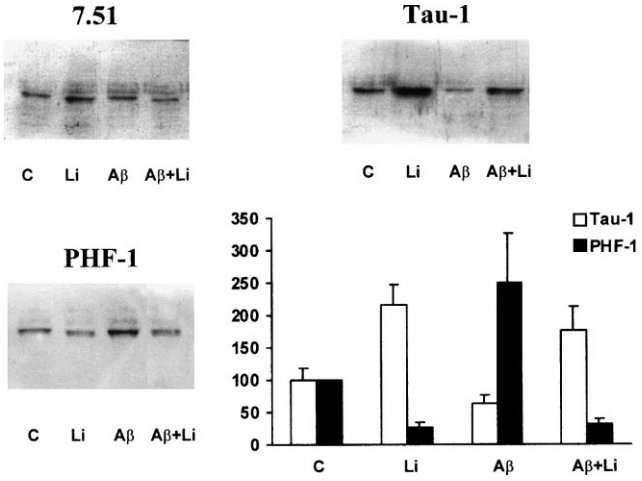

Fig. 2. Lithium prevents β-amyloid peptide-induced tau protein phosphorylation in cultured rat cortical neurons. Neurons were cultured for 9 days in vitro and then treated with vehicle (C), 10 mM LiCl (Li), 25 μM Aβ (Aβ) or 25 μM Aβ in the presence of 10 mM LiCl (Aβ+Li) for 2 h. Cell extracts were prepared, electrophoresed, blotted and probed with antibodies 7.51, Tau-1 and PHF-1. Panels show the blots corresponding to a typical experiment. The immunoreactivities of tau protein to antibodies Tau-1, which recognizes a phosphorylatable epitope only when it is not phosphorylated, and PHF-1, which reacts with a phosphorylated epitope, were quantitated as densitometry values which were normalized with respect to the immunoreactivities to antibody 7.51, which recognizes a phosphorylation-independent epitope. Lower right diagram represents the mean values and standard deviations ($n=3$) of the normalized signals for antibodies Tau-1 and PHF-1.

by cotreatment with 3 μM MK-801 (data not shown), which is a specific antagonist of NMDA-type glutamate receptors. Thus, in our culture conditions, Aβ-induced neuronal death does not involve glutamate excitotoxicity. Interestingly, no Aβ-induced neuronal cell death was observed 3 h after the addition of Aβ, when the maximal phosphorylation of tau is detected (compare Fig. 1A and B). These data indicate that the increased tau protein phosphorylation observed in Aβ-treated neurons is an early event occurring before cell death.

3.2. Lithium prevents the β-amyloid-induced enhancement of tau protein phosphorylation in cultured neurons

To examine whether lithium affects the increase in tau protein phosphorylation triggered by Aβ, the phosphorylation of tau protein was analyzed by immunoblotting with antibodies Tau-1, which recognizes a phosphorylatable epitope only when it is not phosphorylated, and PHF-1, which reacts with a phosphorylated epitope. Fig. 2 shows that treatment of cultured neurons with 10 mM LiCl for 3 h resulted in an increased Tau-1 and a decreased PHF-1 immunoreactivities, which is consistent with a net dephosphorylation of tau protein at both epitopes. In contrast, treatment of cultured neurons with 25 μM $A\beta_{1-40}$ for 3 h resulted in a decreased Tau-1 and an increased PHF-1 immunoreactivity, which indicates an enhanced phosphorylation of tau protein at both epitopes. When neurons were treated with Aβ in the presence of 10 mM LiCl, the increase in PHF-1 and the decrease in Tau-1 immunoreactivities were abolished. Accordingly, no enhancement of tau protein phosphorylation occurred in cultured neurons treated with Aβ in the presence of lithium.

3.3. Lithium protects cultured neurons from β-amyloid-induced cell death

Since lithium blocks an early event triggered by Aβ, its consequences on Aβ-induced neurodegeneration were also examined. Fig. 3 shows that treatment with 25 μM $A\beta_{1-40}$ results in 18.06 ± 1.69% cell death 24 h later. However, only 3.4 ± 1.81% cell death is observed when cultured neurons are treated with Aβ in the presence of 10 mM lithium, which indicates a 81.1% protection (see Fig. 3E). Thus, lithium significantly protects cultured neurons against Aβ neurotoxicity.

Moreover, the neuroprotective effect of lithium does not seem to be merely due to a delay in neuronal cell death as it is still observed 4 days after Aβ treatment. At this time, Aβ treatment of cultured neurons brings about 27.7 ± 2% cell death, which is reduced to 9.85 ± 3.2% in the presence of 2 mM LiCl and 500 μM *myo*-inositol, thus indicating a 64.4% protection (see Fig. 3F). Approximately the same level of neuronal cell death is observed in cultured neurons treated with 2 mM LiCl and 500 μM *myo*-inositol either in the pres-

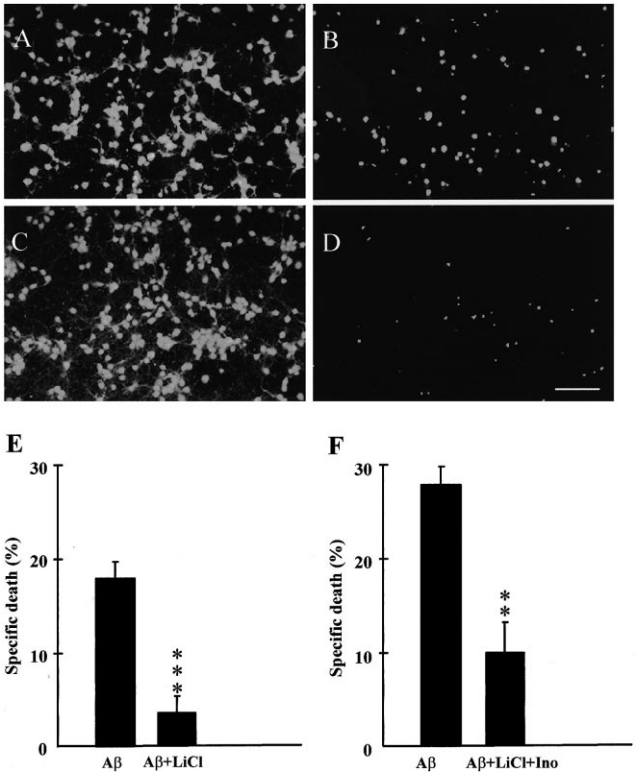

Fig. 3. Lithium protects cultured rat cortical neurons against β-amyloid peptide-induced cell death. Neurons were cultured for 9 days in vitro and then treated with 25 μM Aβ (A and B) or 25 μM Aβ in the presence of 10 mM LiCl (C and D) for 24 h. Viable and dead cells were stained with calcein AM and propidium iodide as described in Section 2 and photographed at a fluorescence microscope. Shown are viable (A and C) and dead (B and D) cells in each treatment. Percentages of specific Aβ-induced cell death were determined after 24 h (E) as described in Section 2. The data represent mean values ± S.E.M. of $n=3$ different experiments performed in triplicate; $^{***}P<0.0005$ (*t*-test). Neurons were cultured for 7 days in vitro and then treated with with 25 μM Aβ (Aβ) or treated with 25 μM Aβ in the presence of 2 mM LiCl and 0.5 mM *myo*-inositol (Aβ+LiCl+Ino) for 96 h. Percentages of specific cell death were determined as described in Section 2 (F). The data represent mean values ± S.E.M. of $n=2$ different experiments performed in triplicate; $^{**}P<0.005$ (*t*-test).

ence or in the absence of Aβ. We used 2 mM instead of 10 mM LiCl because less neuronal cell death was observed after 4 days. It seems therefore that the prolonged treatment of cultured neurons with lithium is slightly toxic to neurons and neurotoxicity is still increased when inositol is omitted (data not shown). This suggests that the depletion of intracellular inositol is presumably important for lithium neurotoxicity. Despite its slight neurotoxicity during prolonged treatment, lithium is still neuroprotective against Aβ-induced neuronal cell death at a concentration similar to that attained clinically (see Fig. 3F).

4. Discussion

Addition of fibrillar Aβ to cultured neurons mimics certain key aspects of neurodegeneration in AD [1]. For this reason, Aβ-treated neuronal cultures are currently employed not only to analyze the molecular mechanisms of Aβ-induced neuronal cell death but also to screen for novel neuroprotective agents [34,35]. In this context, we report here that lithium prevents the Aβ-induced increase in tau protein phosphorylation and significantly inhibits the death of cultured neurons triggered by Aβ. These effects of lithium were observed even in the presence of extracellular inositol, suggesting that they are not due to depletion of intracellular inositol [36]. Indeed, a prolonged treatment of cultured neurons with lithium results in some loss of cell viability, which is reduced by increasing the extracellular inositol concentration. Accordingly, depletion of cellular inositol occurring after inositol monophosphatase inhibition by lithium [37] appears to be toxic for neurons rather than neuroprotective under our culture conditions.

Taken together, these results are consistent with the hypothesis that GSK-3, the putative target for lithium [19–21,38], is involved in the neuropathological cascade triggered by Aβ. The role of GSK-3 in tau protein phosphorylation in AD was previously suggested by the colocalization of one isoform of this kinase (GSK-3β) with hyperphosphorylated tau within the brains of AD patients [39]. Furthermore, a recent report has shown that GSK-3β antisense oligonucleotides inhibit the enhanced phosphorylation of tau protein which is induced by a 10-amino acid fragment of Aβ referred to as $A\beta_{25-35}$ in cultured rat hippocampal neurons [15]. However, the implication of other protein kinases in Aβ-induced tau protein hyperphosphorylation cannot be excluded. In fact, tau protein also becomes hyperphosphorylated in AD at some sites on the molecule which do not seem to be targeted by GSK-3 [16].

Interestingly, lithium not only blocked Aβ-induced tau protein phosphorylation but also inhibited Aβ neurotoxicity. These lithium effects parallel those of insulin and insulin-like growth factor 1 (IGF-1). It has been demonstrated that IGF-1 receptor activation decreases tau protein phosphorylation in a neuronal-like cell line through the inhibition of GSK-3 via the phosphatidylinositol 3-kinase/protein kinase B signaling pathway [40]. Moreover, IGF-1 also protects cultured neurons against amyloid-induced toxicity [35]. Thus, both lithium and IGF-1 are able to inhibit GSK-3, prevent tau phosphorylation and protect from Aβ neurotoxicity. However, we cannot fully rule out the possibility that both IGF-1 and lithium treatments modify an additional unknown target which could be implicated in Aβ-induced neurodegeneration. Even if we assume that GSK-3 is the relevant target for both IGF-1 and lithium, this does not necessarily mean that tau protein phosphorylation is required for Aβ-induced neuronal cell death. The phosphorylation of other proteins in addition to tau may be involved in the activation of a cell death program by GSK-3. In fact, GSK-3 is a multifunctional kinase which modifies many metabolic regulatory enzymes, cytoskeletal proteins and transcription factors [41]. For instance, it has been suggested that the phosphorylation of pyruvate dehydrogenase by GSK-3 may cause mitochondrial dysfunction which might bring about neuronal cell death [42]. In any case, GSK-3 might be a crucial element in the Aβ-triggered molecular cascade leading to neurodegeneration. As lithium treatment does not confer complete protection against Aβ neurotoxicity, it is possible that other intracellular mediators which are not downstream of GSK-3 may also contribute to Aβ-induced neurodegeneration. Alternatively, partial protection may arise from side effects of lithium acting on other targets in addition to GSK-3 as mentioned above.

Of importance in this regard is the fact that similar neuroprotective effects of lithium on cultured neurons subjected to different insults, including suboptimal culture conditions [43–45] and glutamate excitotoxicity [46], have been demonstrated. Whether GSK-3 is the relevant target for lithium in all these cases remains to be established. Supportive of this view is the fact that the overexpression of GSK-3β causes apoptosis, whereas the overexpression of a dominant-negative GSK-3β mutant prevents the apoptosis which is induced following inhibition of phosphatidylinositol 3-kinase in some cell lines [47].

In view of these data, it is tempting to speculate that a chronic low-dose lithium treatment similar to that prescribed for manic-depressive patients [48,49] might also be useful to curb neuronal cell death in AD and possibly in other neurodegenerative disorders.

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