Prion peptide induces neuronal cell death through a pathway involving glycogen synthase kinase 3

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Prion diseases are characterized by neuronal cell death, glial proliferation and deposition of prion peptide aggregates. An abnormal misfolded isoform of the prion protein (PrP) is considered to be responsible for this neurodegeneration. The PrP 106–126, a synthetic peptide obtained from the amyloidogenic region of the PrP, constitutes a model system to study prion-induced neurodegeneration as it retains the ability to trigger cell death in neuronal cultures. In the present study, we show that the addition of this prion peptide to cultured neurons increases the activity of glycogen synthase kinase 3 (GSK-3), which is accompanied by the enhanced phosphorylation of some microtubule-associated proteins including tau and microtubule-associated protein 2. Prion peptide-treated neurons become

progressively atrophic, and die ultimately. Both lithium and insulin, which inhibit GSK-3 activity, significantly decrease prion peptide-induced cell death both in primary neuronal cultures and in neuroblastoma cells. Finally, the overexpression of a dominant-negative mutant of GSK-3 in transfected neuroblastoma cells efficiently prevents prion peptide-induced cell death. These results are consistent with the view that the activation of GSK-3 is a crucial mediator of prion peptide-induced neurodegeneration.

Key words: apoptosis, glycogen synthase kinase 3, lithium, microtubule-associated protein, neurodegeneration, neurotoxicity, prion, spongiform encephalopathy.

INTRODUCTION

Transmissible spongiform encephalopathies, also known as prion diseases, are a group of unusual neurodegenerative disorders, including Creutzfeldt–Jakob disease, new variant Creutzfeldt–Jakob disease, Gerstmann–Straussler–Scheinker syndrome and fatal familial insomnia in humans, as well as scrapie in sheep and bovine spongiform encephalopathy in cattle [1,2]. They are characterized by progressive vacuolation of the neuropil, neuronal degeneration and gliosis. In almost all cases, there is also a marked extracellular accumulation of an amyloidogenic conformer [referred to as the scrapie isoforms of prion protein (PrP^{Sc})] of the normal cellular PrP^C [1–3]. PrP^{Sc} can be distinguished from PrP^C for its high content in β -pleated sheet structure, its tendency to form large fibrillar aggregates and its noteworthy resistance to proteinase digestion [1–3].

Extensive research studies have been focused on understanding how the self-propagating conformation of PrP^{Sc} might be 'infectious' to explain the transmissibility of some prion diseases [1–3]. In contrast, the molecular mechanisms responsible for neuronal degeneration in these disorders have received relatively little attention [4].

Regarding the mechanisms of neuronal cell death in prion diseases, some post-mortem studies have demonstrated that apoptosis may play a significant role [5–10]. Furthermore, the addition of purified PrP^{Sc} to primary neuronal cultures triggers apoptosis [11,12], although these experiments are technically complicated by the presence of detergents during PrP^{Sc} purification [4].

Interestingly, a synthetic peptide corresponding to residues 106–126 of human PrP (PrP 106–126) has also been shown to induce apoptosis in cultured cells, including primary rat hippocampal [13], mouse cortical [14–16] and mouse cerebellar neurons [17], the rat pituitary clonal cell line GH3 [18] and

different human neuroblastoma cell lines [19–21], as well as *in vivo* after injection into mouse retina [22]. This peptide exhibits a prevalent β -pleated sheet structure and forms amyloid fibrillar aggregates that are resistant partially to proteolysis being reminiscent of PrP^{Sc} [23–25]. Moreover, PrP 106–126 is conserved in all the prion isoforms that have been found to accumulate in the brain of patients suffering from prion diseases. As a case in point, a longer synthetic peptide, PrP 82–146, which is identical in sequence to a major amyloid fragment isolated from the brain of patients with Gerstmann–Straussler–Scheinker syndrome, also forms fibrils and is toxic to cultured neurons [26]. Thus PrP 106–126 might constitute a core motif responsible for polymerization into fibrils and neurotoxicity of prion-derived peptides [25].

Accordingly, the ability of PrP 106–126 to induce neuron cell death is considered widely as a valid model to study the molecular mechanisms of prion-induced neurodegeneration. Of particular relevance in this regard is the recent demonstration of significant similarities between the pro-apoptotic cascades activated by PrP 106–126 and β -amyloid peptide A β 1–40 in primary cortical neurons [15,16]. A β 1–40 is a peptide fragment of an integral membrane protein and accumulates as fibrillar aggregates within the brain of patients with Alzheimer's disease, where it may act as a trigger of neuronal cell death [27]. Both PrP 106–126 and A β 1–40 induce caspase activation in cultured cortical neurons, suggesting the existence of shared common mechanisms for neurodegeneration by different amyloidogenic peptides [28]. However, the activation of caspases may be a relatively late event in the apoptotic cascade [29,30]. So it is important to focus on earlier events, which may be triggered by amyloidogenic peptides. In this respect, it has been demonstrated that the treatment of primary neuronal cultures with β -amyloid peptides results in an early activation of glycogen synthase kinase 3 (GSK-3), which is essential for A β -induced neurotoxicity

Abbreviations used: GSK-3, glycogen synthase kinase 3; MAPK, mitogen-activated protein kinase; PrP, prion protein.

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[31,32]. GSK-3 is a multifunctional protein kinase that modifies several transcription factors, metabolic enzymes, cytoskeletal proteins and other regulatory proteins [33,34] and seems to play a very prominent role in the control of neuronal cell survival and apoptosis [35–41].

In the present study, we have examined the effect of PrP 106–126 on GSK-3 in cultured neurons, as well as the requirement of GSK-3 for prion peptide-induced cell death both in cultured neurons and neuroblastoma cells. Our results are consistent with the view that GSK-3 is a critical factor in neurogeneration induced by amyloidogenic peptides.

MATERIALS AND METHODS

PrP peptide 106–126 was synthesized as described previously [19]. The peptide was resuspended in PBS and maintained for 4 h to allow polymerization, which was confirmed by electron microscopy as shown in Figure 1 [19]. We used the peptide (SRC) as a control, which represents a fragment of the amyloid sequence mixed randomly (IMLKGNGASIG).

Cultures of cortical neurons, cerebellar granule neurons and neuroblastoma cell

Cortical neurons were obtained from mouse embryos of E17, basically as described previously [42,43]. Briefly, trypsin-dissociated cells were plated on 100 μ g/ml poly(L-lysine) and 10 μ g/ml laminin-coated plastic dishes or slides (1×10^5 cells/P24 well or 2×10^6 cells/P60 dish) and cultured in Neurobasal-

B27 medium (Gibco, Invitrogen, Barcelona, Spain), supplemented with 25 mM KCl and 1 mM glutamine.

Mouse N2A neuroblastoma cells were grown routinely in Dulbecco's modified Eagle's medium with 10% (v/v) heat-inactivated foetal calf serum containing 2 mM glutamine and antibiotics (penicillin–streptomycin). Before the addition of PrP peptide, the medium was changed to Neurobasal-B27. As lithium chloride is also an inositol monophosphatase inhibitor, we performed all our experiments with lithium along with an excess of *myo*-inositol, to avoid the effect of inositol depletion. Cells were pretreated with 10 mM lithium and 5 mM *myo*-inositol for 2 h before PrP addition. The inhibitor of mitogen-activated protein kinase (MAPK) U0126 (10 μ M) or the inhibitor of the stress-kinase p38 SB203580 (25 μ M) was added 2 h before the PrP addition.

To analyse the influence of insulin addition, the culture medium was modified slightly. Insulin concentration in the B27 supplement was diluted to 1:50, and 30 min before PrP peptide addition the normal amount of insulin was added. In this case, neurons with reduced amount of insulin in the medium were used as control.

Neuronal viability determination

For PrP toxicity experiments, cortical neurons were exposed to 10 μ g/ml PrP for different times. Cell viability was assessed by calcein–propidium iodide uptake [44]. Calcein/acetoxymethyl ester is taken up and cleaved by esterases present in living cells, yielding yellowish-green fluorescence, whereas propidium iodide is taken up only by dead cells which become orange-red fluorescent. Briefly, cells were incubated for 30 min with 8 μ M

Figure 1 GSK-3 activity in PrP-peptide-treated neurons

(A) Primary cultures of cortical neurons were left untreated (C) or treated with 10 μ g/ml PrP peptide 106–126 for 60 min. Cell extracts were prepared and used to quantify GSK-3 activity as described in the Materials and methods section, using GS 1 phospho-peptide as substrate. Total amounts of GSK-3 α and GSK-3 β were determined by Western-blot analysis (lower part of A). Values of GSK-3 activity were normalized with respect to total GSK-3 and expressed in relative units (after setting up the GSK-3 activity of control neurons as 100 units). (B) Primary cultures of cortical neurons were left untreated (C) or treated for 30, 45 and 60 min with 10 μ g/ml PrP peptide 106–126. Total GSK-3 β , as well as phosphotyrosine- and phosphoserine-containing GSK-3 was determined by Western-blot analysis using specific antibodies. (C) The amounts of P-Tyr²¹⁶ and P-Ser⁹ GSK-3 β were quantified by densitometric scanning. The graph shows the ratio P-Tyr/P-Ser of GSK-3 β from cultured neurons at different times after PrP peptide treatment. Error bars represent means $\pm$ S.E.M. (D) The peptide PrP was resuspended at 10 μ g/ml in PBS and maintained for 4 h to permit polymerization, which was confirmed by electron microscopy.

propidium iodide (Sigma) and 1 μ M calcein/acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.). Then, the culture was rinsed once with Hanks balanced salt solution 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 analysed per well (100–200 cells/field) in at least three experiments. Cell viability was expressed as the percentage of calcein-positive cells from the total number of cells.

Quantitative analysis of GSK-3 activity

GSK-3 assays were performed basically as described previously [45,46]. Cell extracts were prepared from neurons at different times after PrP addition and from control cells. Cells were collected with a scraper and homogenized in a buffer of 20 mM Hepes (pH 7.4), containing 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA and 100 nM okadaic acid, with protease inhibitors (COMPLETE™). The soluble fraction was obtained by centrifugation at 13000 *g* for 15 min at 4 °C.

Samples of 5 μ g protein were incubated in a buffer containing 25 mM Tris (pH 7.5), 1 mM dithiothreitol, 10 mM MgCl_2 , with the specific substrate peptide (glycogen synthase) [45] at a final concentration of 0.75 mg/ml, in the presence of [γ - 32 P]ATP. After 1 h, the reaction was stopped by boiling, and the activity was quantified by spotting aliquots on P81 phosphocellulose paper. The difference between the kinase activity in the presence or absence of 20 mM LiCl [46,47] was considered to be a measure of GSK-3 protein kinase activity.

Indirect immunofluorescence

After treatments, cell cultures were fixed with PBS containing 4 % (w/v) paraformaldehyde for 30 min. After several washes with PBS, the cells were preincubated in PBS, containing 0.1 % Triton X-100 and 1 % foetal calf serum (PBS Tx) for 30 min. After a brief wash with PBS, they were incubated overnight at 4 °C with primary antibodies diluted in PBS Tx. Following incubation with the primary antibody, cultures were washed extensively and then incubated with the appropriate secondary antibody, conjugated either with Fluorescein or Texas Red (Jackson Laboratories) for 45 min. After washing, they were mounted immediately with Fluoromount (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A.), examined and photographed under a Labophot (Nikon-AG, Switzerland) or Axiophot (Zeiss, Madrid, Spain) microscope.

Antibodies and Western-blot analysis

Monoclonal antibody against β tubulin (1 : 1000) was purchased from Sigma. Antibodies against microtubule-associated proteins 2, 305 and HM2 were used as described previously [43]. Tau monoclonal antibody AD2 that recognizes phosphorylated Ser³⁹⁶ (1 : 30) was a gift from Dr C. Mourton-Gilles (Institut de Biotechnologie en Immunoanalyse et Pharmacologie, CNRS, Montpellier, France). Monoclonal antibody against total tau 751, a gift from Dr Wischik (MRC, Cambridge, U.K.), was also used [48]. Rabbit polyclonal antibodies against GSK-3 α / β PY^(279/216) and against GSK-3 α / β PS^(21/9) were both purchased from Biosource (Nivelles, Belgium).

Cell extracts were prepared from cultured neurons either treated or untreated with PrP for different times. Cells were washed with PBS and then resuspended in a buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA and protease inhibitors cocktail

(COMPLETE™), and homogenized using a Polytron. The soluble fraction was obtained by centrifugation at 13000 *g* for 10 min at 4 °C.

Proteins (25–50 μ g) were separated on SDS/PAGE gels and electrotransferred to a nitrocellulose filter. Nitrocellulose sheets were blocked with 10 % (v/v) non-fat milk in PBS and 0.1 % Tween 20 (PBST). The nitrocellulose was then incubated with primary antibodies overnight at 4 °C. Filters were rinsed three times in PBST buffer and then incubated with the corresponding peroxidase-conjugated secondary antibody (diluted 1 : 5000; Amersham) for 1 h at room temperature (22 $\pm$ 1 °C). Immunoreactivity was visualized by the enhanced chemiluminescence (ECL®, Amersham) detection system.

Densitometric analysis was performed on different Western-blot lanes (3–5 replicates) and the results processed with an imaging densitometer (GS-6470 model; Bio-Rad Laboratories, Hercules, CA, U.S.A.). Results were analysed with Molecular Analyst software. The densitometric values were normalized with respect to the values obtained for a control antibody to correct for any deviation in the loaded protein. Values are expressed as means $\pm$ S.D. The results were analysed by either one-way ANOVA or Student's *t* test, whichever was appropriate, using STATVIEW and STATISTI software. Differences were considered to be significant at *P* < 0.05.

Plasmids and transfections

XGSK-3 negative dominant (R-85) was generously provided by Dr I. Dominguez (Harvard Medical School, Boston, MA, U.S.A.) [49] and modified as described previously [50]. The expression vector for green fluorescent protein, pEGFP, was obtained from ClonTech (Palo Alto, CA, U.S.A.). Neuro2A cells were transfected transiently using the method of transferrin receptor endocytosis as described previously [50] (DuoFect 80; QUANTUM, Molecular Dynamics, Sunnyvale, CA, U.S.A.). This transfection method yielded more than 50 % transfection efficiency under optimal conditions. In the co-transfection experiments, total amount of DNA was kept constant (3.2 μ g), and 2.56 μ g of R85 or plasmid without cDNA insert was mixed with 0.64 μ g of pEGFP. Cells, 30 h after transfection, were processed for viability assay as described previously [50].

RESULTS

Activation of GSK-3 in cultured neurons treated with PrP peptide

In view of the crucial role of GSK-3 in different models of neurodegeneration [31,32, 35–41,51], we analysed whether prion peptide affects GSK-3 activity. Figure 1(A) shows that the treatment of cultured mouse cortical neurons with 10 μ g/ml PrP 106–126 for 1 h results in a 2-fold increase in GSK-3 activity, which was measured using a specific synthetic peptide as substrate.

Immunoblotting demonstrates the presence of both GSK-3 α and GSK-3 β in extracts from cultured mouse cortical neurons. Prion peptide treatment affects neither the amount nor the relative proportions of GSK-3 α and GSK-3 β [see Figure 1(A), lower panel]. Several studies have indicated that tyrosine phosphorylation (Tyr²⁷⁹ in GSK-3 α and Tyr²¹⁶ in GSK-3 β) is necessary for GSK-3 activity [52] and is augmented in cells subjected to distinct apoptotic stimuli [36]. Thus we determined the phosphorylation state of GSK-3 with a phosphotyrosine-specific antibody. Figure 1(B) shows that treatment of cultured neurons with PrP peptide leads to a rapid and marked increase in GSK-3 tyrosine phosphorylation (which is maximal

30 min after the addition of PrP peptide to the culture medium).

Serine phosphorylation of GSK-3 (at Ser²¹ in GSK-3 α and Ser⁹ in GSK-3 β) was also assayed with a phosphoserine-specific antibody, since this modification is involved in the inactivation of GSK-3 [33,34]. Figure 1(B) shows that PrP administration results in a modest increase in serine phosphorylation of GSK-3, which is not as significant as the enhancement of tyrosine phosphorylation. Thus the relative ratio of phosphotyrosine GSK-3 with respect to phosphoserine GSK-3 is augmented by PrP peptide treatment of cultured neurons (see Figure 1C). These results are consistent with a net activation of GSK-3 in response to PrP peptide treatment, as indicated previously in Figure 1(A). This activation occurs when the PrP peptide was polymerized previously, as shown in Figure 1(D). The addition of the control peptide (scramble) was devoid of effect (results not shown).

To confirm further the PrP-induced GSK-3 activation in cultured neurons, we evaluated the phosphorylation state of tau and microtubule-associated protein 2, which have been demonstrated previously to be good GSK-3 substrates within neurons [32,42,43,53]. Figure 2(A) shows that the addition of PrP peptide to the culture medium of mouse cortical neurons increases the phosphorylation of the site recognized by antibody 305 on microtubule-associated protein 2 A–C. The quantification of the data, normalized with respect to β tubulin, indicates a 2-fold increase in the phosphorylation of microtubule-associated protein 2, in the treated cells, at the site recognized by antibody 305 (Figure 2C).

Figure 2(B) demonstrates that PrP peptide treatment also results in an increased phosphorylation of tau protein at the site recognized by antibody AD2. The quantification of the data, normalized against total tau (monoclonal antibody 751), showed almost a 2.5-fold increase in phosphorylation in this phospho-epitope.

In both cases, preincubation with lithium, which is an inhibitor of GSK-3 [47,54] and may inhibit some other enzymes (see for a review [54a]) in the presence of inositol monophosphate, for 2 h before PrP addition abolished the up-phosphorylation of both microtubule-associated protein 2 and tau proteins. These results indicate strongly that administration of PrP peptide to culture neurons stimulates the activation of GSK-3, which phosphorylates several neuronal proteins including microtubule-associated proteins 2 and tau.

Figure 2 Phosphorylation of microtubule-associated proteins 2 and tau in PrP-peptide-treated neurons

(A) Primary cultures of cortical neurons were treated with 10 μ g/ml (lanes 3 and 5) or 20 μ g/ml PrP peptide 106–126 (lanes 2 and 4) for different times. Additionally, neurons were treated with PrP peptide for 60 min after being pretreated for 2 h with 10 mM LiCl (lanes 4–6). Lane 1 represents untreated control neurons. Cell extracts were prepared and analysed by Western blotting with antibody 305, which recognizes phosphorylated microtubule-associated protein 2 A–C. An antibody against β -tubulin was used as a loading control. (B) Similar amounts of cell extracts, from treatments described above, were also analysed by Western blotting with antibodies to a phosphorylation-independent (7.51) and phosphorylation-dependent (AD2) epitopes on tau protein. (C) Quantification data obtained from the densitometric analysis of microtubule-associated proteins 2 and tau phospho-epitope modifications after PrP treatments. Lane 1 represents untreated control neurons, and 2–6 as indicated above. Data from three different experiments of microtubule-associated protein 2 A–B or microtubule-associated protein 2 C phosphorylation were quantified and normalized with respect to tubulin. The ratio of each phospho-epitope to tubulin in the control condition was considered to be 1 relative unit (RU). The line on each black bar represents the standard deviations of the mean value. Similarly, data ($n = 3$) from tau phosphorylation in the phospho-epitope AD2 was quantified and normalized with respect to total tau (7.51). The ratio of each phospho-epitope with respect to total tau (7.51) in the control condition was considered to be 1 relative unit (RU). The line on each black bar represents the standard deviation of the mean value.

Figure 3 Primary cultures of cortical neurons treated with 10 μ g/ml PrP peptide 106–126 for 60 min

Before the peptide addition, we pretreated the primary cultures for 2 h either with MAPK inhibitor (U0126) or with p38 kinase inhibitor (SB203580) (A). Cell extracts were prepared and analysed by Western blotting with antibody 305, which recognizes phosphorylated microtubule-associated protein 2 C. From three different experiments, the results of microtubule-associated protein 2 C phosphorylation was quantified and normalized with respect to tubulin (B).

Figure 4 Morphological changes in PrP-peptide-treated neurons

Cortical neurons were left untreated (**A, B**) or treated with 10 $\mu\text{g/ml}$ PrP peptide for 1 h (**C, D**). The immunofluorescence microscopy of cells with antibodies HM2 reacting with a phosphorylation-insensitive epitope on microtubule-associated protein 2 (**A, C**) and antibody 305, which recognizes a phospho-dependent epitope on microtubule-associated protein 2 are shown (**B, D**).

To test whether other kinases also play a role in the phosphorylation of the phospho-epitopes analysed in microtubule-associated proteins 2 and tau, we used pharmacological inhibitors of MAPK (U0126) and stress-kinase p38 (SB203580). When each inhibitor was added before the addition of PrP, phosphorylation induced by PrP peptide was not abolished, as shown in Figures 3(A) and 3(B).

PrP peptide triggers morphological changes in cultured neurons

Recent results have suggested that dendritic damage may constitute one of the earliest pathological events causing neuronal dysfunction in prion diseases [10,15,16]. In the present study, we have examined whether PrP peptide is capable of modifying the morphology of cultured cortical neurons. Figure 4 demonstrates that this is indeed the case, as PrP-treated neurons exhibit a certain degree of dendritic atrophy. Dendrites were visualized by immunofluorescence with antibodies to both phosphorylation-dependent (antibody 305) and -independent (antibody HM2) epitopes on microtubule-associated protein 2 [42,43]. Thus PrP-treated neurons have less primary processes (2 ± 1 in comparison with 5 ± 2 processes found in control untreated neurons, $n = 50$). Furthermore, dendritic processes are significantly shorter, less branched and contain less filopodial-like lateral extensions (70–80%). In some cases, growth cones appeared collapsed and neuritic retraction was evident (Figures 4C and 4D). Cell bodies of neurons with severe dendritic atrophy exhibited an intense immunoreactivity towards antibody 305, which recognizes a phospho-epitope on microtubule-associated protein 2 (Figure 4). Thus there might be a correlation between PrP-induced GSK-3-mediated hyperphosphorylation of microtubule-associated protein 2 and dendritic dysfunction.

Figure 5 Lithium and insulin inhibit PrP peptide cell death in mouse cortical neurons and mouse neuroblastoma N2A cells

(**A**) Mouse neuroblastoma N2A cells left untreated (1) or treated with 10 $\mu\text{g/ml}$ PrP peptide (2) and 20 $\mu\text{g/ml}$ PrP peptide (3). The cell viability was recovered when we added 10 mM LiCl, before the addition of either 10 $\mu\text{g/ml}$ (4) or 20 $\mu\text{g/ml}$ PrP peptide (5). Lane 6 represents the cell viability when we added only 10 mM LiCl. After 72 h of treatment, cell viability was determined as indicated in the Materials and methods section. (**B**) Primary cultures of mouse cortical neuron cells left untreated (1) or treated with 10 $\mu\text{g/ml}$ PrP peptide (2), 20 $\mu\text{g/ml}$ PrP peptide (3) or 10 $\mu\text{g/ml}$ PrP peptide in the presence of 10 mM LiCl (4). After 24 h, cell viability was determined as indicated in the Materials and methods section. (**C**) Primary cultures of mouse cortical neuron cells, in a medium with low amount of insulin (see the Materials and methods section) were left untreated (1) or treated with 10 $\mu\text{g/ml}$ PrP peptide (2), 5 $\mu\text{g/ml}$ insulin (3) or 10 $\mu\text{g/ml}$ PrP peptide in the presence of 5 $\mu\text{g/ml}$ insulin (4). After 24 h, cell viability was determined as indicated in the Materials and methods section.

Inhibition of GSK-3 prevents PrP peptide neurotoxicity

Since GSK-3 activation is an early event triggered by PrP peptide, we evaluated the consequences of inhibiting GSK-3 in cultured cells subjected to PrP treatment.

Figure 5 shows that PrP peptide causes cell death both in primary cultures of mouse cortical neurons and in a mouse neuroblastoma cell line, which agrees well with earlier

Figure 6 Overexpression of R85 GSK-3 β inhibits PrP-peptide-induced cell death in transfected neuroblastoma N2A cells

N2A cells were transfected with R85 GSK-3 β cDNA and pEGFP, as indicated in the Materials and methods section. At 30 h post-transfection, N2A cells were either treated with PrP peptide for 24 h (A), or untreated (B). Cell viability was determined as indicated in the Materials and methods section. N2A cells containing GSK-3 β R85 (green cells) remain viable after incubation with PrP peptide whereas most non-transfected cells die (red staining).

reports [13–16,18,20,21]. Interestingly, the presence of lithium, which inhibits GSK-3 [47,54] significantly, protects from PrP-induced cell death both in cultured rat cortical neurons (Figure 5B) and in mouse neuroblastoma N2A cells (Figure 5A).

The effect of insulin on PrP neurotoxicity was also tested as insulin treatment leads to the inactivation of GSK-3 through serine phosphorylation [33,34]. Figure 5(C) shows that insulin (supplemented in a medium with low amount of insulin, see the Materials and Methods section) also decreases PrP-induced neuron cell death.

All these results suggested that inhibition of GSK-3 by either lithium or insulin treatments might be neuroprotective against PrP peptide. To test this possibility further, mouse neuroblastoma N2A cells were transfected with an expression plasmid coding for GSK-3 β R85 (as inactive kinase mutant which may exhibit an inhibitory effect on endogenous GSK-3 β) [49,50,55]. Figure 6 demonstrates that more than 90 % of transfected N2A cells overexpressing GSK-3 β R85 remain viable after incubation with PrP peptide, whereas most non-transfected cells die as shown previously in Figure 5(A). These results support the view that activation of GSK-3 (particularly GSK-3 β) is a crucial step in PrP peptide-induced cell death.

DISCUSSION

Our studies have shown that the activation of GSK-3 is an early and crucial step in the degeneration of cultured neurons triggered by the PrP 106–126 peptide, which is considered widely as an acceptable model system for prion-induced neurodegeneration [4,56]. Thus PrP peptide treatment leads to the activation of GSK-3, which is accompanied by an increased phosphorylation at Tyr²⁷⁹ in GSK-3 α and Tyr²¹⁶ in GSK-3 β . Furthermore, PrP peptide-induced cell death is prevented by treatment with lithium, a well-known GSK-3 inhibitor [47,54], or insulin, which leads to the inactivation of GSK-3 [33,34]. Finally, overexpression of an inactive mutant, GSK-3 β R85, which may inhibit endogenous GSK-3 β [49,50,55], also protects from PrP peptide-induced cell death.

Little is known about the initial events triggered by PrP peptide aggregates in target cells. These peptide aggregates may not only interact with a variety of cell-surface receptors but might also

penetrate directly into cell membranes [56]. Despite the exact mechanisms suggested, it may be hypothesized that membrane alterations could lead to dysfunction of signal-transduction cascades resulting in the activation of GSK-3, among other intracellular alterations. Curiously, GSK-3 activation not only occurs in response to PrP peptide but is also elicited by β -amyloid peptide [31,32,51], which supports the existence of shared common mechanisms for neurodegeneration by amyloidogenic peptides [15,16]. Of interest in this regard is the observation that PrP 106–126 and β -amyloid peptides trigger identical signal-transduction pathways, including the activation of protein tyrosine kinases and intracellular calcium release, in cultured microglia [57]. Similar alterations might be responsible for GSK-3 activation in neurons, since it has been reported that tyrosine kinases and transient increases in cytoplasmic calcium levels may enhance GSK-3 activity [36,58,59]. However, the precise molecular mechanisms whereby amyloidogenic peptides result in GSK-3 activation remain to be established.

Another point worthy of special consideration is the role played by GSK-3 in amyloidogenic peptide-induced neurodegeneration. Both PrP 106–126 and β -amyloid peptides induce apoptosis in cultured neurons [15,16], and this is prevented significantly by GSK-3 inhibition [32]. Accordingly, GSK-3 seems to be a crucial enzyme in apoptosis induction, which is supported by previous evidence concerning other models of neuronal cell death [35–41]. Nevertheless, the molecular targets responsible for apoptosis induction after GSK-3 activation remain to be investigated [34].

In the present study, we have emphasized that GSK-3 activation in PrP-treated neurons leads to hyperphosphorylation of microtubule-associated proteins 2 and tau. This modification of microtubule-associated proteins might facilitate the breakdown of the microtubule cytoskeleton, thus leading to the disorganization of neurites [60]. In particular, microtubule-associated protein 2 hyperphosphorylation might specifically contribute to dendrite dysfunction [61], which may constitute one of the earliest pathological events detectable in prion diseases [10]. It is thus plausible that PrP peptide induces an early neurite dysfunction, which may be partly due to the hyperphosphorylation of microtubule-associated proteins by GSK-3, and later triggers the induction of apoptosis, in which case GSK-3 could also be implicated through the phosphorylation of still unknown targets.

According to this view, GSK-3 should be considered as a relevant target for therapeutic intervention in neurodegenerative disorders like Alzheimer's or prion diseases, in which aggregates of amyloidogenic peptides are accumulated, since it might contribute to both early neuronal dysfunction and late cell death.

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