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PrP(106–126) activates neuronal intracellular kinases and Egr1 synthesis through activation of NADPH-oxidase independently of PrPc

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Abstract Prion diseases are characterised by severe neural lesions linked to the presence of an abnormal protease-resistant isoform of cellular prion protein (PrPc). The peptide PrP(106–126) is widely used as a model of neurotoxicity in prion diseases. Here, we examine in detail the intracellular signalling cascades induced by PrP(106–126) in cortical neurons and the participation of PrPc. We show that PrP(106–126) induces the activation of subsets of intracellular kinases (e.g., ERK1/2), early growth response 1 synthesis and induces caspase-3 activity, all of which are mediated by nicotinamide adenine dinucleotide phosphate hydrogen-oxidase activity and oxidative stress. However, cells lacking PrPc are similarly affected after peptide exposure, and this questions the involvement of PrPc in these effects.
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1. Introduction

Infectious prion pathologies are characterised by profound neural lesions linked to amyloid deposits of an abnormal isoform (PrPsc or PrPres) of cellular copper-binding cellular prion protein (PrPc) [1,2]. Neuronal death in prion encephalopathy is believed to be triggered by the activation of amyloid plaque formation and glial reactivity [3]. A synthetic peptide homologous to region 106–126 of PrPc has been used in vitro and in vivo to elicit toxic effects to cells [4–6]. Several kinases, along with the generation of reactive oxygen species (ROS), are involved in this process in a number of cell types [7,8]. However, the relevance of PrP(106–126) as a model of prion-induced cell effects and the role of PrPc expression in PrPsc and PrP(106–126)-induced effects have both been subject to debate [5,9–13]. Thus, in this study we examined intracellular signalling cascades, the participation of PrPc and the relevance of ROS production in the neurotoxic effects induced by exposure to PrP(106–126) in cortical cultures with normal PrPc levels and in *Prnp*$^{-/-}$ cells. Our results indicate that high doses (80 μM) of PrP(106–126) activate phosphoinositide-3 kinase (PI3K)/Akt, extracellular signal-regulated kinase (ERK1/2) and glycogen synthase kinase 3 (GSK3) kinases, but not c-Jun N-terminal kinase (JNK) or P38, and also increase synthesis of the early transcription gene early growth response 1 (Egr1). However, low doses of PrP(106–126) switched on GSK3 without any notable activation of ERK1/2. These intracellular events are typical hallmarks of oxidative stress. Indeed, we show that ROS generation in which nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-oxidase activity plays an important role after PrP(106–126) exposure is responsible for activation of intracellular kinases and cell death. Lastly, we found that these intracellular effects are not linked to the cellular expression of PrPc.

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Abbreviations: Ac-DEVD-AFC, Ac-Asp-Glu-Val-Asp-7amino 4trifluoromethyl coumarin; AkT, protein kinase B; Egr1, early growth response 1; EMMA, European mouse mutant archive; ERK1/2, extracellular signal-regulated kinase; FYN, protein tyrosine kinase p59; GSK3, glycogen synthase kinase 3; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, Mapk Erk-activating kinase; NAC, *N*-acetyl-L-cysteine; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; PI3K, phosphoinositide-3 kinase; Prnp, prion protein gene; PrPc, cellular prion protein; PrPres, protease-resistant form of prion protein; PrPsc, prion protein scrapie form; RIPA buffer, radioimmunoprecipitation lysis buffer 1; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SFK, Src-family kinase; TPA, phorbol-12-myristate-13-acetate

2. Materials and methods

2.1. Mice

PrP-deficient mice (*Prnp*$^{-/-}$) were purchased from EMMA (Italy). Sixty-two OF-1 pregnant mice (Iffa Credo, France) were also used. Genotyping of *Prnp*$^{-/-}$ mice was done using two PCRs (A and B), as indicated by Bueler and co-workers [14]. Mating day was considered to be embryonic day 0 (E0). All procedures were performed in accordance with the guidelines of the Spanish Ministry of Science and Technology, following European standards.

2.2. Antibodies

Antibodies were obtained from the following sources: Akt antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA), while Akt pserine-473, ERK1/2 pthreonine-202/ptyrosine-204, JNK, JNK-pthreonine-183/ptyrosine-185, p38 and p38 pthreonine-180/ptyrosine-182 were from Cell Signaling Technology (Beverly, USA). panERK was from Transduction Laboratories (Lexington, USA)

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and GSK3 (clone 4G-1E), GSK3 ptyrosine-279/216 (clone 5G-2F) and GSK3 pserine-9 (clone 2D3) were from Upstate Biotechnology Inc. (Lake Placid, USA). Tau was from Calbiochem (Missouri, USA) and PHF-1 and 9G3 antibodies against Tau pserine-396/404 and Tau ptyrosine-18 were gifts from Jesús Avila (CBM-UAM, Spain) and Gloria Lee (Iowa, USA), respectively. The SAF61 monoclonal antibody was from Spi-Bio&Cayman Chemical (Massy Cedex, France). A monoclonal antibody against class βIII-tubulin isoform (TUJ-1, Babco, USA), was used to detect the cytoskeletal protein expressed by post-mitotic neurons.

2.3. Primary embryonic neuronal cultures

E15-16 mouse embryo brains were dissected and washed in ice-cold 0.1 M PBS containing 6.5 mg/ml glucose. The meninges were removed and the cortical lobes isolated. Tissue pieces were trypsinized for 15 min at 37 °C. After addition of horse serum and centrifugation, cells were dissociated by trituration in 0.1 M PBS containing 0.025% DNAse with a polished pipette. Dissociated cells were plated at ~3000 cells/mm^2 on plates (Nunc, Denmark) coated with poly-D-lysine (Sigma, UK). The culture medium was Neurobasal supplemented with 2 mM glutamine, 6.5 mg/ml glucose, antibiotics and B27 (Invitrogen-Life Technologies, Belgium). Cultures contained up to 95% neurons (TUJ-1+) and were used after 4–7 days in vitro.

2.4. Immunocytochemical procedures

For immunocytochemistry primary cortical cultures were left to grow for 4–7 days on coverslips (12 mm ∅) coated with poly-L-Ornitine and Laminin (Sigma–Aldrich) in serum-free medium. Cultures were fixed with 2% phosphate-buffered paraformaldehyde for 1 h at 4 °C. Coverslips were then processed for the immunocytochemical detection of TUJ-1 and ERK1/2 pthreonine-202/ptyrosine-204 using Alexa-Fluor 488 and 568 tagged secondary antibodies (Molecular Probes, USA). After rinsing, cell nuclei were stained with Bisbenzimide (Hoechst 32444, 1 μM in PBS 0.1 M, 10 min) and the coverslips were mounted in Fluoromount (Vector Labs, Burlingame, USA).

2.5. Specific kinase inhibitors and activators

Src-family kinase (SFK) tyrosine kinase inhibitors, PP2, Emodin and Herbimycin A, SU6656 came from Calbiochem, as did Mapk Erk-activating kinase (MEK)1/2 inhibitor PD98509. MEK1/2 inhibitor U0126 was obtained from Promega (Madison, USA). PI3K inhibitors LY294002 and Wortmannin were from Sigma. Tyrosine kinase inhibitor Genistein was also from Sigma, as were NADPH-oxidase inhibitor DPI, *N*-acetyl-L-cysteine (NAC) and phorbol-12-myristate-13-acetate (TPA), a PKC activator.

2.6. Prion peptide treatments, caspase-3 activity assay and immunoblotting

PrPsc was prepared from the brains of terminally ill RML-inoculated mice. Human prion protein fragment 106–126 and scrambled peptide were from Sigma. Peptides (1 μg/μl) were dissolved in 0.1 M PBS and left to aggregate at room temperature for between 5 and 24 h. After aggregation, fibrillar PrP(106–126) or scrambled peptides were added to the cultures for various time periods (from 5 min to 4 days) or with 0.1% brain homogenates for 15 min to 4 days. RML-treated cells were collected after 15 min or 4, 10 and 15 days in vitro. In contrast, PrP(106–126)-treated cells were collected after peptide treatments. Cells were then scraped in radioimmunoprecipitation lysis (RIPA) buffer containing 1× protease inhibitor cocktail and phosphatase inhibitors. The caspase-3 activity assay was performed as previously described [15], with Ac-Asp-Glu-Val-Asp-7amino 4trifluoromethyl coumarin (Ac-DEVD-AFC) (Sigma) as substrate. Cell extracts were boiled in Laemmli sample buffer at 100 °C for 5 min, followed by 6–10% SDS–PAGE electrophoresis, electrotransferred to nitrocellulose membranes for 6 h at 4 °C and processed for immunoblotting using primary antibodies and the ECL-plus kit (Amersham-Pharmacia Biotech, UK). In our experiments, each nitrocellulose membrane was used for detecting both phosphorylated and total kinase levels.

2.7. Determination of GSK3 activity

GSK3 assays were carried out as described elsewhere [16]. Cultured cell extracts were prepared after peptide treatments. Cells were collected with a scraper and homogenized in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate and 5 mM EDTA. The soluble fraction was immunoprecipitated with the GSK3 antibody (see above). Samples of 10 μl were incubated in a buffer containing 25 mM HEPES, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$ and a specific GSK3 substrate peptide (pGSK3 peptide-2, Upstate Biotechnology Inc) at a final concentration of 0.75 mg/ml, and in the presence of γ^{32}P-ATP. After 30 min, the reaction was stopped with 1% H_3PO_4. The difference between kinase activity in the presence or absence of the GSK3 inhibitor LiCl (20 mM) was considered to reflect GSK3 kinase activity.

3. Results

3.1. PrP(106–126) activates stress-associated kinases in primary cortical cultures

Peptide aggregation prior to treatment was corroborated by electron microscope observations (see Fig. 3C). PrP(106–126) (80 μM) induced a strong stimulation of ERK1-2 and a low, but significant, activation of PI3K/Akt 15 and 30 min after peptide treatment (Fig. 1); there was no such effect on JNK (Fig. 1A) or P38 (not shown) (Fig. 1A and B). Similar treatment with scrambled peptide did not activate any of the studied kinases (not shown). In contrast, doses lower than 40 μM did not induce any notable ERK1/2 (Fig. 2B), PI3K, JNK or P38 kinase activation (not shown). In parallel experiments, cultures incubated with brain extracts obtained from RML-inoculated mice also increased ERK1/2 phosphorylation after 15 min (Fig. 1C), while brain extracts from healthy control animals did not (Fig. 1C). Neither PrP(106–126) nor RML brain extracts induced the accumulation of detectable levels of Proteinase-K resistant PrP in our cultures at 4, 10 and 15 days (not shown). Elk1 and the early gene transcription factor Egr1 are direct targets of ERK1/2 [17]. We showed that whereas a slight increase in pElk1 was observed at 30 min, Egr1 protein levels increased at 30 min and, especially, one day after treatment (Fig. 1D). Double immunolabelling using pERK1/2 and TUJ-1 antibodies revealed that pERK1/2 increased in dying neurons (TUJ-1+), disrupted neurites being displayed after treatment with 80 μM of PrP(106–126) (Fig. 1E–G).

We next determined the participation of the ERK1/2 pathway in the activation or otherwise of PI3K/Akt (Fig. 1H-I). PrP(106–126)-induced serine phosphorylation of Akt was impeded by the presence of Ly294002 (10 μM) + Wortmannin (10 μM), but not when incubated with MEK1/2 inhibitors (PD98509 (50 μM) and U0126 (50 μM)) (Fig. 1). Conversely, PD98509 + U0126 treatment blocked ERK1/2 induction, but not Akt phosphorylation after PrP(106–126) treatment. Thus, Akt activation paralleled but did not overlap with ERK1/2 activation after PrP(106–126) incubation, and ERK1/2 activation induced by the peptide may therefore be dependent on MEK2 activity. Parallel pharmacological studies using SFK inhibitors pointed to members of the Src kinase family (Yes, Lyn and probably Fyn) as being responsible for ERK1/2 activation after PrP(106–126) peptide incubation (see Suppl. Fig. 1 for details).

3.2. GSK3 activation and Tau phosphorylation are induced by PrP(106–126) in a concentration-dependent manner

The intracellular kinase GSK3 plays a crucial role in several neurodegenerative diseases [18,19]. Its activity depends on the tyrosine and serine phosphorylation state of the kinase [20]. We explored both types of GSK3 phosphorylation after

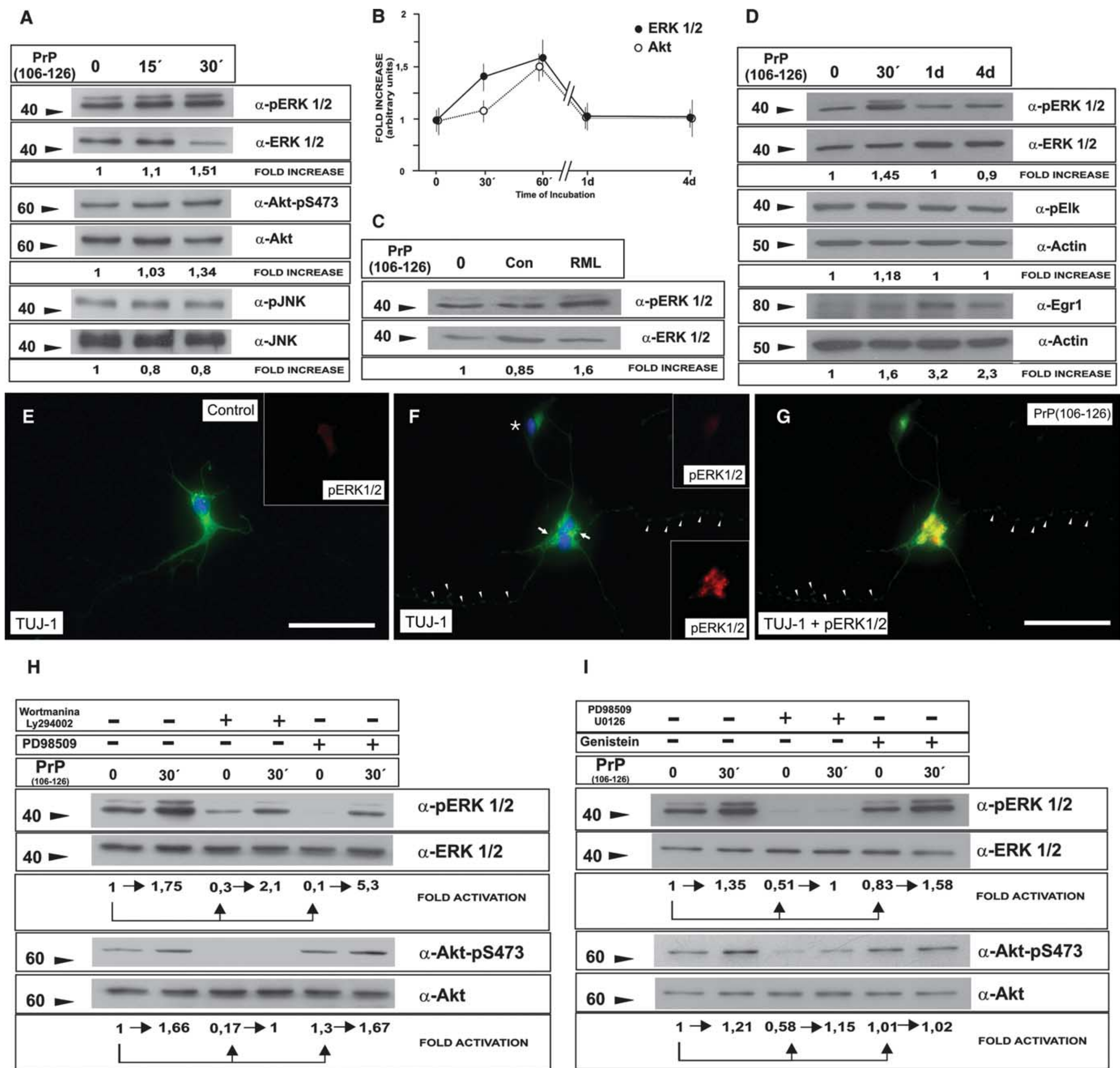

Fig. 1. Activation of ERK1/2 and Akt by PrP(106–126) in primary cortical cultures. (A–D) Cells were treated with 80 μM PrP(106–126) (A,B,D) for the indicated time periods or with 0.1% brain homogenate containing PrPsc (RML) or non-inoculated brain extract (Con) for 15 min; the phosphorylated forms of kinases, Elk and protein levels of Egr1 were analysed by Western blotting. Membranes were re-probed with antibodies against total ERK, Akt JNK or actin for standardisation. Quantitative results from three experiments are shown at the bottom as the fold increase from time 0 and in the graph in (C). (E,F) Examples of double-labelled neurons (TUJ-1 + pERK1/2) in control (E) and after 80 μM PrP(106–126) exposure (F,G). Whereas healthy neurons (asterisk in F) have pale pERK1/2 labelling, dying neurons (arrows) display disrupted neurites (open arrows) and notable pERK1/2 labelling. (H and I) Cortical primary cultures were incubated for 30 min as in (A), in the presence or absence of kinase inhibitors (e.g., 10 μM Wortmannin + 10 μM Ly294002) before and during PrP(106–126) treatment. Densitometric values after pre-incubation with kinase inhibitors at time 0 were standardised with time 0 in untreated cultures. The fold density modification after PrP(106–126) treatment for 0–30 min in each case is then shown. Scale bars in (E) and (F) are 30 μM.

treatment with PrP(106–126) peptide. Our results show that 80 μM PrP(106–126) increased the ratio of tyrosine/serine phosphorylation by 40% after 30 min (Fig. 2A). Surprisingly, this increase does not correlate with higher levels of phosphorylated Tau measured with the PHF1 antibody in Western blots (Fig. 2A). In contrast, 5 μM of PrP(106–126) increased GSK3 activity, as measured by both enzymatic assay and densitometry after Western blot (Fig. 2B and C) and Tau phosphorylation (Fig. 2B), without any apparent ERK1/2 kinase activation (Fig. 2B).

3.3. *PrP(106–126)-induced intracellular kinase activation is independent of PrPc*

It is assumed that $Prnp^{-/-}$ mice develop intrinsic resistance to prion infections and cell death (e.g., [21]). However, recent studies have reported that PrPsc are neurotoxic in $Prnp^{-/-}$

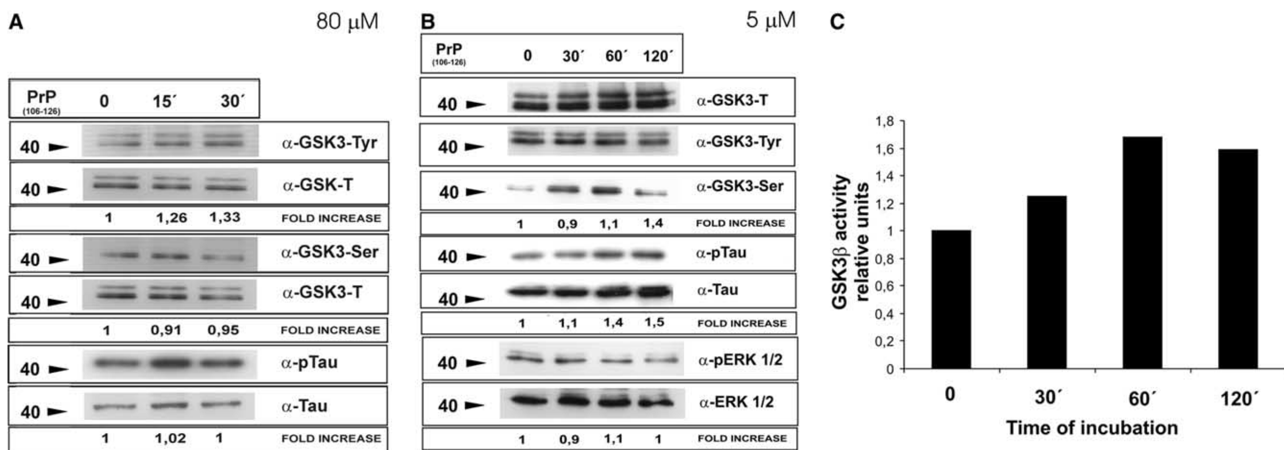

Fig. 2. (A,B) GSK3 activation and Tau phosphorylation after 80 (A) or 5 (B) μM PrP(106–126) incubation. Cortical cultures were treated with the peptide for indicated time periods. The activation of ERK1-2, GSK3 and the phosphorylation of Tau were analysed by Western blotting, as indicated in Fig. 1. Membranes were re-probed with antibodies against total GSK3, ERK1-2 and Tau for protein standardisation. Note the increase of GSK3 activity and Tau phosphorylation in the absence of notable ERK1-2 activation in (B). (B) Histogram illustrating the phosphorylation levels of a GSK3-specific substrate (see Section 2 for details of the enzymatic assay) in neuronal cultures treated with 5 μM PrP(106–126) for 0–120 min. GSK3 activity is the difference between the kinase activities in the presence or absence of LiCl. GSK3 activity rises shortly after PrP(106–126), and the increased activity lasts for 120 min.

cultures [13]. Thus, we explored whether the intracellular cascade activation induced by PrP(106–126) is PrPc-dependent, using cell cultures from *Prnp*$^{-/-}$ embryos. *Prnp*$^{-/-}$ genotype of cultured cells was corroborated by individual PCR genotyping (Fig. 3A). Surprisingly, ERK1-2 and the PI3K/Akt signalling cascades were also activated after 30 and 60 min of peptide exposure in *Prnp*$^{-/-}$ derived cortical cultures (Fig. 3B). Thus, the absence of PrPc does not prevent or modify the intracellular events induced by exposure to PrP(106–126).

On the other hand, PrPc aggregation at the cell surface by antibodies (e.g., SAF61) activated Fyn and other ERK1/2 kinases in vitro [22,23]. Our previous experiments indicated that PrP(106–126)-mediated intracellular effects are independent of PrPc. To corroborate whether PrPc-induced intracellular signalling pathways are different to those mediated by PrP(106–126) exposure, we treated *Prnp*$^{+/+}$ cultures with the SAF61 antibody. Our results showed that ERK1-2 kinases became activated 15 min after treatment with the SAF61 antibody, but that there was no effect on PI3K/Akt activity (Fig. 3C). Moreover, it has been reported that Fyn activation induces phosphorylation of the tyrosine/18 residue of Tau, which is detected by the 9G3 antibody [24]. We showed that the SAF61 antibody induced specific and transient phosphorylation of Tau tyrosine/18. In contrast, Tau phosphorylation revealed by the 9G3 antibody was not observed after PrP(106–126) treatment, which suggests that the notable activation of Fyn in 80 μM PrP(106–126) is unlikely and reinforces the hypothesis of different signalling mechanisms (Fig. 3D).

3.4. *NADPH-oxidase and ROS production regulate intracellular kinase activation induced by PrP(106–126)*

It is known that oxidative stress and other cell insults activate members of SFK (e.g., Yes or Lyn), as well as other intracellular cascades [25–28]. We incubated primary cortical cultures with the fluorescent probe Dihydroethidium (DIE, Sigma, 1 μg/ml concentration) to monitor ROS generation after PrP(106–126) treatment [29]. PrP(106–126) increased ROS generation in treated cultures, assessed by the number of DPI-labelled nuclei (Fig. 4A–E). In addition, 30 μM DPI (a NADPH-oxidase inhibitor) decreased and 20 mM NAC treatment completely blocked both ERK1/2 and PI3K/Akt kinase induction (Fig. 4F), while 20 mM NAC inhibited GSK3 activation (not shown) 30 min after 80 μM PrP(016-126) treatment. This ERK1/2 inhibition was corroborated by a decrease in Egr1 protein levels (Fig. 4G). These results demonstrate that NADPH-oxidase and ROS generation are crucial to both kinase activation and Egr1 synthesis induced by PrP(106–126). We next studied whether ROS-dependent kinase activation led to any considerable caspase-3 activation and the participation of PrPc in this process. PrP(106–126) exposure increased caspase-3 activity after 4 days of treatment in both *Prnp*$^{+/+}$ and *Prnp*$^{-/-}$ cultures (Fig. 4H), which correlates with the appearance of picnotic nuclei in treated cells (not shown). Indeed, incubation of 20 μM NAC 3 days impeded caspase-3 activity induced by 80 μM PrP(106–126) (not shown). In parallel experiments, we observed that the incubation of SU6656 (30 nM) or U0126 (50 nM) for 3 days also reduced caspase-3 activity after 80 μM PrP(106–126) treatment (Fig. 4J). Both inhibitors blocked ERK1/2 activation in acute (Suppl. Fig. 1) and/or long-duration PrP(106–126) incubation experiments (Fig. 4I). Taken together, these results indicate that ROS generation with the participation of NADPH-oxidase is responsible for ERK1/2 and Akt activation and further Egr1 synthesis after PrP(106–126) exposure. In addition, our results suggest that the ERK1/2 kinase pathway plays an important role in the caspase-3 activation and cell death observed after four days of 80 μM PrP(106–126) treatment in primary cortical cultures.

4. Discussion

4.1. *PrP(106–126) peptide induces intracellular kinase activation independently of PrPc-expression*

There is evidence that neurons derived from *Prnp*$^{-/-}$ mice are resistant to the toxicity of PrP(106–126) [30], and that the toxicity of PrPsc and PrPc fragments depends on neuronal

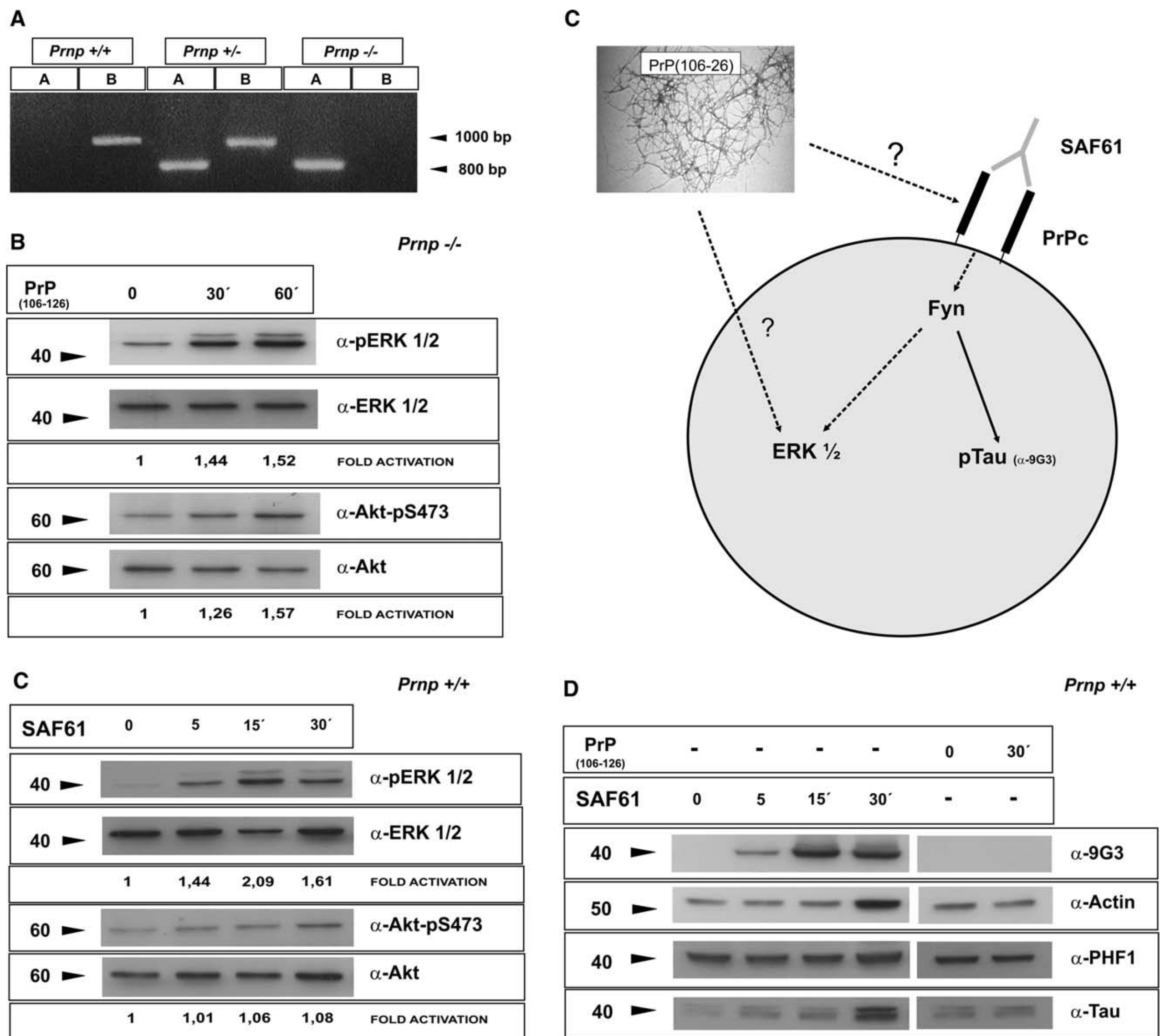

Fig. 3. (A) PCR identification of *Prnp* gene expression in tissue samples from embryos used in the experiments (see [14] for genotyping details and primer sequences). (B) $Prnp^{-/-}$ cultures were treated with 80 μM PrP(106–126) for indicated time periods and kinases were analysed by Western blotting. (C,D) Cells from wild-type ($Prnp^{+/+}$) mice were incubated with the SAF 61 antibody and kinases were analysed by Western blotting as above. Quantitative results of band analysis and fold increase are as in Fig. 1. (D) Cells from $Prnp^{+/+}$ mice were incubated with SAF61 or with 80 μM PrP(106–126). The phosphorylated form of Tau recognised by the 9G3 antibody (Fyn-dependent) or the PHF1 antibody were analysed by Western blotting. No labelling was obtained with the 9G3 antibody after PrP(106–126) incubation. Membranes were re-probed with antibodies against actin and total Tau for standardisation.

expression of PrPc [5,21,31]. Thus, a protein–protein interaction between PrP(106–126) or PrPsc with PrPc, which may render cells more susceptible to oxidative stress by impairing copper homeostasis, was postulated [32]. Our results showed that kinase activation, caspase-3 activation and cell death occur in $Prnp^{-/-}$ cultures, which argues against any participation of PrPc in the toxic effects induced by 80 μM PrP(106–126). In this respect, contradictory data about PrPc-expression levels and PrP(106–126) or PrPsc-associated toxicity have been reported [9,33,34]. Indeed, PrPsc may cause neurotoxicity in $Prnp^{-/-}$ cells [13]; in addition, neuronal colocalization of PrP(106–126) and PrPc failed in vitro [9]. Moreover, our results show that PrPc- and PrP(106–126)-mediated ERK1/2 activation are different. Thus, not only are the mechanisms involved in prion neurotoxicity still unclear (see [35] for comments), but PrP(106–126) may also reflect the full repertoire of intracellular signals associated with prion diseases.

4.2. High doses of PrP(106–126)-induced kinase activation by oxidative stress

Increased generation of ROS by microglial cells in a PrPc-dependent way has mainly been reported in in vitro experiments using PrP(106–126) [21,36]. However, PrP(106–126) alone increases the generation of hydrogen peroxide and reactive hydroxyl radicals in a cell-free system [37], and reduces glutathione levels and glutathione reductase or copper/zinc superoxide dismutase activity, thus increasing susceptibility to oxidative insults, in cultured cells [38,39]. Although 80 μM

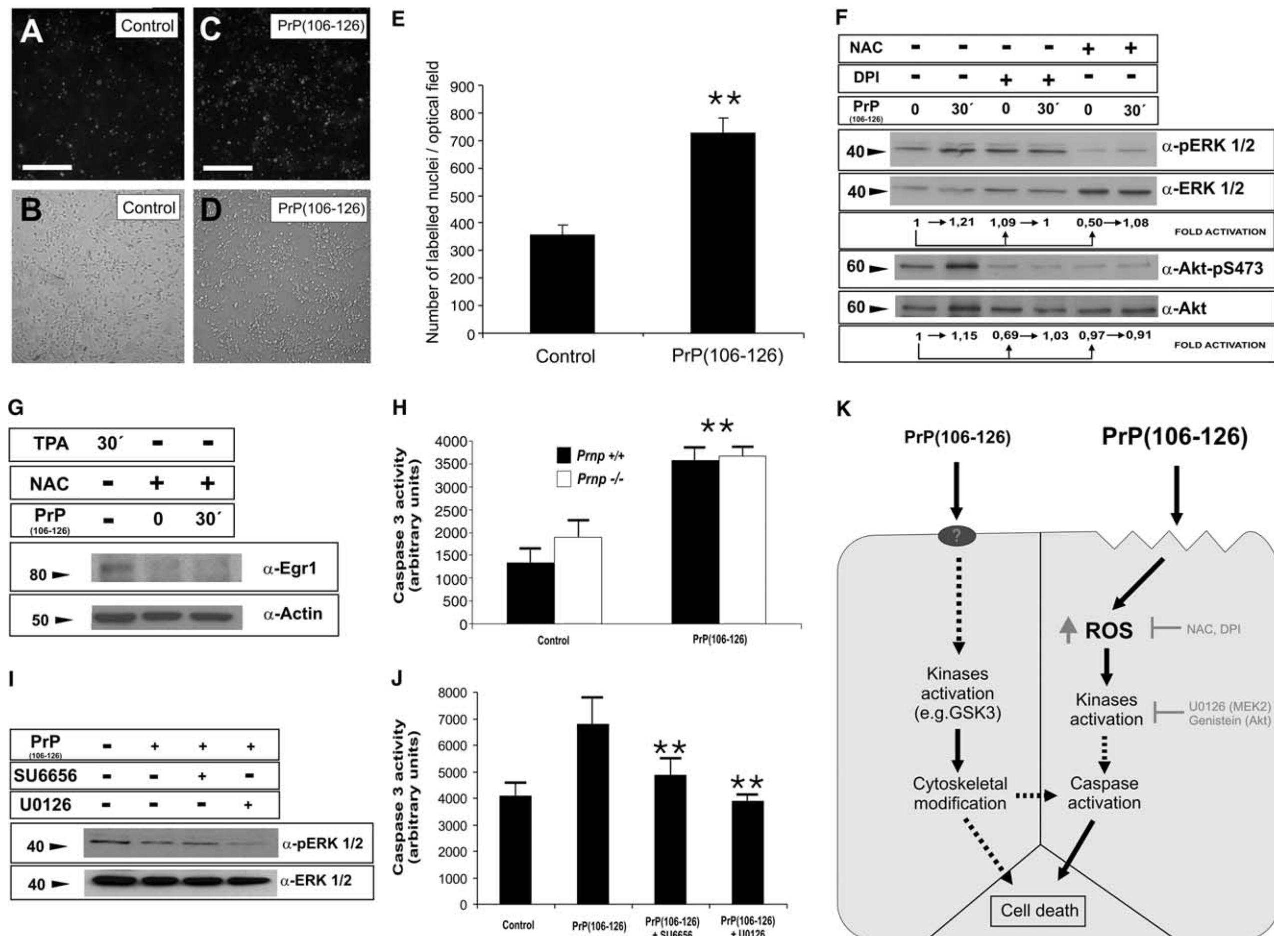

Fig. 4. (A–D) Fluorescence (A–C) and parallel phase contrast views (B,D) of cultures incubated with dihydroethidium (1 μg/ml) in control (A,B) and after PrP(106–126) treatment (C,D). (E) Histogram illustrating quantitative results of (A). Histograms represent the mean ± SEM of three independent experiments. (F,G) Cortical primary cultures were incubated with PrP(106–126), except that cells were pre-incubated in the presence or absence of the NADPH-oxidase inhibitor DPI (F), NAC (F,G) and PrP(106–126). Densitometry values after pre-incubation with inhibitors at time 0 are standardised with time 0 in untreated cultures. The variation after PrP(106–126) treatment from 0 to 30 min in each case is then shown. (H–J). Analysis of caspase-3 activation in both culture types, as determined by fluorimetry. Caspase-3 activation is expressed as an arbitrary unit and represents mean ± S.D. of duplicates from two independent experiments (in H and J). (I) Cortical cultures from wild-type were incubated with PrP(106–126) for 4 days in the presence of 10 nM SU6656 and 50 nM U0126. (K) Model of PrP(106–126)-mediated intracellular effects after interaction with cell membrane. The main results of the pharmacological inhibitory treatments are also indicated. Lower PrP(106–126) concentrations increase GSK3 activity leading to cell death, as indicated [50], by interacting with unknown membrane proteins or receptors (A) where the participation of PrPc is unknown. In contrast, high concentrations of PrP(106–126) leading to large β-sheet aggregates may induce a destabilization of the cellular membrane leading to cell death through different mechanisms that are likely to be associated with oxidative stress. Asterisks in E,H,J indicate statistical significance ($^{**}P < 0.01$, ANOVA).

PrP(106–126) is less neurotoxic than H_2O_2 [38], both treatments activate similar kinases in different cell types such as PC-12 cells or neuronal cultures [40]. In spite of this, we showed that 80 μM PrP(106–126) increases ROS production that further activates ERK1/2 (MEK2-dependent) and PI3K/Akt, increases Egr1 levels and induces GSK3 activation. The participation of NADPH-oxidase is relevant in these processes. In this regard, the MEK2 pathway has been linked to NADPH-oxidase and superoxide production under cellular stress [41], and oxidative stress has been reported to induce apoptosis by ERK1/2 activation through a Ras/Raf-dependent mechanism [42], which may support our data.

We also found that prolonged aggregation times of PrP(106–126) peptides at concentrations higher than 13 μM increased caspase-3 activation and cell death, as reported by Brown and co-workers using a MTT colorimetric assay [5]. In addition, filtered PrP(106–126) did not affect cell viability in our experiments (not shown). In contrast, the activation of p38 in neuroblastoma cells without apparent fibril formation has been described recently [43], and, surprisingly, PrP(106–126) has been considered non-neurotoxic by some authors [10]. Our data indicate that the aggregated peptide is toxic in primary neuronal cultures, as shown in other culture types or neurons [7,44–47]. However, we believe that the neurotoxic effects of PrP(106–126) depend on both its conformation and membrane interaction, as reported for other amyloid peptides including PrPsc or Aβ [5]. For example, both different neurotoxic potential and binding properties of Aβ gith membrane proteins depend on its aggregation form [48,49]. Moreover, low doses (5 μM) of PrP(106–126) activate GSK3 that, in turn, hyperphosphorylates Tau, which may lead to cell death without apparent activation of ERK1/2 [50] (see also present

results). This indicates that cell signalling elicited by different concentrations of PrP(106–126) may trigger different neurotoxic cascades in affected neurons (see Fig. 4K for hypothesis). Recent experiments by Zambrano and co-workers [51] demonstrate that oxidative stress induced by H_2O_2 decreases Tau phosphorylation through enhanced phosphatase activity, which could be prevented by NAC treatments. These findings may explain our different Tau phosphorylation results at several doses of PrP(106–126). 80 μM PrP(106–126), in contrast to lower doses, induced cell membrane destabilization and increased intracellular ROS production, thereby initiating both kinase and phosphatase (such as PP1) activities [52]. However, another possibility is that GSK3 might be activated by unknown signalling mechanisms at lower PrP(106–126) concentrations (e.g., by binding of the peptide to particular membrane receptor/s, as suggested elsewhere [53]), although this condition would be insufficient to activate cellular phosphatases and further Tau dephosphorylation, which may induce abnormal neuronal functioning and cell death. In conclusion, taking together, we believe the use of high doses of PrP(106–126) in vitro should not be regarded per se as a good experimental model for prion research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.06.037.

References

[1] Checler, F. and Vincent, B. (2002) Alzheimer's and prion diseases: distinct pathologies, common proteolytic denominators. Trends Neurosci. 25, 616–620.
[2] Aguzzi, A. (2000) Molecular pathology of prion diseases. Vox. Sang. 78 (Suppl. 2), 25.
[3] Hur, K., Kim, J.I., Choi, S.I., Choi, E.K., Carp, R.I. and Kim, Y.S. (2002) The pathogenic mechanisms of prion diseases. Mech. Ageing Dev. 123, 1637–1647.
[4] Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O. and Tagliavini, F. (1993) Neurotoxicity of a prion protein fragment. Nature 362, 543–546.
[5] Brown, D.R. (2000) Prion protein peptides: optimal toxicity and peptide blockade of toxicity. Mol. Cell. Neurosci. 15, 66–78.
[6] Ettaiche, M., Pichot, R., Vincent, J.P. and Chabry, J. (2000) In vivo cytotoxicity of the prion protein fragment 106–126. J. Biol. Chem. 275, 36487–36490.
[7] White, A.R., Guirguis, R., Brazier, M.W., Jobling, M.F., Hill, A.F., Beyreuther, K., Barrow, C.J., Masters, C.L., Collins, S.J. and Cappai, R. (2001) Sublethal concentrations of prion peptide PrP106–126 or the amyloid beta peptide of Alzheimer's disease activates expression of proapoptotic markers in primary cortical neurons. Neurobiol. Dis. 8, 299–316.
[8] Brown, D.R. (2004) Metallic prions. Biochem. Soc. Symp., 193–202.
[9] Fioriti, L., Quaglio, E., Massignan, T., Colombo, L., Stewart, R.S., Salmona, M., Harris, D.A., Forloni, G. and Chiesa, R. (2005) The neurotoxicity of prion protein (PrP) peptide 106–126 is independent of the expression level of PrP and is not mediated by abnormal PrP species. Mol. Cell. Neurosci. 28, 165–176.
[10] Kunz, B., Sandmeier, E. and Christen, P. (1999) Neurotoxicity of prion peptide 106–126 not confirmed. FEBS Lett. 458, 65–68.
[11] Singh, N., Gu, Y., Bose, S., Kalepu, S., Mishra, R.S. and Verghese, S. (2002) Prion peptide 106–126 as a model for prion replication and neurotoxicity. Front. Biosci. 7, a60–a71.
[12] Brown, D.R. (1998) Prion protein-overexpressing cells show altered response to a neurotoxic prion protein peptide. J. Neurosci. Res. 54, 331–340.
[13] Marella, M., Gaggioli, C., Batoz, M., Deckert, M., Tartare-Deckert, S. and Chabry, J. (2005) Pathological prion protein exposure switches on neuronal mitogen-activated protein kinase pathway resulting in microglia recruitment. J. Biol. Chem. 280, 1529–1534.
[14] Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M. and Weissmann, C. (1993) Mice devoid of PrP are resistant to scrapie. Cell 73, 1339–1347.
[15] Gurtu, V., Kain, S.R. and Zhang, G. (1997) Fluorometric and colorimetric detection of caspase activity associated with apoptosis. Anal. Biochem. 251, 98–102.
[16] Sayas, C.L., Moreno-Flores, M.T., Avila, J. and Wandosell, F. (1999) The neurite retraction induced by lysophosphatidic acid increases Alzheimer's disease-like Tau phosphorylation. J. Biol. Chem. 274, 37046–37052.
[17] Beckmann, A.M. and Wilce, P.A. (1997) Egr transcription factors in the nervous system. Neurochem. Int. 31, 477–510, discussion 517-6.
[18] Bhat, R.V., Budd Haeberlein, S.L. and Avila, J. (2004) Glycogen synthase kinase 3: a drug target for CNS therapies. J. Neurochem. 89, 1313–1317.
[19] Kaytor, M.D. and Orr, H.T. (2002) The GSK3 beta signaling cascade and neurodegenerative disease. Curr. Opin. Neurobiol. 12, 275–278.
[20] Bhat, R., Xue, Y., Berg, S., Hellberg, S., Ormo, M., Nilsson, Y., Radesater, A.C., Jerning, E., Markgren, P.O., Borgegard, T., Nylof, M., Gimenez-Cassina, A., Hernandez, F., Lucas, J.J., Diaz-Nido, J. and Avila, J. (2003) Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. J. Biol. Chem. 278, 45937–45945.
[21] Brown, D.R., Schmidt, B. and Kretzschmar, H.A. (1996) Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. Nature 380, 345–347.
[22] Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J.L., Lehmann, S., Launay, J.M. and Kellermann, O. (2000) Signal transduction through prion protein. Science 289, 1925–1928.
[23] Schneider, B., Mutel, V., Pietri, M., Ermonval, M., Mouillet-Richard, S. and Kellermann, O. (2003) NADPH oxidase and extracellular regulated kinases 1/2 are targets of prion protein signaling in neuronal and nonneuronal cells. Proc. Natl. Acad. Sci. USA 100, 13326–13331.
[24] Lee, G., Thangavel, R., Sharma, V.M., Litersky, J.M., Bhaskar, K., Fang, S.M., Do, L.H., Andreadis, A., Van Hoesen, G. and Ksiezak-Reding, H. (2004) Phosphorylation of tau by fyn: implications for Alzheimer's disease. J. Neurosci. 24, 2304–2312.
[25] Crossthwaite, A.J., Hasan, S. and Williams, R.J. (2002) Hydrogen peroxide-mediated phosphorylation of ERK1/2, Akt/PKB and JNK in cortical neurones: dependence on Ca(2+) and PI3-kinase. J. Neurochem. 80, 24–35.
[26] Cao, H., Sanguinetti, A.R. and Mastick, C.C. (2004) Oxidative stress activates both Src-kinases and their negative regulator Csk and induces phosphorylation of two targeting proteins for Csk: caveolin-1 and paxillin. Exp. Cell Res. 294, 159–171.
[27] Sun, X., Wu, F., Datta, R., Kharbanda, S. and Kufe, D. (2000) Interaction between protein kinase C delta and the c-Abl tyrosine kinase in the cellular response to oxidative stress. J. Biol. Chem. 275, 7470–7473.
[28] Torres, M. and Forman, H.J. (1999) Activation of several MAP kinases upon stimulation of rat alveolar macrophages: role of the NADPH oxidase. Arch. Biochem. Biophys. 366, 231–239.
[29] Bindokas, V.P., Jordan, J., Lee, C.C. and Miller, R.J. (1996) Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. J. Neurosci. 16, 1324–1336.

[30] Brown, D.R., Herms, J. and Kretzschmar, H.A. (1994) Mouse cortical cells lacking cellular PrP survive in culture with a neurotoxic PrP fragment. Neuroreport 5, 2057–2060.
[31] Brown, D.R., Schmidt, B. and Kretzschmar, H.A. (1996) A neurotoxic prion protein fragment enhances proliferation of microglia but not astrocytes in culture. Glia 18, 59–67.
[32] Brown, D.R. and Sassoon, J. (2002) Copper-dependent functions for the prion protein. Mol. Biotechnol. 22, 165–178.
[33] Bueler, H., Raeber, A., Sailer, A., Fischer, M., Aguzzi, A. and Weissmann, C. (1994) High prion and PrPSc levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. Mol. Med. 1, 19–30.
[34] Brandner, S., Isenmann, S., Raeber, A., Fischer, M., Sailer, A., Kobayashi, Y., Marino, S., Weissmann, C. and Aguzzi, A. (1996) Normal host prion protein necessary for scrapie-induced neurotoxicity. Nature 379, 339–343.
[35] Aguzzi, A. (2005) Cell biology. Prion toxicity: all sail and no anchor. Science 308, 1420–1421.
[36] Brown, D.R., Schulz-Schaeffer, W.J., Schmidt, B. and Kretzschmar, H.A. (1997) Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. Exp. Neurol. 146, 104–112.
[37] Turnbull, S., Tabner, B.J., Brown, D.R. and Allsop, D. (2003) Generation of hydrogen peroxide from mutant forms of the prion protein fragment PrP121–231. Biochemistry 42, 7675–7681.
[38] White, A.R., Collins, S.J., Maher, F., Jobling, M.F., Stewart, L.R., Thyer, J.M., Beyreuther, K., Masters, C.L. and Cappai, R. (1999) Prion protein-deficient neurons reveal lower glutathione reductase activity and increased susceptibility to hydrogen peroxide toxicity. Am. J. Pathol. 155, 1723–1730.
[39] Perovic, S., Schroder, H.C., Pergande, G., Ushijima, H. and Muller, W.E. (1997) Effect of flupirtine on Bcl-2 and glutathione level in neuronal cells treated in vitro with the prion protein fragment (PrP106–126). Exp. Neurol. 147, 518–524.
[40] Nair, V.D., Yuen, T., Olanow, C.W. and Sealfon, S.C. (2004) Early single cell bifurcation of pro- and antiapoptotic states during oxidative stress. J. Biol. Chem. 279, 27494–27501.
[41] Bhunia, A.K., Han, H., Snowden, A. and Chatterjee, S. (1997) Redox-regulated signaling by lactosylceramide in the proliferation of human aortic smooth muscle cells. J. Biol. Chem. 272, 15642–15649.
[42] Lee, H.T., Xu, H., Ota-Setlik, A. and Emala, C.W. (2003) Oxidant preconditioning protects human proximal tubular cells against lethal oxidant injury via p38 MAPK and heme oxygenase-1. Am. J. Nephrol. 23, 324–333.
[43] Corsaro, A., Thellung, S., Villa, V., Principe, D.R., Paludi, D., Arena, S., Millo, E., Schettini, D., Damonte, G., Aceto, A., Schettini, T. and Florio, T. (2003) Prion protein fragment 106–126 induces a p38 MAP kinase-dependent apoptosis in SH-SY5Y neuroblastoma cells independently from the amyloid fibril formation. Ann. N. Y. Acad. Sci. 1010, 610–622.
[44] Della-Bianca, V., Rossi, F., Armato, U., Dal-Pra, I., Costantini, C., Perini, G., Politi, V. and Della Valle, G. (2001) Neurotrophin p75 receptor is involved in neuronal damage by prion peptide-(106–126). J. Biol. Chem. 276, 38929–38933.
[45] Stewart, R.S. and Harris, D.A. (2001) Most pathogenic mutations do not alter the membrane topology of the prion protein. J. Biol. Chem. 276, 2212–2220.
[46] Thellung, S., Florio, T., Corsaro, A., Arena, S., Merlino, M., Salmona, M., Tagliavini, F., Bugiani, O., Forloni, G. and Schettini, G. (2000) Intracellular mechanisms mediating the neuronal death and astrogliosis induced by the prion protein fragment 106–126. Int. J. Dev. Neurosci. 18, 481–492.
[47] Thellung, S., Villa, V., Corsaro, A., Arena, S., Millo, E., Damonte, G., Benatti, U., Tagliavini, F., Florio, T. and Schettini, G. (2002) p38 MAP kinase mediates the cell death induced by PrP106–126 in the SH-SY5Y neuroblastoma cells. Neurobiol. Dis. 9, 69–81.
[48] Verdier, Y. and Penke, B. (2004) Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. Curr. Protein Pept. Sci. 5, 19–31.
[49] Verdier, Y., Zarandi, M. and Penke, B. (2004) Amyloid beta-peptide interactions with neuronal and glial cell plasma membrane: binding sites and implications for Alzheimer's disease. J. Pept. Sci. 10, 229–248.
[50] Perez, M., Rojo, A.I., Wandosell, F., Diaz-Nido, J. and Avila, J. (2003) Prion peptide induces neuronal cell death through a pathway involving glycogen synthase kinase 3. Biochem. J. 372, 129–136.
[51] Zambrano, C.A., Egana, J.T., Nunez, M.T., Maccioni, R.B. and Gonzalez-Billault, C. (2004) Oxidative stress promotes tau dephosphorylation in neuronal cells: the roles of cdk5 and PP1. Free Radic. Biol. Med. 36, 1393–1402.
[52] Dupiereux, I., Zorzi, W., Lins, L., Brasseur, R., Colson, P., Heinen, E. and Elmoualij, B. (2005) Interaction of the 106–126 prion peptide with lipid membranes and potential implication for neurotoxicity. Biochem. Biophys. Res. Commun. 331, 894–901.
[53] Thellung, S., Florio, T., Villa, V., Corsaro, A., Arena, S., Amico, C., Robello, M., Salmona, M., Forloni, G., Bugiani, O., Tagliavini, F. and Schettini, G. (2000) Apoptotic cell death and impairment of L-type voltage-sensitive calcium channel activity in rat cerebellar granule cells treated with the prion protein fragment 106–126. Neurobiol. Dis. 7, 299–309.