Altered toxicity of the prion protein peptide PrP106–126 carrying the Ala¹¹⁷ → Val mutation

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The inherited prion diseases such as Gerstmann–Sträussler–Scheinker syndrome (GSS) are linked to point mutations in the gene coding for the cellular isoform of the prion protein (PrP^C). One particular point mutation A117V (Ala¹¹⁷ → Val) is linked to a variable pathology that usually includes deposition of neurofibrillary tangles. A prion protein peptide carrying this point mutation [PrP106–126(117V)] was generated and compared with a peptide based on the normal human sequence [PrP106–126(117A)]. The inclusion of this point mutation increased the toxicity of PrP106–126 which could be linked to an increased β -sheet content. An assay of microtubule formation in the presence

of tau indicated that PrP106–126 decreased the rate of microtubule formation that could be related to the displacement of tau. PrP106–126 carrying the 117 mutation was more efficient at inhibiting microtubule formation. These results suggest a possible mechanism of toxicity for protein carrying this mutation via destabilization of the cytoskeleton and deposition of tau in filaments, as observed in GSS.

Key words: calcium, cerebellar, Gerstmann–Sträussler–Scheinker, microtubule, tau.

INTRODUCTION

Most cases of human prion disease arise ‘spontaneously’ with no known cause. However, the inherited prion diseases such as Gerstmann–Sträussler–Scheinker Syndrome (GSS) and Fatal Familial Insomnia and some forms of Creutzfeldt–Jakob Disease (CJD) are linked to distinct point mutations within the gene coding for the cellular isoform of the prion protein (PrP^C) [1–3]. The point mutations in the human *PRNP* gene include the codons 102, 105, 117, 145, 198, 217 (all GSS) as well as 178, 200 and 210 (mostly CJD) [4,5]. In CJD or GSS patients PrP^C accumulates as the abnormal scrapie isoform of the prion protein (PrP^{Sc}). Patients with GSS are normally diagnosed with the disease in the fifth decade of life, the first signs of disease usually being motor disturbances. The form of prion disease in GSS is characterized by multicentric amyloid plaques in cerebellum and cortex. The extent and nature of neuropathological changes and clinical signs is extremely variable even when patients have the same mutation, and also with patients from the one family. Such variability suggests that prion ‘strains’ associated with artificial inoculation of mice have little relevance to naturally occurring prion diseases.

Some of the mutations associated with GSS occur only in a small number of families. Whereas GSS with the P102L (Pro¹⁰² → Leu) mutation occurs in greater than 30 known families, the A117V (Ala¹¹⁷ → Val) mutation occurs in only three [6–8]. The A117V mutation requires two changes in the genetic code to produce the amino acid change, possibly suggestive of a rare occurrence. Some forms of GSS are associated with neuropathology inclusive of neurofibrillary tangles formed from abnormally deposited tau protein. Although this is mostly associated with the F198S (Phe¹⁹⁸ → Ser) [9] and Q217R (Gln²¹⁷ →

Arg) mutations it is also known to occur in one case with A117V [10].

The A117V mutation lies within a region of the protein conserved in every known mammalian sequence of the prion protein (PrP) [11]. The high conservation of this region suggests that it is important that no mutation be introduced into the sequence either to preserve PrP^C function or to prevent pathological consequences. Previous detailed analysis of peptides spanning this region (PrP106–126) allows analysis of the effect of this mutation on this peptide.

PrP106–126 applied to cultured cells has been used as a model for the neurotoxicity of PrP^{Sc}. In culture both PrP^{Sc} [12] and PrP106–126 [13,14] require neuronal expression of PrP^C and the presence of activated microglia in order to exert a toxic effect. PrP106–126 causes a reduction in the resistance of neuronal cells to oxidative stress [15], possibly through inhibiting incorporation of copper into superoxide dismutase [16] and possibly other enzymes. PrP106–126 also inhibits astrocytic glutamate uptake [17], which may have consequences for neuronal survival in the latter stages of prion disease [18] as a result of the induction of astrocyte proliferation [19].

In this study it was shown that synthesizing PrP106–126 with the substitution of valine for alanine at amino acid residue 117 altered the peptide making it more neurotoxic, altering its β -sheet content and enhancing its ability to inhibit tau-stimulated microtubule assembly. This study suggests some possibilities as to how the A117V mutation can result in a pathological phenotype.

METHODS

Unless stated, pharmacological agents were purchased from Sigma. Calciseptine was from Latoxan (Rosans, France).

Abbreviations used: CJD, Creutzfeldt–Jakob Disease; GSS, Gerstmann–Sträussler–Scheinker syndrome; LLME, L-leucine methyl ester; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PrP, prion protein; PrP^{Sc}, scrapie isoform of PrP; PrP^C, cellular isoform of PrP; PrP106–126, PrP peptide consisting of amino acids 106–126 of the human PrP sequence; PrP106–126(117A), wild-type sequence of PrP106–126; PrP106–126(117V), PrP106–126 carrying a valine substitution at position 117; Prnp^{0/0}, PrP knockout mice.

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Animals

The PrP knockout mice (Prnp^{0/0}) used in this study were those generated by Büeler et al. [20]. The wild-type mice used were descendants of an F1-generation mouse produced by interbreeding the original parental strains [C57BL/6J and 129/Sv(ev) mice] used to generate the Prnp^{0/0} mice.

Neuronal cell culture

Preparation of cerebellar cells from 6-day-old mice (P6) was as described previously [14]. Briefly, the cerebella were dissociated in Hanks medium (Gibco) containing 0.5% trypsin (Sigma) and plated at $1\text{--}2 \times 10^6$ cells/cm² in 24-well trays (Falcon) coated with poly-D-lysine (50 µg/ml; Sigma). Cultures were maintained in Dulbecco's minimal essential media (Gibco) supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine and 1% (v/v) antibiotics (penicillin, streptomycin, fungizone; Gibco). Cultures were maintained at 37 °C with 6% CO₂ for 10 days. Peptides were added to cultures initially and on day three. The peptide concentration was maintained at 80 µM. Cell survival was determined on day seven. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) was diluted to 200 µM in Hanks solution and added to the cultures for 2 h at 37 °C. The MTT formazan product was released from the cells by the addition of DMSO (Sigma) and measured at 570 nm in a Unicam UV2 spectrophotometer (ATI Unicam). Relative survival in comparison with untreated control could then be determined.

For co-culture experiments, cerebellar cells were plated as normal in 24-well trays. Microglia were plated in tissue-culture inserts (Falcon) with 0.4 µm pores. Peptides could then be applied directly to the lower wells and to the insert. After peptide treatments, the inserts were removed and MTT assays carried out on the cerebellar cells as described above.

Glia cell culture

Microglia were isolated as described previously [19]. Briefly, cortices from newborn mice were dissociated with trypsin and seeded into tissue-culture flasks (Falcon). When cultures were confluent, microglia were isolated by rapid shaking for 2 h. The floating cells were collected and plated for 30 min. The non-adhering cells were discarded. The adhering cells were identified as pure cultures of microglia by immunostaining for ferritin as described previously [19].

Peptides

Peptides used in these experiments included PrP106–126(117A) with the sequence KTNMKHMAGAAAAGAVVGGLG, derived from amino acid residues 106–126 of the human PrP sequence, and the mutant version of the peptide, PrP106–126(117V), based on the human form of the sequence found in GSS patients, KTNMKHMAGAAVAGAVVGGLG. Peptides were produced in house by the Protein and Nucleic Acid Chemistry facility or produced as described previously [14].

Analysis of fibril formation

The ability of PrP peptides to form fibrils was checked by negative staining with phosphotungstic acid and transmission electron microscopy. Briefly, a droplet of 10 µM peptide was incubated on a formvar-coated electron microscopy grid for 2 min. Excess solution was removed and the grid allowed to air dry. Analysis of triplicate preparations of the peptides was performed on a JOEL transmission electron microscope at 80 kV.

CD analysis

CD spectra were recorded for PrP peptides in a CD6 spectropolarimeter (Jobin Yvon, Division d'Instrumente S.A.), calibrated with ammonium d-camphor-10-sulphonate by a method similar to that described previously [20a]. Peptide samples (2 mg/ml) prepared in 10 mM sodium phosphate (pH 7.4) were measured in cuvettes with a 0.1 mm pathlength. The spectrum from 195–250 nm was analysed with a step resolution of 0.5 nm at a temperature of 23 °C. Five scans were averaged and the background from buffer was subtracted. Spectra are presented as molar ellipticity $[\theta]_M$.

Tubulin polymerization assays

Two assays were used to assess tubulin polymerization. The first was a tau-independent assay. This assay was as described previously [21]. Tubulin (Sigma) was added to the reaction at 0.5 mg/ml (55 nM). The assay ran for 45 min while measuring the turbidity at 350 nm in a spectrophotometer. The second assay was a tau-dependent assay. This assay was similar to one described by Yoshida and Ihara [22]. The polymerization buffer contained 100 mM Mes (pH 6.5), 1 mM EGTA, 0.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM GTP and 0.1 mg/ml tau (ICN). The assay was started by the addition of 1.0 mg/ml tubulin (110 nM). The assay ran for 45 min while measuring the turbidity at 350 nm in a spectrophotometer.

Measurements of intracellular Ca²⁺

Cerebellar cell cultures were loaded with fura-2/AM (Molecular Probes) to assess changes in intracellular calcium levels as described previously [23]. Cells were examined after 24 h treatment with PrP106–126 peptides using epifluorescence stimulation and the level of intracellular calcium determined as described previously [23]. For the assessment of $[\text{Ca}^{2+}]_i$, fluorescence signals were collected from a 15 µm diameter area. This window was centred on the cell soma.

RESULTS

Fibril formation

The ability of the two forms of PrP106–126 to form fibrils was compared. PrP106–126 was dried on to electron microscopy grids and stained with phosphotungstic acid. The resulting fibrils, as seen via electron microscopy, are shown in Figure 1. There was no apparent differences in the ability of the peptides to form fibrils.

CD analysis

CD analysis of the PrP peptides used in this study was carried out. The results of the analysis appear in Figure 2. Both peptides were analysed in phosphate buffer as in previous investigations [20a]. The peptides were prepared freshly and analysed immediately. Both PrP106–126(117A) and PrP106–126(117V) showed a major trough at approx. 197 nm consistent with a random-coil structure. The spectra of both peptides also show a trough at 219–224 nm. This trough is indicative of β -sheet content. However, this trough is much greater for PrP106–126(117V) suggesting that β -sheet is a predominant characteristic

Figure 1 Electron microscopic photomicrographs of peptides prepared in PBS and stained with phosphotungstic acid

Long fibrils in matrices can be seen in the photomicrographs. (A) PrP106–126(117A), (B) PrP106–126(117V). Magnification $\times 50000$.

of its structure under these conditions. These results suggest a major difference in the secondary structure of PrP106–126(117V) compared with PrP106–126(117A).

Toxicity

A comparison was made between the mechanism of toxicity of PrP106–126(117A) and the mutant peptide, PrP106–126(117V). PrP106–126 is known to be toxic to neurone-rich cultures such as those produced by postnatal mice. Cerebellar cell cultures from 5–6-day-old wild-type mice were prepared and exposed to various concentrations of PrP106–126(117A) and PrP106–126(117V). After 7 days of treatment the survival of the cultures was compared using an MTT assay. As seen in Figure 3A PrP106–126(117V) was far more toxic than PrP106–126(117A). PrP106–126(117V) showed significant levels of toxicity even at 10 μM (Student's *t* test, $P < 0.05$).

The dependence of PrP106–126(117V) toxicity on PrP^C expression was investigated by application of the peptides to cerebellar cells derived from mice deficient in PrP^C expression (Prnp^{0/0}). At concentrations of 20 μM and above PrP106–126(117V) was toxic to Prnp^{0/0} cerebellar cells while PrP106–126(117A) was not (Figure 3B). This suggests that the toxicity of PrP106–126(117V) was partly independent of PrP^C expression.

Figure 2 CD study of peptides

Peptides were prepared freshly in 10 mM phosphate buffer, pH 7.4, at 2 mg/ml. The CD spectrum of PrP106–126(117V) (—) was compared with that of PrP106–126(117A) (·····). Spectra are presented as molar ellipticity (θ).

Figure 3 Toxicity of PrP peptides to cerebellar cells

(A) Wild-type cerebellar cells from 5–6-day-old mice were exposed to increasing concentrations of either PrP106–126(117A) (○) or PrP106–126(117V) (●) for 7 days. After this time an MTT assay was carried out to determine relative survival. Values for treated cells were compared with those of untreated cells as a percentage (control value = 100% survival). Both peptides were toxic; however, PrP106–126(117V) was far more toxic than PrP106–126(117A). (B) Prnp^{0/0} cerebellar cells were exposed to increasing concentrations of either PrP106–126(117A) (○) or PrP106–126(117V) (●) for 7 days. After this time an MTT assay was used to measure relative survival. PrP106–126(117V) was toxic to Prnp^{0/0} cerebellar cells but PrP106–126(117A) was not. Values shown are the means $\pm$ S.E.M. for a minimum of four experiments.

The dependence of PrP106–126(117V) toxicity on the presence of microglia in the cerebellar cell cultures was also examined. Wild-type cerebellar cells were treated with 50 μM L-leucine methyl ester (LLME), an agent that is selectively toxic to

Figure 4 The requirement of microglia for PrP106–126 toxicity

(A) LLME was applied to wild-type cerebellar cells at 50 μ M for 2 h before treatment with 80 μ M PrP106–126 peptides. Some LLME-treated cultures were co-cultured with microglia (10^4 cells/well) applied in inserts. After 7 days treatment an MTT assay was carried out on the cerebellar cells only. LLME treatment blocked the toxicity of PrP106–126(117A) but only partially inhibited the toxicity of PrP106–126(117V). Addition of microglia increased the toxicity of both peptides. Open bars, control; grey bars, LLME treated; black bars, LLME treated and microglia co-cultured. (B) Prnp^{0/0} cerebellar cells were co-cultured with an increasing number of wild-type microglia. PrP106–126 (117V) was applied at 80 μ M to Prnp^{0/0} cerebellar cells with and without microglial co-culture. An MTT assay was carried out after 7 days treatment to determine relative survival. Addition of microglia did not enhance the toxicity of this peptide. Values shown are the means $\pm$ S.E.M. for a minimum of four experiments.

microglia (Figure 4A). Although the toxicity of PrP106–126(117V) was greatly reduced by this treatment (Figure 4A) it was not abolished. In comparison, the toxicity of PrP106–126(117A) was abolished. Addition of purified microglia in co-culture with LLME-treated wild-type cerebellar cells enhanced the toxicity of both PrP106–126(117A) and PrP106–126(117V). This suggests that, similar to PrP106–126(117A), the toxicity of the mutant peptide is also dependent on microglia. However, the failure of LLME treatment to abolish this toxicity suggests that some component of the toxicity of PrP106–126(117V) is microglia independent.

The microglial dependence of the toxicity of PrP106–126 (117V) to Prnp^{0/0} cerebellar cells was tested. Prnp^{0/0} cerebellar cells were co-cultured with an increasing number of wild-type microglia. Treatment with 80 μ M PrP106–126(117V) followed. The effect of microglial co-culture did not enhance the toxicity of PrP106–126(117V) to Prnp^{0/0} cerebellar cells suggesting that the toxicity of this peptide to these cells was independent of microglia (Figure 4B).

The above results suggest a strong difference in the mechanism of toxicity of PrP106–126(117V) to cerebellar cells compared with PrP106–126 containing the wild-type sequence. These results also indicate that a component of the toxicity that is different is independent of PrP^C expression and also independent of the presence of microglia. Therefore further experiments were carried

out on Prnp^{0/0} cerebellar cells to further characterize this novel component of the peptide's toxicity.

Prnp^{0/0} cerebellar cells treated with 80 μ M PrP106–126(117V) were treated with increasing concentrations of a variety of agents. The antioxidant *N*-acetyl cysteine did not block the toxicity of PrP106–126(117V) suggesting that the toxicity of this peptide does not involve oxidative stress (Figure 5A). MK801, an *N*-methyl-D-aspartate ('NMDA') receptor antagonist, also did not inhibit the toxicity of this peptide (Figure 5B) suggesting that calcium entry through these receptors is not involved in the toxicity of PrP106–126(117V). However, two inhibitors of L-type calcium channels, verapamil and calciseptine, both inhibited the toxicity of PrP106–126(117V) at low concentrations (Figure 5C) suggesting that activation of these channels is involved in the toxicity of this peptide.

The intracellular calcium concentration of Prnp^{0/0} cerebellar neurones was assessed using the fura-2-based imaging technique. As suggested previously [23], treatment with 80 μ M PrP106–126(117A) had no effect on the intracellular calcium concentration ($n = 25$; Figure 5D). However, PrP106–126(117V) significantly increased the intracellular calcium concentration ($P < 0.05$) in Prnp^{0/0} cerebellar neurones ($n = 25$) compared with untreated cells ($n = 20$). This suggests that the toxicity of PrP106–126(117V) to Prnp^{0/0} neurones may be mediated via a calcium-dependent mechanism.

Inhibition of tubulin polymerization

A previous report has suggested that PrP106–126 can bind to microtubules [24]. The ability of PrP106–126 to inhibit tubulin polymerization in a cell-free system was investigated. Both tau-dependent and tau-independent polymerization of tubulin was assayed using a spectrophotometric assay of turbidity. The presence of 50 μ g/ml tau caused rapid polymerization of tubulin (Figure 6A) over a 45 min period, as indicated by increased turbidity. The presence of 10 μ g/ml PrP106–126(117A) in this reaction inhibited the reaction slightly but significantly ($P < 0.05$). However, addition of 10 μ g/ml PrP106–126(117V) inhibited the reaction much more and the effect was significantly different to that of PrP106–126(117A) ($P < 0.05$; Figure 6A). The final absorbance readings in this assay were reduced, suggesting that the peptide inhibited the achievable level of polymerized tubulin and not necessarily the rate of polymerization.

A different assay of tubulin polymerization not dependent on tau was also used (Figure 6B). In this assay neither peptide inhibited the polymerization of tubulin suggesting that PrP106–126(117V) did not prevent tubulin polymerization. The effect of the peptides in the tau-dependent assay was re-assessed by examining the effects of the peptides on the rate of the reaction. According to Figure 6(A) the reaction was linear within the first 10 min. Therefore the change in absorbance over the initial 10 min was assessed in the presence of 0.1 mg/ml tau and increasing concentrations of peptide (Figure 6C). Both peptides significantly ($P < 0.05$) decreased the rate of tubulin polymerization having a maximal effect at 1 μ g/ml. PrP106–126(117V) had a much greater effect than PrP106–126(117A). With concentrations above 1 μ g/ml there was a slightly decreased effect possibly due to aggregation of the peptide. Using 1 μ g/ml peptide the effect of tau on the rate of tubulin polymerization in the tau-dependent assay was assessed (Figure 6D). As expected tau on its own greatly enhanced the rate of tubulin polymerization in a dose-dependent manner. This effect was diminished by the presence of peptide and especially that of PrP106–126(117V).

Figure 5 The mechanism of toxicity of PrP106–126(117V) to Prnp^{0/0} cerebellar cells

Cerebellar cells were treated with 100 μM PrP106–126(117V) for 7 days in combination with a variety of agents at increasing concentrations. Relative survival was measured using an MTT assay. (A) *N*-Acetylcysteine did not inhibit the toxicity of PrP106–126(117V); (B) MK801 did not inhibit the toxicity of PrP106–126(117V); (C) Verapamil (●) and calciseptine (○) both inhibited the toxicity of PrP106–126(117V). (D) Intracellular calcium concentrations, $[\text{Ca}^{2+}]_i$, of Prnp^{0/0} cerebellar neurones with and without peptide treatment for 24 h, as determined by imaging using the dye fura-2/AM. PrP106–126(117V) greatly increases the intracellular calcium concentration. Values shown are the means $\pm$ S.E.M. for a minimum of four experiments.

These results suggest that PrP106–126(117V) inhibited tau-dependent polymerization of tubulin by preventing the interaction of tau with tubulin.

Further experiments were carried out to test whether destabilization of the cytoskeleton was implicated in the toxicity of these peptides. Two stabilizers were tested, taxol, which stabilizes microtubules, and phalloidin, which stabilizes actin filaments. Increasing concentrations of taxol inhibited the toxicity of 20 μM PrP106–126(117V) but did not inhibit the toxicity of 80 μM PrP106–126(117A) (Figure 7A). Furthermore taxol blocked the toxicity of PrP106–126(117V) completely on Prnp^{0/0} cerebellar cells (Figure 7B). Phalloidin had no effect on the toxicity of either peptide (Figure 7C). These results suggest that the mechanism of toxicity of PrP106–126(117V) involves destabilization of microtubules causing increased calcium entry via L-type calcium channels.

DISCUSSION

This study examined the effect of a known human point-mutation in the PrP gene on the neurotoxicity *in vitro* of the PrP peptide, PrP106–126. The A117V point mutation of GSS probably arose through two distinct point mutations occurring within the one codon (GCA changing to GTG) [4]. However, this is the only known mutation within the sequence of PrP106–126. This region is very highly conserved between mammalian species [11] and also between birds and mammals [25]. This would suggest that any mutation within this region may have extremely deleterious consequences. Patients carrying this mutation may not present with symptoms until late in life implying that the mutation can

lie silent until after reproductive life is past. This might not be the case for any other mutation in this region.

This single change in the amino acid sequence of PrP106–126 altered the toxicity of the peptide greatly. The toxicity of PrP106–126 without the mutation has been studied in detail [13–15,18]. In cerebellar cell cultures, this neurotoxicity requires neuronal expression of PrP^C and microglial release of reactive oxygen species. PrP^{Sc} (murine) applied to the same culture system has the same basic neurotoxic mechanism [12]. In comparison PrP106–126 carrying the A117V mutation does not have this mechanism of toxicity.

The mechanism of neurotoxicity of PrP106–126(117V) showed two components. The first component of the neurotoxicity was the same as that of PrP^{Sc} and requires the presence of microglia and neuronal PrP^C expression for toxicity to neurones in cerebellar cell cultures. However, the second component was novel and did not require neuronal PrP^C expression. Furthermore, this component of the toxicity was not enhanced by the presence of extra microglia. This second component could be inhibited by L-type calcium-channel blockers and taxol. The finding that this peptide can inhibit microtubule polymerization suggests that the second element of the toxic mechanism involves destabilization of the cytoskeleton leading to cell death via calcium entry. The difference in the toxic mechanism of the mutant peptide is supported by the finding that the PrP106–126(117V) has a different secondary structure to that of the wild-type peptide.

The role of calcium influx through L-type calcium channels has been suggested for PrP106–126 from previous experiments [23,26,27]. PrP106–126 effects the kinetics of calcium entry into synaptosomes [26], neurones [27] and astrocytes [27], all of which

Figure 6 Assays of tubulin polymerization were carried out using spectrophotometric determination of turbidity

(A) Tau-dependent assay. Polymerization without the addition of tau was slow (○). Polymerization was accelerated by the presence of the microtubule-binding tau protein (●). PrP106-126(117A) inhibited this reaction slightly (□), but PrP106-126(117V) inhibited this reaction greatly (■). (B) Tau-independent assay. In the absence of tau neither PrP106-126(117V) (■) nor PrP106-126(117A) (□) inhibited tubulin polymerization and the reaction occurred at the same rate as it did without the addition of peptide (●). (C) Rate of tubulin formation in the tau-dependent assay in the presence of a set amount of tau (as in A) and increasing concentrations of either PrP106-126(117V) (■) or PrP106-126(117A) (□). PrP106-126(117V) inhibited the rate of tau-dependent tubulin polymerization in a dose-dependent manner and its effect was greater than PrP106-126(117A). (D) Rate of tubulin formation in the tau-dependent assay in the absence of peptide (●) or with 1 μg/ml PrP106-126(117V) (■) or PrP106-126(117A) (□). The rate of tubulin polymerization was greatly enhanced by tau in a dose-dependent manner; however, this was inhibited by PrP106-126(117V). Values shown are the means ± S.E.M. for three experiments per time point.

can be inhibited by L-type calcium-channel blockers. However, PrP106-126(117A) is not toxic to neurones lacking expression of PrP^C [13,14] and, as reported previously [23], does not cause an increase in intracellular calcium in PrP^C-deficient cells. PrP106-126(117V), in contrast, does not require PrP^C expression to have a toxic effect. Nevertheless, similar to the effect of PrP106-126 on wild-type cells, the toxicity of PrP106-126(117V) can be inhibited by blockers of L-type calcium channels and application of the peptide caused an increase in intracellular calcium. Changes in microtubule stability have been shown to cause changes in calcium entry via L-type channels [28]. Therefore as PrP106-126 can bind to microtubules [24] it is possible that the changes in intracellular calcium measured here are a result of PrP106-126(117V) binding to microtubules.

The peptide PrP106-126 has many similarities to PrP^{Sc} in that it is protease resistant, forms fibrils, is neurotoxic and has relatively high β -sheet content. However, the finding that a modification of PrP106-126 alters the toxic mechanism *in vitro* suggests that there could be heterogeneity in the mechanism of neurotoxicity of PrP^{Sc}. The mechanism of toxicity of any 'type' of PrP^{Sc} would then depend on the sequence of the protein. Clearly patients from families with different inherited mutations in the PrP gene have quite distinct pathologies [4]. However, this issue is confused by there being considerable variability in pathology among patients carrying the same mutation. However, if there are distinct 'types' of PrP^{Sc} with different toxic

mechanisms then this has broad implications for any treatment of the disease(s).

One important distinction of some forms of GSS that has emerged in recent years is the finding that neurofibrillary tangles are associated with some of the point mutations, especially Y145stop [29], F189S [30] and Q217R [30]. Some cases of A117V also show tangles [10]. It has recently been shown that PrP106-126 can bind tubulin [24] in a cell-free assay. Additionally analysis of cellular uptake of biotinylated PrP106-126 with electron microscopy has confirmed that PrP106-126 can enter the cytosol and bind to microtubules in neurones when applied to cultured cerebellar cells [31]. In the current article PrP106-126(117V) inhibited tau-mediated tubulin polymerization. However, the peptide did not inhibit spontaneous polymerization. This implies that microtubules may form in cells but that tau might be displaced. An accumulation of tau in cells may then result in the formation of paired helical filaments and thus tangles. This intriguing possibility might explain the occurrence of tangles in this form of GSS. However the identification PrP^{Sc} binding to microtubules in the cells of GSS patients would be necessary before this could be confirmed.

In summary, a neurotoxic PrP peptide carrying an amino acid substitution mimicking the A117V mutation of GSS has a different toxic mechanism and secondary structure to that of the peptide with the wild-type sequence. This peptide causes destabilization of cytoskeleton by inhibiting tau-dependent tubulin

Figure 7 Effect of cytoskeletal-stabilizing agents on the toxicity of PrP peptides

(A) Wild-type cerebellar cells were exposed to 80 μ M PrP106–126(117A) (○) or 20 μ M PrP106–126(117V) (●) for 7 days. Taxol treatment inhibited the toxicity of PrP106–126(117V) only, as determined by an MTT assay. (B) Prnp^{0/0} cerebellar cells were treated for 7 days with 20 μ M PrP106–126(117V). Taxol co-treatment fully blocked the toxicity of this peptide, as determined by an MTT assay. (C) Wild-type cerebellar cells were exposed to 80 μ M PrP106–126(117A) (○) or 20 μ M PrP106–126(117V) (●) for 7 days and co-treated with phalloidin. An MTT assay after this time indicated that phalloidin did not inhibit the toxicity of either peptide. Values shown are the means $\pm$ S.E.M. for a minimum of four experiments.

polymerization. These results suggest that there may be heterogeneity in the neurotoxicity of PrP^{Sc} dependent on its amino acid sequence.

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