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The role of Bax and caspase-3 in doppel-induced apoptosis of cerebellar granule cells

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Abbreviations: PrP, prion protein; PrP^{C}, cellular isoform of PrP; PrP^{Sc}, scrapie isoform of PrP; Dpl, doppel

Doppel (Dpl) protein is a paralog of the prion protein (PrP^{c}) that shares 25% sequence similarity with the C-terminus of PrP^{c}, common N-glycosylation sites and a C-terminal signal peptide for attachment of a glycosylphophatidyl inositol anchor. Whereas PrP^{C} is highly expressed in the central nervous system (CNS), Dpl is detected mostly in testes and its ectopic expression in the CNS leads to ataxia as well as Purkinje and granule cell degeneration in the cerebellum. The mechanism through which Dpl induces neurotoxicity is still debated. In the present work, primary neuronal cultures derived from postnatal cerebellar granule cells of wild-type and PrP-knockout FVB mice were used in order to investigate the molecular events that occur upon exposure to Dpl. Treatment of cultured cerebellar neurons with recombinant Dpl produced apoptosis that could be prevented by PrP co-incubation. When primary neuronal cultures from Bax-deficient mice were incubated with Dpl, no apoptosis was observed, suggesting an important role of Bax in triggering neurodegeneration. Similarly, cell survival increased when recDpl-treated cells were incubated with an inhibitor of caspase-3, which mediates apoptosis in mammalian cells. Together, our findings raise the possibility that Bax and caspase-3 feature in Dpl-mediated apoptosis.

Introduction

Prion diseases are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease, fatal familial insomnia and Gerstmann-Sträussler-Scheinker disease in humans. Prions also cause bovine spongiform encephalopathy in cattle, scrapie in sheep and chronic wasting disease in deer, elk and moose. The crucial event in these maladies is a post-translational transition, in which the host-encoded cellular prion protein (PrP), denoted PrP^{C}, is transformed into a pathological conformer (PrP^{Sc}).[1] PrP^{Sc} is highly enriched with β-sheet structures.[2]

Encoded by *Prnp* gene, PrP^{C} is a glycoprotein highly expressed in the CNS. Although it is evolutionarily conserved among different classes of organisms, its function is still elusive. The generation of PrP-null mice ($Prnp^{0/0}$) failed to show any gross phenotypes.[3] Several PrP-knockout lines were produced and some (Ngsk, Rcm0, ZrchII and Rikn) developed late-onset ataxia accompanied by cerebellar neurodegeneration.[4-6] Further analysis demonstrated that the gene-knockout process employed in the generation of such lines resulted in the ectopic expression of a gene located 16 Kb downstream of *Prnp* locus.[5] This gene, later named *Prnd*, encodes a PrP^{C} paralog protein called doppel (Dpl), most likely derived from the duplication of a single ancestral gene.[7]

Dpl resembles an N-terminally truncated form of PrP and has been found to share several structural and biochemical features with PrP^{C}.[8,9] Although Dpl primary sequence has only 25% homology with the C-terminus of PrP^{C}, both proteins are structurally similar as confirmed by NMR studies.[10] Their structures are characterized by three α-helices and two short antiparallel β-strands.[11,12] While PrP^{C} structure is stabilized by a single disulfide bond (Cys 178–213, mouse numbering), Dpl tertiary structure presents two disulfide bonds: one at Cys 109–143 and the other between Cys 94 and 148. The higher stability conferred by the presence of an additional disulfide bond may explain why Dpl does not undergo the conformational change seen in PrP.[13] Moreover, both proteins have two N-glycosylation sites and a glycosylphosphatidyl inositol (GPI) anchor that allows proper localization in lipid rafts. Despite these biochemical similarities, the expression patterns of PrP^{C} and Dpl are extremely different. While PrP^{C} is highly abundant in the CNS, Dpl localizes mainly in non-nervous tissues, especially in testes.[14] In fact, Dpl seems to be involved in spermatogenesis and

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Table 1. MoDpl induces cell death in granule cells

MoDpl (μM)	wt FVB	FVB/*Prnp*$^{0/0}$
0	101.7 ± 0.7	92.8 ± 3.8
0.3	76.7 ± 2.4	88.3 ± 4.7
3	39.0 ± 1.0	46.7 ± 1.0
6	22.6 ± 9.4	29.9 ± 4.3

Primary granule neurons from wt FVB and FVB/*Prnp*$^{0/0}$ mice were incubated with increasing concentrations of MoDpl (0, 0.3, 0.6 and 6 μM) and assessed for cell viability by calcein AM assay. Survival is expressed as a percentage of viable cells relative to medium-treated controls. Data represent mean and standard error, respectively, from at least three independent measurements.

sperm-egg interaction since transgenic mice lacking *Prnd* gene exhibited infertility due to impaired acrosomal function.[15]

Since Dpl was discovered, much interest has been shown in Dpl research. In particular, the study of Dpl-induced cerebellar neurodegeneration has elucidated the importance of the N-terminal domain of PrP in neuroprotection. In fact, expression of N-terminally truncated PrP [PrP(Δ32–121) or PrP(Δ32–134)] in *Prnp*$^{0/0}$ mice[16] leads to ataxia and Purkinje cell loss in the same fashion as Dpl ectopic expression.[17] The cytotoxic effects of ΔPrP and Dpl are counteracted by full-length PrP,[18-20] suggesting common molecular mechanisms, most likely interfering in some cellular pathways essential for cell survival in which full-length PrP is involved.[21,22] The same results were recapitulated in several neuronal cell models. Human SH-SY5Y and murine neuroblastoma (N2a) cell lines transiently expressing Dpl or PrP(Δ32–121) showed similar apoptotic features, fully rescued by full-length PrP co-expression.[23-25]

The deletion of N-terminal residues 23–88 from PrP seems to interfere with the rescue of ΔPrP/Dpl-induced neurodegeneration.[26] Moreover, *Prnp*$^{0/0}$ mice expressing a chimeric protein composed of Dpl and the PrP N-terminus (1–124) failed to exhibit any neurodegenerative signs.[27] The same fusion protein, expressed in PrP-defective HpL3–4 cells, conferred resistance against serum deprivation-induced apoptosis.[28] Taken together, these data suggest a crucial role for the octapeptide repeat region and hydrophobic region for the anti-apoptotic activity of PrP.

Although Dpl-induced apoptosis in the cerebellum has been well-characterized histopathologically, the molecular mechanisms that occur in Purkinje and granular cells expressing Dpl are still controversial. For this reason, we set up an in vitro system to investigate molecular mechanisms of Dpl-induced cerebellar neurodegeneration and dissect the pathways involved in the process. Primary cell cultures of cerebellar neurons from both wild-type (wt) FVB and FVB/*Prnp*$^{0/0}$ mice were tested for cell viability after incubation with mouse (Mo) Dpl (26–155), full-length MoPrP(23–230), or truncated MoPrP(89–230). Dpl-specific apoptosis in cerebellar neurons was confirmed, as well as its rescue upon co-incubation with full-length PrP, but not truncated PrP(89–230). The role of Bax in triggering apoptotic signals, as well as the involvement of caspase-3 in Dpl-induced apoptosis were also demonstrated.

Results

Analysis and characterization of MoDpl and MoPrP proteins. Monomeric MoDpl and MoPrP proteins were expressed in *E. coli* using high-density culture fermentations. The proteins, localized in the inclusion bodies, were purified and characterized as described in the Material and Methods section. SDS-PAGE followed by silver staining and mass spectrometry was performed for estimating protein quality. All proteins revealed, from the gel analysis, a single band at the expected molecular weight and were folded predominantly in an α-helical conformation as determined by CD spectroscopy (data not shown).

Dose-dependent neurotoxic effect of MoDpl on cerebellar granule neurons. To study Dpl-induced apoptosis and the relationship between PrP and Dpl, we elected to use in vitro primary cell cultures of cerebellar granule cells. Cerebellar cultures have extensively been used as a model to study apoptotic mechanisms[29] as well as to test the effects of anti-prion molecules in primary neuronal cultures.[30] Cultures from both wt FVB and FVB/*Prnp*$^{0/0}$ mice (P6) were incubated for 3 d with increasing concentrations of MoDpl, up to 100 μg/mL (~6 μM). Cell survival, tested by calcein AM dye assay, was inversely dependent on the MoDpl dose in both cell lines (**Table 1**). This toxic effect was most evident at the higher concentrations of the protein, 50 and 100 μg/mL (~3–6 μMDpl). Cells incubated for 3 d with similar concentrations of MoPrP(23–230) showed no toxic effects.

The apoptotic effect of Dpl was confirmed using TUNEL assay, according to manufacturer's protocol (**Fig. S1**). Cerebellar granule cells of both wt FVB and FVB/*Prnp*$^{0/0}$ mice were incubated for 2 d in either media alone, 4 μM MoDpl or 4 μM MoPrP(23–230), stained for TUNEL and counterstained with DAPI to visualize total chromatin. Green-labeled cells were classified as apoptotic cells and blue cells were classified as healthy cells. A third category of cells was classified as "indeterminate" as some cells were clearly neither labeled green nor blue. Stained cells were assessed under a microscope, using a counting 1 mm^2 graticule, to ensure all cells were analyzed. In both wt FVB and FVB/*Prnp*$^{0/0}$ cell cultures incubated with MoDpl, > 75% of cells were apoptotic and < 20% of cells were classified as healthy. Upon incubation with MoPrP(23–230), wt FVB and FVB/*Prnp*$^{0/0}$ apoptotic cells ranged from 10% to 20%, comparable to those obtained incubating with media alone. A certain amount of cell death in control conditions is probably due to enzymatic and mechanical stresses induced by the techniques used to dissociate neuronal cells. Moreover, the slight differences between the two assays depend on the fact that they look at different phenomena. While calcein AM discriminates between cells with and without a functional cell membrane which is still capable of actively transporting the dye inside the cytosol, TUNEL detects DNA fragmentation in the nuclei of cells undergoing apoptosis.

To assess whether Dpl-induced apoptosis was specific to granule cell neurons, primary cultures of hippocampal neurons from both wt FVB and FVB/*Prnp*$^{0/0}$ were incubated with 3 μM of MoDpl(26–155), MoPrP(23–230) or MoPrP(89–230) for 5 d, then assessed for cell survival (**Fig. S2**). None of the recombinant

proteins appeared to influence the survival of the hippocampal neurons when compared with the media control.

Differential rescue by co-incubation with MoPrP(23–230). In 2001, Moore et al.[31] demonstrated in transgenic mice that full-length PrP rescued Dpl-induced neurotoxicity in vivo. In order to investigate the interaction between the two proteins, ELISA experiments were performed. We found that MoDpl bound to immobilized MoPrP in a direct ELISA (**Fig. S3**) as detected using a rabbit polyclonal antibody against Dpl. Furthermore, both MoPrP(23–230) and MoPrP(89–230) bound to immobilized MoDpl, as detected by a rabbit polyclonal antibody to PrP (**Fig. S3**). Interestingly, full-length MoPrP(23–230) showed a greater binding capacity to Dpl compared with MoPrP(89–230), which lacks the octapeptide region. The absence of the octarepeat sequences in MoPrP(89–230) therefore could account for the lower binding to MoDpl and may be influential in the ability of PrP to rescue Dpl-induced apoptosis.

To validate the data obtained with recombinant proteins expressed in bacteria, another source of MoPrP, a dimeric form of PrP expressed in a eukaryotic system [murine neuroblastoma (N2a) cells], MoPrP-Fc,[32] was used. MoPrP-Fc protein bears all the post-translational modifications occurring in PrP in vivo, such as glycosylation. Interestingly, MoPrP-Fc bound to Dpl as effectively as full-length, monomeric MoPrP(23–230), confirming our previous results (**Fig. S3**). In addition, these ELISA data are supported by surface plasmon resonance (SPR) data as published by Benvegnù et al.[33]

We also tested whether MoPrP(23–230) could rescue Dpl toxicity in primary cell cultures of granule neurons. When cerebellar neurons were exposed to both MoPrP(23–230) and MoDpl(26–155), a significant increase in cell survival (**Fig. 1A**, $p < 0.01$) was observed. Cells were incubated with Dpl alone (3 μM or 9 μM) or with 3 μM PrP, then assessed for cell viability with calcein AM. Cell survival with 3 μM and 9 μM of Dpl alone was ~60 and ~50%, respectively, which was fully rescued after co-incubation with 3 μM MoPrP(23–230). As a control, anisomycin was used. Anisomycin is an inhibitor of DNA synthesis, which is known to be toxic to cells in a PrP^{C}-independent manner. When MoPrP(89–230), which lacks the N-terminal sequence, was co-incubated with MoDpl(26–155), no rescue was observed (**Fig. 1B**). Thus, full-length MoPrP(23–230) appears to be critical for the rescue of Dpl-induced toxicity of cerebellar neurons.

Dissecting the mechanism of Dpl-induced apoptosis. To define and dissect the pathway involved in Dpl toxicity, we studied the role of Bax in mediating the apoptotic process. We prepared primary cultures from cerebellar granule neurons from wt Bax and $Bax^{-/-}$ mice. Cultures were incubated for 5 d, with MoDpl(26–155) and assessed for cell survival by calcein AM. Cerebellar granule neurons from wt Bax mice revealed dose-dependent cell death upon incubation with MoDpl(26–155) (**Fig. 2**, shaded bars). In comparison, granule cells derived from $Bax^{-/-}$ mice were unaffected by the same concentrations of MoDpl (**Fig. 2**, open bars). Anisomycin, a potent inducer of apoptosis through the Bax pathway,[34] was used as positive control. Therefore, in the absence of the Bax gene, apoptosis induced by either Dpl or anisomycin was prevented (**Fig. 2**).

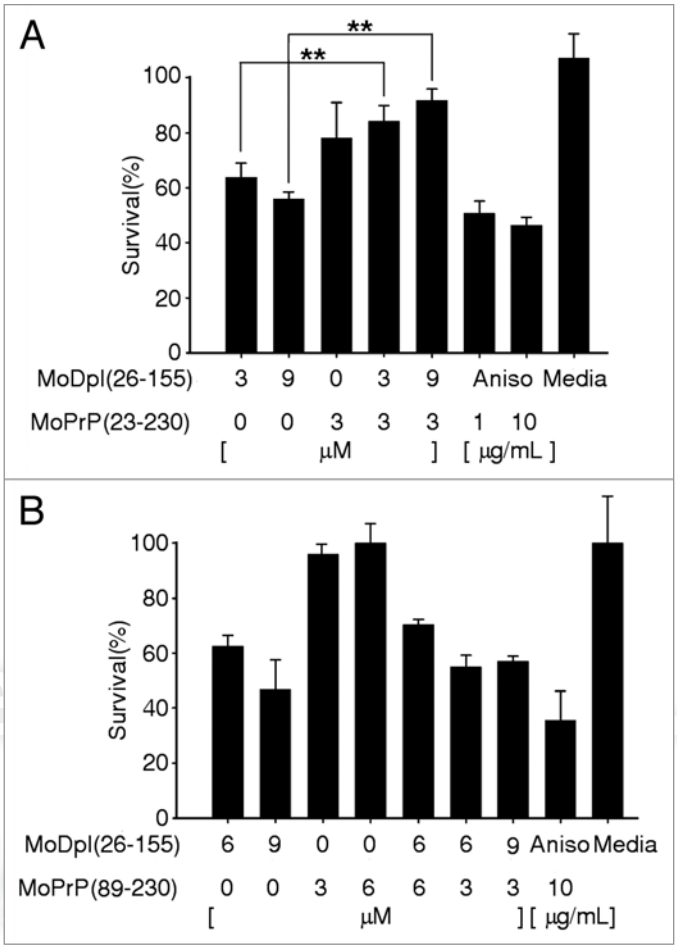

Figure 1. Dpl-induced toxicity is rescued by full-length PrP. Primary granule cell cultures were co-incubated with equimolar and sub-equimolar concentrations of MoDpl and full-length MoPrP (A) or truncated MoPrP (B). After 5 d, cell survival was assessed by calcein AM. Full-length MoPrP was able to fully rescue Dpl-induced apoptosis whereas no rescue was detected using truncated MoPrP. As positive and negative controls, anisomycin and media alone were used, respectively. The data are from at least three independent experiments (**$p < 0.01$).

Involvement of caspase-3 in Dpl-induced apoptosis. The apoptotic process can follow two different pathways downstream of Bax: caspase-dependent and caspase-independent route. Caspase-3 has been identified as a key molecule in mediating apoptosis in mammalian cells.[35] A selective and irreversible pharmacological inhibitor of caspase-3, AC-DEVD-CMK, was used to assess its involvement in Dpl-induced apoptosis (**Fig. 3**).

For cerebellar cultures from wt FVB mice (**Fig. 3A**), cell survival in the presence of 9 μM of MoDpl was ~40%. With the addition of the caspase-3-inhibitor, cell survival increased significantly to 55% ($p < 0.05$). Similar observations were made for cerebellar cultures derived from FVB/$Prnp^{0/0}$ mice (**Fig. 3B**). As a positive control, camptothecin was used. This inhibitor of DNA synthesis is known to be toxic to cells. These results demonstrate that inhibition of caspase-3 increased the survival of cerebellar neurons, suggesting that caspase-3 plays a role in Dpl-induced apoptosis.

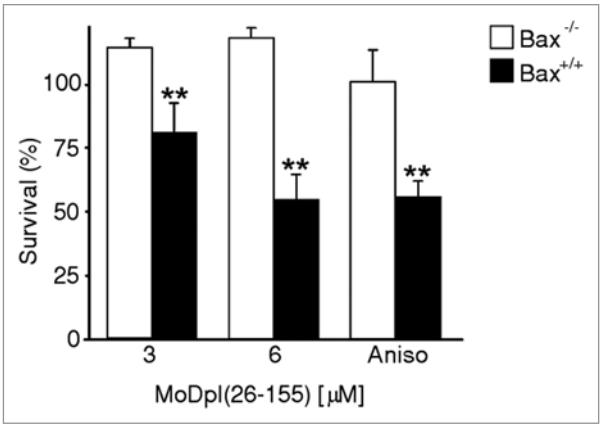

Figure 2. The role of Bax in Dpl-mediated toxicity. Primary granule cell cultures from wt Bax (shaded bars) and Bax$^{-/-}$ (open bars) mice were incubated with 3 or 6 μM MoDpl and tested for cell viability by the calcein AM assay. Cells derived from wt Bax mice showed a dose-dependent, Dpl-induced apoptosis whereas cells from Bax-knockout mice failed to show any cytotoxic features. Anisomycin was used as positive control. The data represent means from at least three independent experiments (**$p < 0.01$).

Discussion

Although the pro-apoptotic function of Dpl has been well assessed in Purkinje and granule cells of cerebellum, the molecular basis of the process remains still controversial. The understanding of Dpl mechanisms may help in elucidating the neuroprotective role of PrP since the two molecules share a high similarity in their tertiary structures. In order to dissect the Dpl-activated pathways in the cerebellum, we set up an in vitro assay using primary post-natal mouse cerebellar granule cells from wt FVB and FVB/*Prnp*$^{0/0}$ animals. We utilized an *E. coli*-expressed, monomeric MoDpl(26–155) polypeptide refolded in α-helical conformation and lacking post-translational modifications such as glycosylation.

First, we showed that incubation of granule cells with MoDpl (3–6 μM) induced a diffuse apoptosis that was completely rescued by co-incubation with full-length MoPrP(23–230) but not with N-terminally truncated MoPrP(89–230), consistently with data derived from transgenic mouse models.[36] We probed the specificity of Dpl activity on cerebellum by incubating hippocampal primary neurons with MoDpl (3 μM). No neurotoxic effects were detected upon Dpl exposure. The differential response between these two neuronal populations toward Dpl had been analyzed previously to some extent in the pionieristic work of Legname et al. 2002.[32] The authors used a chimeric form of both Dpl and PrP—fused to the Fc domain of an immunoglobuline—to search for their physiological ligands within the CNS. Immunohistochemical analyses highlighted that both PrP-Fc and Dpl-Fc restrictly bind to granule cells in the cerebellum. These data are compatible with the presence of a receptor for Dpl and PrP on granule cell bodies, which is not expressed in hippocampal neurons. This hypothesis is corroborated by the fact that the concentrations at which Dpl induces apoptosis are low, within the micromolar range. This evidence suggests that its molecular mechanism of neurotoxicity is not due to accumulation of the protein in the extracellular compartment but, most likely, to specific interactions with a ligand on the cell surface of granule neurons. According to our ELISA data, Dpl binds to PrP and this interaction has been recently confirmed either by SPR or by in vivo experiments.[33,38] Moreover, Dpl has been shown to interact distinctively with a plasma metalloproteinase inhibitor, the α-2-macroblobulin (α_2M), in the granule cell layer of the cerebellum.[32,33] According to the proposed mechanism, in the absence of PrP, Dpl could bind and sequester α_2M and the withdrawal of proteinase inhibitors may eventually lead to cerebellar degeneration. The presence of PrP would prevent Dpl-α_2M binding and the subsequent apoptotic processes.

Our data are in agreement with this proposed mechanism, which also might explain why in our system, Dpl induces apoptosis both in wt and PrP-deficient cells. This finding is apparently in contrast with previous data, which showed an effect on survival only in cells from PrP-knockout mice.[37] Indeed, when recombinant Dpl is in excess, all endogenous PrP in wt cells is virtually bound; in this state of PrP inactivity, remaining Dpl is available to sequester α_2M and promote neurotoxicity. Presumably, genetic overexpression of Dpl in mouse cerebella with a wt background for PrP could not be sufficient to bind all the endogenous PrP. Hence, those mouse models did not show any pathological phenotype. Interestingly, N-terminally truncated PrP showed lower affinity to Dpl compared with full-length PrP in SPR experiments and also lacked the ability to rescue Dpl-induced apoptosis in our cultured-cell experiments. These observations support the involvement of the N-terminal domain in neuroprotection as indicated in previous genetic experiments,[39] suggesting that the N-terminus of PrP may exert its function by sequestering Dpl and rendering it unavailable to interact with metalloproteinase inhibitors.

Second, we investigated the role of Bax in Dpl-mediated neurodegeneration. Bax is a pro-apoptotic member of the Bcl-2 family that regulates cell death in many cell types. Bax can form homodimers or heterodimers with Bcl-2 itself. Heterodimerization with Bcl-2 no longer protects the cell from programmed cell death and inevitably leads to apoptosis.[40] Previous studies focusing on Purkinje cells (PCs) showed controversial results. The deletion of Bax in Ngsk/*Prnp*$^{0/0}$ mice resulted in a partial rescue of PCs number, suggesting that the ectopic expression of Dpl induces both Bax-dependent and Bax-independent pathways of cell death.[41] The same authors showed that the pro-apoptotic effects of Dpl on PCs can be partially counteracted by Bcl-2 overexpression.[42] Recently, they have also described the upregulation of autophagic markers as well as extensive accumulation of autophagosomes in PCs of Ngsk mice, proposing that a progressive dysregulation of autophagy could contribute to PCs loss by triggering apoptotic cascades.[43,44] In contrast, other authors failed to detect any ameliorating effect on PCs derived from Bax inactivation in Tg(Dpl) mice in which Dpl is ectopically expressed in the CNS driven by the neural-specific enolase promoter.[45] Interestingly, also truncated PrP-induced neurodegeneration in mice expressing PrP(Δ32–134) seems to be mediated by Bax. In particular, a double-step mechanism takes place in which Bax-related

pathways mediate only the early neurotoxic actions and Bax-independent pathways are co-responsible for the overall neurodegeneration induced by the truncated form.[46]

Our data presented here indicate that the absence of Bax abolishes Dpl-induced apoptosis in granule cells occurring through the activation of caspase-3. The possible discrepancies in the sensitivity of PCs and granule cells toward Bax deletion are not surprising. Different death pathways might be activated in these two neuronal cell populations upon exposure to Dpl, reflecting the expression of cell type-specific Dpl co-receptors at the level of cellular membrane. The availability of an in vitro model for Dpl-induced neurodegeneration will nevertheless assist in the isolation of these putative binding partners.

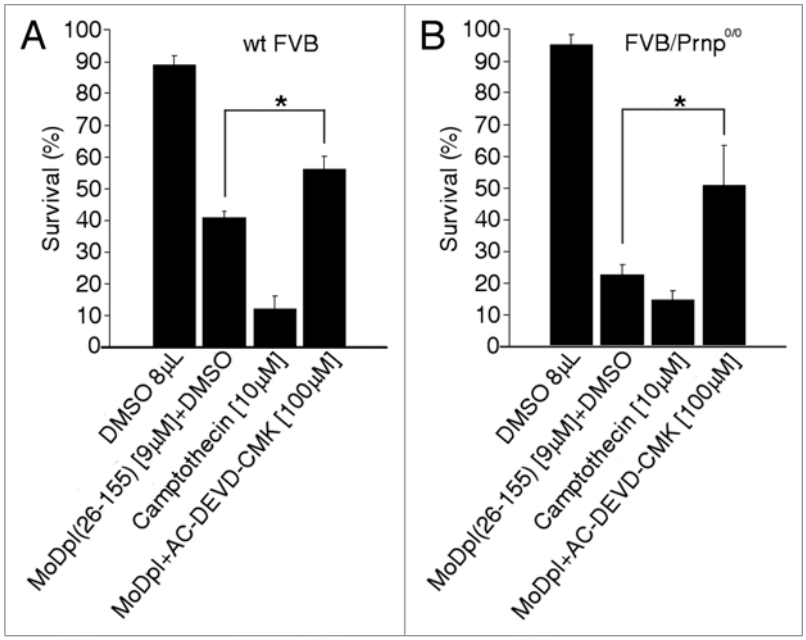

Figure 3. The role of caspase-3 in Dpl-mediated apoptosis. Granule cell cultures from wt FVB (A) and FVB/*Prnp*$^{0/0}$ (B) mice were co-incubated for 5 d with 9 µM MoDpl and 100 µM AC-DEVD-CMK (an inhibitor specific for caspase-3) diluted in DMSO. The cells co-incubated with AC-DEVD-CMK showed a significant increase in cell survival compared with cells incubated with MoDpl only, suggesting that caspase-3 may be involved in mediating Dpl-induced apoptosis. Camptothecin (10 µM) and DMSO (8 µL) alone were used as positive and negative controls, respectively. The data represent means from at least three independent experiments. (*$p < 0.05$).

Materials and Methods

Recombinant protein production and purification. Generation and purification of all proteins used in this study were as described previously, in detail in reference 47 and 48. To increase expression levels of recombinant, full-length MoPrP(23–230), an alanine was added after the initiator methionine.

Recombinant full-length MoPrP(23–230) was expressed from pET11a plasmid in *E. coli* BL21 (DE3) (Novagen) in minimal media containing 100 µg/mL ampicillin. The bacterial pellet was resuspended in 25 mM TRIS-HCl, 5 mM EDTA (pH 8.0) and processed twice in a Microfluidizer M-110EH (Microfluidics Corp.). Inclusion bodies were collected by centrifugation and solubilized in five volumes of 8 M Urea, 10 mM MOPS (pH 7.0) by agitation overnight at room temperature (RT). The protein was purified by column chromatography using carboxyl methyl sepharose (Amersham Bioscience) followed by C4 reverse-phase media (Phenomenex).

Recombinant truncated MoPrP(89–230) was expressed in *E. coli* host 27C7 from plasmid pNT3A as described in reference 47. Insoluble inclusion bodies that contained PrP were extracted, solubilized and purified by various chromatographic procedures as described by Mehlhorn and colleagues.[49]

For the production of MoDpl(26–155), a bacterial construct for the expression of MoDpl(26–155) in the expression vector pET11a (Novagen) was generated by standard working procedures. After transformation into *E. coli* (DE3) cells, fermented cultures were processed in a Microfluidizer as described for MoPrP. The protein was then purified using a Mono S FPLC column with the peak fractions lyophilized and stored until use.

MoPrP-Fc was constructed by cloning the MoPrP sequence (23–230) between *Ndel* and *Xbal* sites of pSecTag plasmid (Invitrogen) containing the human IgG1-Fc region. As a control, Fc expressing pSecTag plasmid was also constructed. A FLAG (DYK DDD DK) epitope tag was cloned into the 3' end of either MoPrP-Fc or Fc constructs using the Quick-change PCR mutagenesis kit (Strategene). Neuroblastoma (N2a) cells were transiently or stably transfected with DNA constructs (5 or 10 µg) using the DOTAP DNA transfection kit (Boehringer Mannheim). Stably transfected N2a cells with either MoPrP-Fc or Fc constructs were maintained as previously described in reference 32, with the media supplemented with zeocin at 200 µg/mL.

To check the transfection efficacy, cells were lysed in buffer T, which contains 10 mM TRIS-HCl, pH 8.0; 0.5% deoxycholate; 0.5% Nonidet P-40; 150 mM NaCl. Cell lysates or cell-conditioned media containing the fusion protein were resolved by SDS-PAGE. Samples were blotted onto PVDF membranes and blocked with 5% (w/v) non-fat milk protein in Tris-buffered saline with 0.05% Tween-20 (TBST). The Fc portion of the protein was detected using anti-human Fc antibodies conjugated to horseradish peroxidase (SIGMA) at various concentrations in TBST. Blots were developed with the enhanced chemiluminescence (ECL) reagent (Amersham) for 1 min and exposed to ECL hypermax film (Amersham).

Purification of the fusion proteins was performed as follows. After transfection, cells were cultured for a minimum of 72 h with the conditioned media containing the secreted fusion proteins. The media was centrifuged at 3,500x g to remove cellular debris and loaded onto a column of anti-FLAG M2 affinity gel (SIGMA). Affinity gel with the immobilized protein was washed with 20 column volumes of PBS. Bound protein was then eluted with 5 column volumes of PBS containing 100 µg/mL FLAG peptide.

Purity of the proteins. Purity of the recombinant proteins was estimated by SDS-PAGE followed by silver staining[50] and mass spectrometry (data not shown). Structural conformation of purified protein was also analyzed using CD spectroscopy (data not shown) as described previously in reference 48. Immunochemical analysis of the fusion proteins was performed by SDS-PAGE followed by western blotting using a panel of antibodies directed against various sites of both the PrP-Fc fusion protein and Fc domain. Recombinant MoDpl(26–155), MoPrP(23–230) and MoPrP(89–230) were solubilized in either distilled water or refolded in 20 mM sodium acetate buffer (pH 5.5) and stored at 4°C until needed.

Primary cell cultures. For preliminary experiments, which were performed to develop and evaluate the assay in a murine model, primary cell cultures were obtained from both cerebellar granule cell layer and hippocampal layer of wt FVB and FVB/*Prnp*$^{0/0}$ mice[51] at postnatal day 6 (P6). In addition to wt FVB and FVB/*Prnp*$^{0/0}$ mice, cultures were obtained from wtBax and Bax$^{-/-}$ mice[52] at P6. All experiments were performed in accordance with European regulations [European Community Council Directive, November 24, 1986 (86/609/EEC)], and approved by the local authority veterinary service.

Cell cultures were prepared as follows. Working as quickly as possible and under sterile conditions, the P6 mice were decapitated and the heads placed immediately into a Petri dish containing ice-cold Hank's BSS, Ca^{2+}/Mg^{2+}-free, without Phenol Red (Invitrogen) solution containing a high concentration of penicillin/streptomycin to slow metabolism and decrease external contamination. The whole brain was removed and placed again into ice-cold Hank's solution. Under a stereomicroscope (Nikon SMZ 1500), the structures of interest were dissected, cleansed of meninges if necessary (to avoid glial contamination), minced and transferred to a sterile, 15-mL conical tube (BD Biosciences). This was then centrifuged at 228 g in a Beckmann GS-6 centrifuge for 5 min. The supernatant was carefully aspirated, mixed thoroughly with a solution containing 20 units of papain (Worthington) and 0.005% DNase (Worthington) and incubated at 37°C for 20–45 min, depending on the tissue volume. Tissues were then mechanically dissociated using 5 mL and 1 mL pipette tips. Dissociated cells were passed through a 40 μm cell strainer (BD Falcon) further spun at 228 g and resuspended in 3 mL of Neurobasal-A media (P6) containing B-27 supplement; fetal bovine serum (1%); glutamax-1 (2 mM); penicillin and streptomycin G (100 U/mL). The proportion of viable cells was determined by staining with trypan blue (Sigma), visualized with a hematocytometer under a light microscope and plated in complete Neurobasal media (± A) at 1×10^5 cells per 12 mm^2 coverslip or per well of a 96 well plate. All coverslips or wells had been coated with poly-d-lysine at 250 μg/mL.

Incubation with recombinant proteins. Approximately 24 h after plating of cells, 0.1% Arabinose C was added to fresh media to suppress non-neuronal proliferation. Recombinant proteins were initially resuspended in dH_2O and diluted to 5 mg/mL. The solution was then diluted to 1 mg/mL with 8 M guanidine, and left to stand at RT for 30 min before rapid dilution to 0.1 mg/mL with Tris buffer (pH 8.0), dialysed against 20 mM sodium acetate buffer at 4°C, and finally passed through a 0.22 μm filter. For each experiment, the proteins were added exogenously to each coverslip or well, using media as the vehicle, and incubated for up to 5 d depending upon the experiment.

Cell survival assay. Cell survival was assessed using calcein AM, according to manufacturer's protocol (Invitrogen). In brief, 1 mg of calcein AM was dissolved in 1 mL of DMSO to make a stock solution. This solution was then diluted to 1:50 in Ca^{2+}/Mg^{2+}-free Dulbecco's PBS (Invitrogen). After 24 h, cells that had been plated at 10^5 in 100 μl of Neurobasal-A medium on black, 96-well plates (BD Bioscience) were incubated between 2–5 d with the recombinant proteins at 37°C. Cells were washed three times using Ca^{2+}/Mg^{2+}-free Dulbecco's PBS before being incubated 1:10 with 100 μl of calcein AM in DMSO per well for 30 min. Viability of the cells was then read on a fluorescence microplate reader (Tecan, USA) using an excitation filter of 490 ± 10 nm and an emission filter of 530 ± 15 nm.

TUNEL staining. Apoptosis was determined using the terminal deoxynucleotidyltransferase nick end-labeling assay (TUNEL). Two days after treatment with MoPrP and MoDpl, cells were fixed in 4% paraformaldehyde for 30 min. Before pre-incubation and permeabilization with a solution containing 0.1% sodium citrate (Sigma) and 0.1% triton-X-100 (Sigma) for 10 min, cells were washed in PBS. After permeabilization, cells were further washed for 5 min before staining using the Roche Kit. Permeabilized cells were incubated 1 h at 37°C with 45 μL of stain per coverslip. An additional two washes in PBS were performed before mounting on slides in Vectashield media containing DAPI (Vector Laboratories). Cells were viewed at x20 magnification on a Leica DMRB fluorescence microscope with filters specific for DAPI and FITC at 490 nm.

Quantification of apoptosis. Quantification was performed manually with a counting graticule (Ted Pella). Blue-labeled nuclei indicated "healthy cells"; green-labeled nuclei indicated apoptotic cells. For nuclei labeled both blue and green, a third classification (indeterminate) was included to denote that cells were putatively undergoing apoptosis.

Protein-protein interaction assay. To study Dpl and PrP interaction, an ELISA assay was performed. Wells were pre-incubated at 4°C for 1 h with a saturating solution containing 0.25% bovine serum albumin and 0.05% Tween-20 in Ca^{2+}/Mg^{2+}-free Dulbecco's PBS. For each well, a defined amount of protein was diluted in 100 μL of 0.1 M sodium bicarbonate solution and incubated overnight at 4°C. After nine washes with 1x TBST, wells were blocked using the saturating solution for 1 h at RT. All subsequent incubations for protein binding were performed at RT.

In general, indicated amounts of PrP were diluted in the saturated solution and incubated for 2 h. Nine repeated washes between incubations were performed with 1x TBST. For PrP detection, either a 2 μg/mL of humanized anti-PrP HuM-D18 antibody fragment (Fab),[53] or a 1:1,000 dilution of a rabbit polyclonal anti-PrP R073,[54] was added and incubated for 1 h. For Dpl detection, a 1:1,000 dilution of the rabbit polyclonal

anti-Dpl antibody E6977,[32] was added and incubated for 1 h. The appropriate secondary antibody, goat anti-human Fab (1:1,000 dilutions) conjugated to AP to detect the Fab fragment antibody or an anti-rabbit IgG conjugated to AP, was further incubated with the proteins for 1 h. PrP-Fc was detected using a goat polyclonal anti-human Fc antibody conjugated to AP at 1:1,000.

Caspase inhibitor assay. AC-DEVD-CMK, a specific caspase-3 inhibitor, was purchased from Calbiochem and dissolved in DMSO to the desired concentration. It was added at the start of the experiment and co-incubated with MoDpl(26–155) for up to 5 d. Camptothecin was used as positive control.

Statistical analysis. For cell survival studies, means and standard deviations were calculated for each group of experiments. The differences between the experiments were compared using the unpaired Student's t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
www.landesbioscience.com/journals/prion/article/20026

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