Cytosolic PrP can participate in prion-mediated toxicity

Alana M. Thackray, Chang Zhang, Tina Arndt, and Raymond Bujdoso#

University of Cambridge, Department of Veterinary Medicine,
Madingley Road, Cambridge, CB3 OES, UK

#Corresponding author
(Tel: 44-1223-337655; Fax 44-1223-337610; email: rb202@cam.ac.uk)

Running title: Cytosolic PrP transgenic Drosophila

Word count: Abstract; 231, Main text; 5,693
Prion diseases are characterized by a conformational change in the normal host protein PrPC. While the majority of mature PrPC is tethered to the plasma membrane by a glycosylphosphatidyl-inositol anchor, topological variants of this protein can arise during its biosynthesis. Here we have generated Drosophila transgenic for cytosolic ovine PrP in order to investigate its toxic potential in the fly in the absence and presence of exogenous ovine prions. While cytosolic ovine PrP expressed in Drosophila was predominantly detergent insoluble and showed resistance to low concentrations of Proteinase K, it was not overtly detrimental to the fly. However, Drosophila transgenic for cytosolic PrP expression exposed to classical or atypical scrapie prion inocula showed an accelerated decrease in locomotor activity compared to similar flies exposed to scrapie-free material. The susceptibility to classical scrapie inocula could be assessed in Drosophila transgenic for pan neuronal expression of cytosolic PrP whereas susceptibility to atypical scrapie required ubiquitous PrP expression. Significantly, the toxic phenotype induced by ovine scrapie in cytosolic PrP transgenic Drosophila was transmissible to recipient PrP transgenic flies. These data show that while cytosolic PrP expression does not adversely affect Drosophila, this topological PrP variant can participate in the generation of transmissible scrapie-induced toxicity. These observations also show that PrP transgenic Drosophila are susceptible to classical and atypical scrapie prion strains and highlight the utility of this invertebrate host to model mammalian prion disease.
Importance

During prion diseases, the host protein PrPC converts into an abnormal conformer PrPSc, a process coupled to the generation of transmissible prions and neurotoxicity. While PrPC is principally a glycosylphosphatidyl-inositol-anchored membrane protein, the role of topological variants, such as cytosolic PrP, in prion-mediated toxicity and prion formation is undefined. Here we have generated *Drosophila* transgenic for cytosolic PrP expression in order to investigate its toxic potential in the absence and presence of exogenous prions. Cytosolic ovine PrP expressed in *Drosophila* was not overtly detrimental to the fly. However, cytosolic PrP transgenic *Drosophila* exposed to ovine scrapie showed a toxic phenotype absent in similar flies exposed to scrapie-free material. Significantly, the scrapie-induced toxic phenotype in cytosolic transgenic *Drosophila* was transmissible to recipient PrP transgenic flies. These data show that cytosolic PrP can participate in the generation of transmissible prion-induced toxicity and highlight the utility of *Drosophila* to model mammalian prion disease.
**Introduction**

Prion diseases, or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders of humans and various other mammalian species (1). These conditions include scrapie of sheep, Bovine Spongiform Encephalopathy (BSE) of cattle, Creutzfeldt–Jakob disease (CJD) of humans and Chronic Wasting Disease (CWD) of cervids. Susceptibility to prion disease requires expression of the host-encoded protein PrPC (2-5). Furthermore, prion diseases are associated with conversion of PrPC, the normal form of the prion protein, into an abnormal conformer PrPSc in a template-directed manner (6, 7). Misfolding of PrP is associated with an increase in β-sheet content of the protein, which accumulates principally in the central nervous system of affected individuals. There is now considerable evidence to suggest that the transmissible prion agent comprises PrPSc (8-14). However, despite intensive investigation, the molecular mechanisms of PrPC to PrPSc conversion and of prion-mediated neurodegeneration remain unknown.

Although PrPC is highly conserved amongst different mammalian species its physiological functions remain elusive. PrPC is a glycosylphosphatidyl-inositol (GPI)-anchored sialoglycoprotein, principally located in lipid rafts on the outer leaf of the cell membrane (15, 16). Nascent PrPC is synthesized as a pre-protein of approximately 250 amino acid residues in length. The N-terminal leader peptide is cleaved as PrP enters the endoplasmic reticulum (ER) and the C-terminal signal sequence is cleaved upon attachment of the GPI anchor that holds the protein to the membrane (15, 17, 18). Inside the ER lumen PrPC undergoes additional post translational modification with the addition of carbohydrate structures at two asparagine residues (19). In addition, a di-sulfide bond forms within the C-terminal globular domain (20). During its biosynthesis, PrP may undergo aberrant translocation since leader peptide inefficiency prevents all of the nascent protein entering into the lumen of the ER. As a consequence, subpopulations of PrP are either retained fully in the cytosol (PrPcyt) or produced as a membrane-bound protein with either N- or C-terminal residues exposed to the cytosol (21-24). ER misfolded and aberrantly translocated proteins are targeted for degradation by the ubiquitin proteasome system (UPS), the major cellular proteolytic pathway, or via the autophagic / lysosomal system. While normal levels of cytosolic and aberrantly translocated PrP are usually metabolized by the cell, these forms of PrP have been reported to be neurotoxic when present in elevated amounts (25-28). Increased cellular levels of cytosolic PrP may arise as a consequence of PrPSc-mediated inhibition of the
catalytic activity of the proteasome in cells (29-31). The role of cytosolic PrP in the generation of infectious prions has yet to be determined.

Ovine scrapie is an important model of prion disease, not only for the natural host but for mammalian species in general (32, 33). Polymorphisms within ovine PrPC correlate with susceptibility to different types of scrapie in sheep. Four major polymorphisms in the ovine prion protein, located at amino acid residues 136, 141, 154 and 171 are associated, in some cases relatively (34, 35), with susceptibility to two classifications of scrapie disease (36-38). Sheep that express A136L141R154Q171 (termed ARQ, where A, L, R and Q stand for alanine, leucine, arginine and glutamine, respectively) or V136L141R154Q171 (termed VRQ, where V stands for valine) ovine PrP are susceptible to classical scrapie, a transmissible prion disease within the natural host (39). In contrast, a different ovine prion disease, referred to as atypical or Nor98 scrapie, has been reported in classical scrapie-resistant PrP genotypes including A136L141R154R171 (termed ARR), A136F141R154Q171 (termed AFRQ, where F stands for phenylalanine) and A136L141H154Q171 (termed AHQ, where H stands for histidine) (38). It is considered that atypical scrapie is a spontaneous disorder of PrP folding and/or metabolism (38, 40), although transmission by the oral route cannot yet be excluded (41-44). We have begun to model sheep scrapie in Drosophila in order to develop a more tractable model of mammalian prion disease. In doing so, we have previously generated Drosophila transgenic for polymorphic variants of ovine PrP expressed with a GPI anchor sequence [PrP(GPI)] (45). Furthermore, we have shown that Drosophila transgenic for AHQ(GPI) ovine PrP show a significant reduction in median survival time compared to flies transgenic for VRQ(GPI). It has yet to be established whether the toxic potential of AHQ prion protein is mediated by a cytosolic variant of this particular genotype of ovine PrP and whether cytosolic PrP per se can participate in prion-mediated toxicity.

Here we have generated Drosophila transgenic for polymorphic variants of cytosolic ovine PrP in order to investigate for the first time its toxic potential in the fly in the absence and presence of exogenous ovine prions. While cytosolic ovine PrP expressed pan neuronally in Drosophila was predominantly detergent insoluble and showed protease resistance to low concentrations of Proteinase K (PK), it was not overtly detrimental to the fly. In contrast, Drosophila transgenic for cytosolic PrP expression exposed to classical or atypical scrapie prion inocula showed an accelerated decrease in locomotor activity compared to similar flies exposed to scrapie-free material. The susceptibility to classical ovine scrapie was evident in Drosophila transgenic for pan neuronal cytosolic PrP whereas susceptibility to atypical ovine
scrapie required ubiquitous expression. Significantly, the toxic phenotype induced by ovine scrapie in cytosolic transgenic *Drosophila* was transmissible to PrP transgenic recipient flies. These data show that while cytosolic ovine PrP is not inherently neurotoxic in *Drosophila*, this topological variant can participate in the generation of a transmissible toxicity induced by scrapie prion inocula. These novel observations highlight the utility of *Drosophila* to model mammalian prion disease.
Materials and Methods

Fly stocks and generation of cytosolic ovine PrP transgenic Drosophila

The UAS-PrP(GPI) fly lines w; M(AHQ-PrP(GPI), 3xP3-RFP.attP)ZH-51D and w; M(ARQ-PrP(GPI), 3xP3-RFP.attP)ZH-51D, that are transgenic for ovine A\textsuperscript{136}H\textsuperscript{154}Q\textsuperscript{171} or A\textsuperscript{136}R\textsuperscript{154}Q\textsuperscript{171} PrP, respectively, expressed with an N-terminal leader peptide and C-terminal glycosylphosphatidylinositol (GPI) signal sequence [AHQ(GPI) and ARQ(GPI), respectively] were generated by PhiC31 site-specific transformation as previously described (45). The UAS-PrP(cyt) fly lines generated here were w; M(AHQ-PrP, 3xP3-RFP.attP)ZH-51D, w; M(ARQ-PrP, 3xP3-RFP.attP)ZH-51D, and w; M(VRQ-PrP, 3xP3-RFP.attP)ZH-51D. The ovine PrP(cyt) transgenes for insertion into the Drosophila genome were prepared by PCR that generated a DNA fragment encoding ovine PrP amino acid residues 25-232. PCR was carried out in the presence of Pfu DNA polymerase (Promega) using substrate plasmid DNA that contained an insert encoding AHQ, ARQ or VRQ ovine PrP amino acid residues 25-252 (45) and oligonucleotide primers P2 (forward primer): 5'-GATGA GAA TTC AAC ATG AAG AAG CGA CCA AAA CCT GGC 3'; and P4 (reverse primer): 5'-ACGATGAA CTC GAG CTA CCC CCT TTG GTA ATA AG 3'. The PCR primers P2 and P4 contained EcoR1 and Xho1 restriction sites, respectively, that allowed directional cloning of the 658bp PCR product into the Drosophila transgenesis vector pUASTattB. A Kozak translation site (46) was incorporated into the forward primer and a stop codon was incorporated into the reverse primer ahead of the Xho1 restriction site. The PCR reaction conditions comprised an initial denaturation at 95 °C for 2 minutes followed by 30 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds and primer extension at 75 °C for 1 minute, and a final extension of the PCR product at 75 °C for 10 minutes. PCR products that contained DNA encoding PrP(cyt) DNA were subsequently ligated into pUASTattB and rescued by transformation in DH5α bacteria. Plasmid DNA was isolated from transformed bacteria by an alkaline lysis method using the Qiagen maxiprep kit and the PrP construct insert verified by DNA sequence analysis. Site-specific transformation of the pUASTattB-PrP constructs into the 51D fly line (y[1] M{vas-int.Dm}ZH-2A w[*]; M(3xP3-RFP.attP)ZH-51D) was performed by Bestgene Inc (California, USA). F1 flies were balanced and viable lines were maintained as balanced stocks by conventional fly crosses. DNA sequence analysis was performed on genomic DNA from each balanced fly line to confirm the presence of the correct PrP transgene at the 51D site. Cre-mediated removal of RFP from the fly genome of VRQ(cyt) PrP was performed by conventional fly crosses. Elav-GAL4 (P{w[+mW.hs]=GawB}elav[C155]) and GMR-GAL4 (w; wg[Sp-1]/CyO; GMR-GAL4,
w+/TM6B) driver lines, and the control 51D (w; M(3xP3-RFP.attP)ZH-51D) fly line were obtained from the Department of Genetics, University of Cambridge, UK. All fly lines were raised on standard cornmeal media (47) at 25 °C and maintained at low to medium density. Flies were used in the assays described below or harvested at various time points and then frozen at -80 °C until required.

**Prion inoculation of Drosophila**

*Primary passage of sheep scrapie (sheep-to-fly):* Drosophila at the larval stage of development were exposed to brain homogenates from confirmed scrapie-positive or known scrapie-negative sheep. The classical scrapie-infected isolates were prepared from terminal scrapie-affected sheep identified by routine statutory surveillance (VRQ/VRQ isolate SE1848/0005; ARQ/ARQ isolate SE1848/0008) (50). The atypical scrapie-infected isolates (n=2) were prepared from terminal AHQ/AHQ sheep challenged intracerebrally with atypical scrapie and that were confirmed positive for the disease (43). New Zealand-derived VRQ/VRQ (n=1), ARQ/ARQ (n=1) or AHQ/AHQ (n=2) scrapie-free brain tissue was used as control material. Two hundred and fifty microlitres of a 1 % brain homogenate prepared in PBS pH 7.4 were added to the top of the cornmeal that contained third instar Drosophila larvae in 3" plastic vials. Flies were transferred to fresh, non-treated vials following eclosion.

*Secondary passage of sheep scrapie (fly-to-fly):* Drosophila brain homogenates were prepared from 30 day old flies that had been exposed at the larval stage to scrapie-positive or scrapie-negative sheep brain material. Two hundred and fifty microlitres of a 10 % dilution (v/v) of the original fly brain homogenate were added to the top of the cornmeal that contained third instar Drosophila larvae in 3" plastic vials. Flies were transferred to fresh, non-treated vials following eclosion.

**Preparation of fly head homogenates**

Whole flies in an eppendorf tube were frozen in liquid nitrogen for 10 minutes followed by 2 minutes of vortexing. Individual fly heads were then isolated and placed in clean eppendorf tubes using a paint brush. Homogenates were prepared by manual grinding of fly heads in eppendorf tubes with sterilized plastic pestles. Homogenates for ELISA or western blot without Proteinase K (PK) digestion of PrP were prepared by processing 20 fly heads in 20 μl of lysis buffer [50 mM Tris pH 7.5, 100 mM NaCl, 0.5 % (v/v) Nonidet P-40 and 1 mM 4-(2-Aminoethyl) benzenesulfonl fluoride (AEBSF)]. In the case of PK digestion of PrP, AEBSF was not added to the lysis buffer. Homogenates for conformational-dependent immunoassay (CDI) were prepared by processing 40 fly heads in 8 μl of 8M GdnHCl, incubated at 18 °C for
15 minutes followed by a 1:50 dilution in assay buffer and assessed as described previously
(48). Homogenates for fly-to-fly transmission (secondary passage samples) were prepared
by processing 150 male and 150 female fly heads per group previously harvested at 30 days
of age. Each group of 300 fly heads was added to 300 µl of PBS (pH 7.4) prior to
homogenization.

Preparation of soluble and insoluble prion protein fractions
PrP fractions were prepared from fly head homogenates using a method adapted from
Fernandez-Funez et al. (49). A volume of fly head homogenate that was equivalent to 20 fly
heads was mixed with 20 µl of 10 % (w/v) Sarkosyl pH 7.4. The sample was shaken at 225
rpm for 10 minutes at 37 °C, 5 units of Benzonase were added and the sample was shaken
at 225 rpm for a further 10 minutes at 37 °C. Sodium phosphotungstic acid (diluted in PBS
pH 7.4) was added to the reaction mix to give 0.3 % w/v final concentration and the tubes
were shaken at 225 rpm for 30 minutes at 37 °C prior to centrifugation at 16,000 X g for 30
minutes at 4 °C. To obtain the soluble and insoluble PrP fractions, the supernatant (soluble
fraction, 40 µl) was transferred to a fresh tube and the pellet (insoluble fraction) was
resuspended in 40 µl of 0.1 % w/v Sarkosyl in PBS pH 7.4.

Proteinase K digestion of fly head homogenate
Fly head homogenates were prepared in 1.5 ml eppendorf tubes by processing 10 fly heads
in 9 µl of lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.5 % Nonidet P-40) using plastic
pestles. A 1 µl volume of PK at x10 the required concentration was added to the homogenate
and the mixture incubated at 37 °C for 15 minutes. Proteolysis was stopped by the addition of
1.1 µl of 10 mM AEBSF and the samples analyzed by SDS-PAGE and western blot to detect
PrP.

SDS-PAGE and western blot
Fly head homogenate was mixed with an equal volume of x2-strength Laemmli loading
buffer, boiled for 10 minutes, cooled on ice and then centrifuged at 13,000 X g for 5 minutes
at 18 °C to remove debris. Fly head homogenate was subjected to SDS/PAGE run under
reducing conditions and western blot as described in detail previously (50) except that the
nitrocellulose membranes were probed with a 1:2000 dilution of anti-PrP monoclonal
antibody Sha31 (51).

Capture-detector ELISA
Duplicate 40 µl aliquots of fly head homogenate were diluted to 100 µl with PBS pH 7.4. PrP was quantified by capture-detector ELISA carried out as described previously (52) except that the capture reagent was anti-PrP monoclonal antibody 245 (53) and the detector antibody was biotinylated SAF32 (51). The equivalent of 10 fly heads were assayed per well in duplicate.

Conformational-dependent immunoassay (CDI)

Head homogenate was prepared as described above and PrP was quantified by CDI as described previously (48) except that the capture reagent was anti-PrP monoclonal antibody 245 (53) and the detector antibody was biotinylated SAF32 (51). The equivalent of 20 fly heads were assayed per well in duplicate.

Survival assay

Newly eclosed flies were allowed to mature and mate for 24 hours before the females were separated and collected for survival assays. One hundred flies of each genotype were housed in groups of 10 and the flies were flipped every 2 to 3 days onto fresh food. The number of dead flies was recorded three times a week (45). Survival curves were calculated using Kaplan-Meier plots and differences between them were analyzed by the log-rank method using Prism (GraphPad Software Inc, San Diego, USA).

Locomotor assay

The locomotor ability of flies was assessed in a negative geotaxis climbing assay as described previously (54). Briefly, age-matched, pre-mated female flies were placed in adapted plastic 25 ml pipettes that were used as vertical climbing columns. The flies were allowed to acclimatize for 30 minutes prior to assessment of their locomotor ability. Flies were tapped to the bottom of the pipette (using the same number and intensity of taps) and then allowed to climb for 45 seconds. At the end of the climbing period the number of flies above the 25 ml mark, the number below the 2 ml mark and the number in between the 2 ml and 25 ml mark were recorded. This procedure was performed three times at each time point. The mean performance index (PI) ± SD for each group of flies was calculated as described (54).

Statistical analysis
Statistical analysis of the data was performed by one-way analysis of variance, together with Tukey highly significant difference (HSD) for post hoc analysis or the unpaired samples t test using Prism (GraphPad Software Inc, San Diego, USA).
Results

Cytosolic ovine PrP expression in *Drosophila*

Here we have generated *Drosophila* transgenic for polymorphic variants of cytosolic ovine PrP in order to investigate the toxic potential of intracellular PrP expression in the absence or presence of exogenous prions. The data in Figure 1 show the western blot detection of cytosolic PrP [PrP(cyt)] expression in *Drosophila*. The analysis in Figure 1a shows that ARQ(cyt), AHQ(cyt) and VRQ(cyt) were all efficiently expressed at a similar level pan neuronally in the fly. The molecular mass of all three genotypes of PrP(cyt) was approximately 27kDa, the same as that of non-glycosylated ovine recombinant PrP. The analysis in Figure 1b shows that AHQ(cyt) and VRQ(cyt) transgenic *Drosophila* expressed significantly higher levels of PrP compared to flies that expressed AHQ or VRQ PrP with a GPI anchor sequence [PrP(GPI)]. The opposite trend was seen with *Drosophila* that expressed the ARQ PrP genotype.

We subsequently used capture-detector ELISA with C-terminal-specific anti-PrP monoclonal antibodies in order to quantify the level of each genotype of cytosolic ovine PrP expressed in *Drosophila*. The data in Figure 2a show that significantly lower levels of pan neuronally expressed ovine PrP(cyt) were recognized by the anti-PrP-specific ELISA compared to ovine PrP(GPI). This observation suggested that cytosolic ovine PrP may adopt a distinct conformation compared to other forms of ovine PrP expressed in *Drosophila* that are recognized by this ELISA (45). In order to test this, we used a conformational-dependent immunoassay (CDI) whereby PrP(cyt) was denatured by guanidine prior to its recognition by capture-detector immunoassay (48). The data in Figure 2b show that all of the genotypes of cytosolic ovine PrP expressed pan neuronally in *Drosophila* were recognized by the denaturant-based CDI.

Cytosolic PrP is predominantly detergent insoluble and displays protease resistance

We next investigated whether the immunobiochemical properties of PrP(cyt) expressed in *Drosophila* correlated with distinct conformers of the ovine prion protein. Figure 3 shows a comparison of ARQ(cyt) and ARQ(GPI) with respect to detergent solubility and relative resistance to proteolytic digest. In order to determine the detergent solubility of cytosolic ovine PrP we extracted fly head homogenates with Sarkosyl to prepare soluble and insoluble fractions for subsequent analysis by western blot with anti-PrP monoclonal antibody Sha31. The data in Figure 3a show that while *Elav*-driven ARQ(cyt) and ARQ(GPI) flies both
displayed a major band of approximately 27kDa in detergent soluble and insoluble head homogenate fractions, the proportion of PrP present in these fractions varied. The level of insoluble prion protein was greater than the level of soluble material in Elav-ARQ(cyt) flies compared to that of the Elav-ARQ(GPI) fly line. The data in Figure 3b show the western blot analysis of PK-digested fly head homogenate from pan neuronal ARQ(cyt) and ARQ(GPI) flies. Pan neuronally expressed ARQ(GPI) PrP was readily cleaved by PK when treated with 3 - 9 µg/ml of proteolytic enzyme. In contrast, ARQ(cyt) was resistant to digestion with PK when treated with the proteolytic enzyme used in the same concentration range and was only susceptible to complete digest when PK was used in excess of 27 µg/ml. All three polymorphic variants of cytosolic ovine PrP showed these trends (data not shown).

Survival of cytosolic ovine PrP transgenic Drosophila

Cytosolic PrP accumulation is toxic to some neurons (26, 55) and may be part of the neurotoxic mechanism associated with prion diseases (25). It was important therefore to determine the effect of cytosolic PrP expression on the general well being of Drosophila prior to prion infectivity studies in these fly lines.

The data in Figure 4 show the survival curves for PrP transgenic fly lines that pan neuronally expressed cytosolic ovine prion protein in comparison with the survival curve for the control Elav-51D fly line. Cytosolic PrP expression did not appear to be overtly detrimental to Drosophila since the survival curve for each of the ovine PrP transgenic fly lines showed a similar profile to that of the non-transgenic 51D control flies. However, log-rank test analysis showed that the survival curves of all three genotypes of prion protein transgenic fly lines were significantly different to that of the 51D control flies (p≤0.002) and this was reflected in differences in median survival times, which were: 51D 86 days; AHQ(cyt) 81 days; ARQ(cyt) 79 days; and VRQ(cyt) 76 days. The general lack of toxicity in Drosophila as a consequence of pan neuronal cytosolic PrP expression was also evident when PrP(cyt) was expressed ubiquitously. For example, the percent survival for β-actin-driven and elav-driven VRQ(cyt) PrP transgenic flies was similar at approximately 90 % and 95 %, respectively, when assessed at 50 days of age.

Cytosolic PrP transgenic Drosophila are susceptible to ovine prion inocula

In order to assess whether cytosolic ovine PrP transgenic Drosophila were susceptible to the toxic effect of exogenous ovine prion inocula, flies at the larval stage of development were exposed to scrapie-infected sheep brain material and the locomotor activity of prion-exposed
flies assessed after eclosion (i.e. hatching). The prion inoculum used here was sheep brain homogenate derived from natural cases of VRQ/VRQ and ARQ/ARQ classical (50) or AHQ/AHQ experimental atypical (43) sheep scrapie. Genotype matched scrapie-free brain homogenates were used as control material and 51D Drosophila were used as the control fly line, which were similarly exposed to scrapie-infected and scrapie-free sheep brain homogenate.

In order to assess the response to classical scrapie prion inocula, β-actin- or Elav-driven VRQ(cyt) Drosophila were exposed at the larval stage to VRQ/VRQ scrapie-infected sheep brain homogenate. Figure 5 shows the climbing ability expressed as a performance index of prion exposed and control flies post-eclosion. The data in Figure 5a show that prion-exposed β-actin-driven VRQ(cyt) Drosophila displayed a significantly accelerated decline in locomotor activity compared to similar flies exposed to genotype-matched control brain homogenate (p<0.001 over the whole assay). In contrast, β-actin-driven 51D flies showed a similar decline in locomotor activity following exposure to scrapie-infected or genotype matched control sheep brain homogenate. The data in Figure 5b show that prion-exposed Elav-driven VRQ(cyt) Drosophila also showed a significantly accelerated decline in locomotor activity compared to similar flies exposed to genotype-matched control brain homogenate (p<0.05 between days 2 and 51 of the assay), which was somewhat reduced compared to that seen by β-actin-driven VRQ(cyt) Drosophila. In contrast, the performance index of elav-driven VRQ(cyt) PrP transgenic Drosophila exposed to ARQ/ARQ scrapie-infected sheep brain homogenate was not significantly different to that of similar flies exposed to scrapie-free ARQ/ARQ sheep brain homogenate (data not shown). Elav-driven 51D flies showed no difference in the decline of locomotor activity following exposure to scrapie-infected or genotype matched control sheep brain homogenate.

We subjected head homogenates from prion-exposed flies to proteolytic digest followed by western blot with anti-PrP monoclonal antibody in order to attempt to detect PK-resistant PrPSc. The data in Figure 6 show that the majority of pan neuronally expressed VRQ(cyt) from prion-exposed and control treated flies was digested with PK at 10 - 30 µg/ml and with similar resultant molecular profiles. At 20 days of age a greater fraction of the VRQ(cyt) was resistant to PK digestion at these concentrations of the proteolytic enzyme.

We subsequently investigated whether the toxic phenotype displayed by prion-exposed VRQ(cyt) Drosophila was transmissible. In order to do so, we prepared homogenates from
the heads of 30 day-old *Drosophila* that had been exposed at the larval stage to either

VRQ/VRQ prion-infected or genotype-matched scrapie-free sheep brain homogenate. Fly

head homogenates were subsequently used to inoculate fresh batches of recipient VRQ(cyt)

*Drosophila* larvae. After hatching, the locomotor activity of fly head homogenate-exposed

*Drosophila* was assessed by a negative geotaxis climbing assay. The data in Figure 7 show

that head homogenate from prion-exposed VRQ(cyt) *Drosophila* induced a significantly

accelerated decline in locomotor activity compared to control fly head homogenates in

VRQ(cyt) recipient flies (*p*<0.01 over the whole assay) (Figure 7a). In contrast, no significant

differences were seen in the locomotor response of recipient VRQ(cyt) *Drosophila* to head

homogenate prepared from non-transgenic 51D flies previously exposed to either scrapie-

infected or scrapie-free sheep brain material (Figure 7b).

In order to assess the response to atypical scrapie prion inocula, *β*-Actin- or *Elav*-driven

cytosolic AHQ(cyt) ovine PrP transgenic *Drosophila* were exposed at the larval stage to

AHQ/AHQ prion-infected or genotype-matched scrapie-free sheep brain homogenate. *β*-Actin-

and *Elav*-driven AHQ(GPI) and *Elav*-driven ARQ(GPI) *Drosophila*, that both express

PrP with a GPI anchor sequence, were included for comparison. The data in Figure 8 show

the climbing ability of *Drosophila* with ubiquitous AHQ expression after exposure to atypical

scrapie-infected sheep brain homogenate. *β*-Actin-driven AHQ(cyt) flies showed an

accelerated decline in locomotor activity following exposure to atypical scrapie-infected

sheep brain homogenate compared to control brain homogenate (*p*=0.0226 between day 8

and day 39) (Figure 8a). Similarly, atypical prion-exposed *β*-Actin-driven AHQ(GPI) flies

showed a significantly enhanced decline in locomotor activity compared to similar flies

exposed to control inocula (*P*=0.0278 over the whole assay) (Figure 8b). *β*-Actin-driven

ARQ(GPI) flies also showed a significantly enhanced decline in locomotor activity following

exposure to AHQ/AHQ scrapie-infected brain homogenate compared to control inocula

(*p*=0.0351 between day 8 and day 39) (Figure 8c). In contrast, *β*-Actin -driven 51D flies

showed the same decline in locomotor activity following exposure to AHQ/AHQ scrapie-

infected or genotype-matched scrapie-free sheep brain homogenate (Figure 8d). Similar

trends were seen with both atypical scrapie inocula (data not shown).

*Elav*-driven AHQ(cyt), AHQ(GPI) or 51D flies showed no difference in decline of locomotor

activity following exposure to AHQ/AHQ scrapie-infected brain homogenate compared to

control brain homogenate. The data in Figure 9 show the climbing ability of *Drosophila* after

exposure to atypical scrapie-infected and control sheep brain homogenate. *Elav*-driven
AHQ(cyt) flies showed a similar decline in locomotor activity following exposure to AHQ/AHQ scrapie-infected or genotype-matched scrapie-free sheep brain homogenate, or PBS (Figure 9a). In a similar manner, Elav-driven AHQ(GPI) flies showed no difference in decline of locomotor activity following exposure to AHQ/AHQ scrapie-infected brain homogenate compared to control inocula (Figure 9b) although a response was seen at day 33 with one atypical scrapie inoculum (data not shown). In contrast to these data, Elav-driven ARQ(GPI) flies showed a significantly enhanced decline in locomotor activity following exposure to AHQ/AHQ scrapie-infected brain homogenate compared to control inocula ($p<0.05$ between day 7 and day 40) (Figure 9c). Elav-driven 51D flies showed the same decline in locomotor activity following exposure to AHQ/AHQ scrapie-infected sheep brain homogenate or control inocula (Figure 9d). Similar trends were seen with both atypical scrapie inocula (data not shown).
Discussion

The pathogenesis that occurs during prion diseases is associated with the conformational change of PrPC into PrPSc and concomitant neurodegeneration (1). However, the mechanism of PrP conversion and its role in neurotoxicity are unknown. While PrPC is primarily attached by a GPI anchor to the external side of the cell membrane, topological variants of the protein can arise during its biogenesis and metabolism (25). Here we have shown that one such variant, namely cytosolic PrP, can participate in the generation of a transmissible toxicity induced by ovine prion inocula.

To do so, we have generated Drosophila that express cytosolic AHQ, ARQ or VRQ ovine PrP. All three cytosolic PrP variants were expressed at a similar level in the fly and comprised predominantly detergent insoluble material that showed resistance to proteolytic digest with relatively low concentrations of Proteinase K enzyme. In addition, epitopes normally exposed in ovine PrPC were either hidden or buried in PrP(cyt) since the latter required denaturation prior to its immunodetection by capture-detector immunoassay. The molecular profile and conformational properties of the PrP(cyt) variants expressed in Drosophila are distinct from those of the same polymorphic variants expressed with a GPI anchor in this host (45). This is likely to be due to the lack of post translational modifications experienced by PrP(cyt) as a consequence of its failure to enter the ER during biosynthesis. The modifications that PrPC normally experiences during its biogenesis include glycosylation and the introduction of a disulfide bond to the polypeptide chain, both of which influence protein folding and thermodynamic stability. Despite the acquisition of properties of misfolded prion protein, the pan neuronal expression of PrP(cyt) in Drosophila was not overtly detrimental to the fly. We have previously shown that Drosophila transgenic for AHQ expressed with a GPI anchor sequence displayed a median life-span that was significantly reduced compared to control 51D flies (45). Ovine AHQ PrP is associated with susceptibility to atypical scrapie in sheep, which is considered to be a spontaneous disorder of PrP folding and/or metabolism (38, 40) rather than an acquired condition (41-43). Our observation here that cytosolic AHQ does not induce a comparable toxicity to AHQ(GPI) suggests that the toxicity associated with AHQ targeted to the cell membrane is a consequence of this protein trafficking through the secretory pathway of the cell. The expression of AHQ variants of ovine PrP in Drosophila provides a novel model system to investigate the potential spontaneous misfolding of this genotype of ovine prion protein.
We assessed the response of PrP(cyt) transgenic *Drosophila* to exogenous ovine prions in a negative geotaxis climbing assay, a versatile and robust method used to assess locomotor defects in fly models of mammalian neurodegenerative conditions (54). *Drosophila* transgenic for VRQ(cyt) or AHQ(cyt) expression showed a decreased climbing ability after exposure at the larval stage to classical or atypical scrapie-infected sheep brain homogenate, respectively. The toxic effect of classical and atypical scrapie in PrP(cyt) transgenic *Drosophila* is suggestive of a prion-mediated effect as it was not induced by exposure to normal sheep brain homogenate and it was PrP dependent, since scrapie-infected sheep brain homogenate was not toxic to non transgenic 51D flies. Importantly, we have shown that the toxic phenotype of prion-exposed PrP(cyt) flies was transmissible. Fly head homogenate from prion-exposed VRQ(cyt) PrP transgenic *Drosophila* efficiently induced a toxic phenotype in recipient flies of the same genotype. This was not due to carry over of scrapie-infected sheep brain inocula in fly head homogenate since no effect was induced in recipient PrP(cyt) flies by prion-exposed non-transgenic 51D fly head inocula. These observations are suggestive of the generation of an infectious moiety, analogous to prion replication, during primary passage of scrapie in VRQ(cyt) flies (i.e. sheep-to-fly transmission) that was subsequently transmitted at secondary passage (i.e. fly-to-fly transmission). However, we were unable to demonstrate an increase in PK-resistant VRQ(cyt) PrP in prion exposed flies of this genotype. In other studies we have shown that protein misfolding cyclic amplification can be used to detect PK-resistant PrPSc in prion exposed VRQ(GPI) transgenic *Drosophila* but not similarly treated VRQ(cyt) flies (Thackray et al. submitted).

A feature of the response by AHQ(cyt) transgenic *Drosophila* to atypical scrapie toxicity was the requirement for ubiquitous PrP expression in the fly. The lack of susceptibility of pan neuronal AHQ(cyt) but not VRQ(cyt) transgenic *Drosophila* to scrapie prions would not appear to be due to the level of ovine PrP expressed in these flies since cytosolic PrP was expressed at a similar level in both Elav-driven fly lines. Furthermore, resistance to atypical scrapie toxicity by *Drosophila* transgenic for pan neuronal expression of AHQ(cyt) did not appear to be due to the topological expression of PrP in this fly line since *Drosophila* transgenic for pan neuronal expression of AHQ(GPI) were also refractive to the same inocula. The need for ubiquitous PrP expression in AHQ PrP transgenic *Drosophila* for susceptibility to atypical scrapie toxicity may reflect a low infectious titre in these particular prion-infected isolates compared to classical scrapie material. Alternatively, atypical scrapie infectivity may be more unstable than its classical scrapie counterpart. It is known that the
PrPSc associated with atypical scrapie is less PK resistant compared to that associated with classical scrapie (56, 57). Whatever the case, ubiquitous expression of PrP in *Drosophila* may provide an environment for enhanced uptake and neuroinvasion of scrapie-infected material and generation of the toxic agent compared to pan neuronal expression, which may be more important for the response to atypical scrapie prion inocula. In mammalian species PrPC is ubiquitously expressed, a feature that plays an essential role in the transmission of prion infectivity in naturally acquired cases of prion disease (58), which may include atypical scrapie (41, 42). However, not all of the ovine PrP transgenic fly lines used here required ubiquitous expression of PrP in order to succumb to atypical scrapie prion inocula. *Drosophila* with pan neuronal expression of ovine ARQ(GPI) showed susceptibility to AHQ/AHQ atypical scrapie-infected sheep brain homogenate, as they do to ARQ/ARQ and VRQ/VRQ classical scrapie prion inocula (59). The promiscuous susceptibility of ARQ(GPI) PrP flies to atypical and classical scrapie-induced toxicity correlates with the high level of ovine prion protein expressed by this fly line (45). It is known that the transmission barrier effect (1) can be circumvented by elevated levels of PrP expression. For example, tg338 mice that express high levels of ovine VRQ PrP are susceptible to atypical scrapie isolates whereas VRQ/VRQ sheep are resistant (34, 42). Collectively, these observations suggest that *Drosophila* engineered for elevated levels of ubiquitous cell surface or cytosolic PrP expression will be susceptible to a greater diversity of scrapie prion isolates and potentially lower quantities of associated toxicity. This suggests that PrP transgenic *Drosophila* could provide the basis of a new animal model to bioassay low levels of infectious toxicity in peripheral tissues and blood of prion-affected animals.

Our studies with cytosolic PrP transgenic *Drosophila* presented here begin to contribute to an understanding of the potential role of topological variants in prion-induced neurotoxicity. While the mechanism of prion toxicity remains to be defined, it is established that PrP expression is required for susceptibility to the neurotoxic agent. The essential requirement for PrP expression in prion-induced neurotoxicity may suggest an intermediate in the conversion of PrPC to PrPSc is the neurotoxic agent (60, 61). An alternative possibility is that neurotoxicity results from PrPSc interference with the normal biosynthesis and metabolism of PrPC (25). PrP can accumulate in the cytosol in a misfolded form when proteasomal activity is compromised (28, 31) and cytosolic PrP has been reported to be neurotoxic in some neurons (26, 55). However, the neurotoxicity of cytosolic PrP *per se* has been debated (62, 63). Our observations here have shown that while cytosolic PrP can adopt a conformation distinct from PrP targeted to the cell membrane, expression of PrP(cyt) in *Drosophila* does
not result in the accumulation of a transmissible toxic moiety without prior exposure of these flies to exogenous prion inocula. Collectively, our data presented here suggest that cytosolic PrP is not overtly toxic to neurons \textit{per se} but may participate in a toxicity mediated by scrapie prion inocula, possibly by acting as a substrate for the generation of PrP-dependent transmissible moiety that initiates or maintains repression of neuronal proteostasis (29, 30, 64-66). The tractable nature of \textit{Drosophila} as a genetically and biochemically well-defined experimental model will allow us to test the validity of this hypothesis.

Acknowledgements

We thank John Roote for useful discussion regarding fly genetics. We thank Drs. Marion Simmons and John Spiropoulos for the supply of sheep atypical scrapie samples. This work was supported by funds from the China Scholarship Council and Cambridge Overseas Trust who provided PhD studentship support for C.Z. We acknowledge funding support from the NC3Rs.
Legends

**Figure 1. Western blot detection of cytosolic ovine PrP expression in Drosophila**

Head homogenates were prepared from 5 day old ovine PrP transgenic Drosophila or 51D control flies crossed with the Elav-GAL4 driver fly line. Samples were analyzed by SDS-PAGE and western blot with anti-PrP monoclonal antibody Sha31. (a) Molecular profile of ARQ(cyt); AHQ(cyt); VRQ(cyt) all at the equivalent of 10 fly heads per track. Mature length ovine VRQ recombinant PrP (rPrP) was used at 10 ng. Molecular mass marker values (kDa) are shown on the left hand side. (b) Comparison of ovine PrP(cyt) and PrP(GPI) expression in Drosophila. Tracks 1 and 2: PrP(cyt); tracks 3 and 4: PrP(GPI); tracks 1 and 3: male flies; tracks 2 and 4: female flies. The equivalent of 5 fly heads were run per track. Molecular mass marker values (kDa) are shown on the left hand side. The ovine PrP genotype is indicated on the right hand side.

**Figure 2. Capture-detector immunoassay analysis of cytosolic ovine PrP expression in Drosophila**

Head homogenates were prepared from 5 day old ovine PrP transgenic Drosophila or 51D control flies crossed with the Elav-GAL4 driver fly line. Samples were analyzed by: (a) ELISA using anti-PrP monoclonal antibody 245 as capture and biotinylated anti-PrP monoclonal antibody SAF32 as detector. The equivalent of 10 fly heads were measured per well and the results shown are OD$_{415nm}$ means ± SD for duplicate wells; (b) Conformational-dependent immunoassay (CDI). Fly head homogenates were treated with 8M GdnHCl prior to immunoassay using anti-PrP monoclonal antibody 245 as capture and biotinylated anti-PrP monoclonal antibody SAF32 as detector. The equivalent of 20 fly heads were measured per well. Mature length ovine ARQ recombinant PrP (rPrP) was used at 122 ng/well. The results are shown as time resolved fluorescence (TRF) counts per second (cps) ± SD for duplicate wells.

**Figure 3. Cytosolic ovine PrP is characterized by reduced solubility and increased PK resistance**

Head homogenates were prepared from 5 day old ovine ARQ(cyt) or ARQ(GPI) PrP transgenic Drosophila crossed with the Elav-GAL4 driver fly line. After various treatments fly head homogenate samples were analyzed by SDS-PAGE and western blot with anti-PrP monoclonal antibody Sha31. The equivalent of 10 fly heads were loaded per track. Molecular mass marker values (kDa) are shown on the left hand side of each gel. (a) Total (T), soluble
(S) and insoluble (I) fractions of PrP were prepared from fly heads as described in the Materials and Methods. Reaction products of fly head homogenates incubated with various concentrations of PK at 37 °C for 30 minutes.

Figure 4. Survival curves for cytosolic ovine PrP transgenic Drosophila
Groups of 100 age-matched Elav-PrP or control Elav-51D flies were selected for survival assays. The number of surviving flies was recorded three times a week as described in the Materials and Methods. Survival curves were calculated using Kaplan-Meier plots and differences between them were analyzed by the log-rank method using Prism (GraphPad Software Inc, San Diego, USA).

Figure 5. Primary transmission of classical ovine scrapie in cytosolic VRQ PrP transgenic Drosophila
VRQ(cyt) PrP transgenic (squares) or 51D control flies (circles) crossed with either the β-actin-GAL4 or Elav-GAL4 driver line were assessed for locomotor activity by a negative geotaxis climbing assay following exposure at the larval stage to VRQ/VRQ scrapie-infected (filled symbols) or scrapie-free (open symbols and dashed lines) sheep brain homogenate. β-actin-GAL4-VRQ(cyt) PrP flies were non RFP. The mean performance index ± SD is shown for three groups of n=15 flies of each genotype per time point (45 flies in total for each group). Statistical analysis of the linear regression plots was performed using one-way analysis of variance (ANOVA) and post hoc Tukey honestly significant difference.

Figure 6. PK digestion of prion-exposed cytosolic VRQ fly head homogenate
Fly head homogenates were prepared from ovine VRQ(cyt) PrP transgenic Drosophila crossed with the Elav-GAL4 driver fly line following exposure at the larval stage to VRQ/VRQ scrapie-free (tracks 1-3) or scrapie-infected (tracks 4-6) sheep brain homogenate. Samples were incubated with 0, 10 or 30 µg/ml PK at 37 °C for 15 minutes and the reaction products analyzed by SDS-PAGE and western blot with anti-PrP monoclonal antibody Sha31. The equivalent of 10 fly heads were loaded per track. Molecular mass marker values (kDa) are shown on the left hand side of each gel. Age of flies (in days) is shown on the right.

Figure 7. Fly-to-fly transmission of prion-induced toxic phenotype
VRQ(cyt) PrP (non RFP) transgenic flies crossed with the β-actin-GAL4 driver line were assessed for locomotor activity by a negative geotaxis climbing assay following exposure at the larval stage to a 10 % (v/v) dilution of head homogenate derived from 30 day old...
*Drosophila* exposed at the larval stage to either scrapie-infected (filled squares) or scrapie-free (open squares and dashed line) sheep brain homogenate. The mean performance index ± SD is shown for three groups of n=15 flies of each genotype per time point (45 flies in total for each group). Statistical analysis of the linear regression plots was performed using one-way analysis of variance (ANOVA) and *post hoc* Tukey honestly significant difference.

**Figure 8. Primary transmission of atypical scrapie in β-actin-driven PrP transgenic *Drosophila***

PrP transgenic or 51D control flies crossed with the β-actin-GAL4 driver line were assessed for locomotor activity by a negative geotaxis climbing assay following exposure at the larval stage to AHQ/AHQ scrapie-infected (filled circles) or scrapie-free (open circles and dashed lines) sheep brain homogenate. The mean performance index ± SD is shown for three groups of n=15 flies of each genotype per time point (45 flies in total for each group).

Statistical analysis of the scrapie-infected and scrapie-free linear regression plots for each fly line was compared by the unpaired samples *t* test.

**Figure 9. Lack of response by Elav-driven AHQ PrP transgenic *Drosophila* to atypical scrapie***

PrP transgenic or 51D control flies crossed with the Elav-GAL4 driver line were assessed for locomotor activity by a negative geotaxis climbing assay following exposure at the larval stage to AHQ/AHQ scrapie-infected (closed squares and continuous line) or scrapie-free (closed circles and dashed line) sheep brain homogenate or PBS (closed triangles and dotted line). The mean performance index ± SD is shown for three groups of n=15 flies of each genotype per time point (45 flies in total for each group). Statistical analysis of the linear regression plots was performed using one-way analysis of variance (ANOVA) and *post hoc* Tukey honestly significant difference.


Figure 1. Western blot detection of cytosolic ovine PrP expression in *Drosophila*

(a) 

(b)
Figure 2. Capture-detector immunoassay analysis of cytosolic ovine PrP expression in *Drosophila*
Figure 3. Cytosolic ovine PrP is characterized by reduced solubility and increased PK resistance
Figure 4. Survival curves for cytosolic ovine PrP transgenic *Drosophila*
Figure 5. Primary transmission of classical ovine scrapie in cytosolic VRQ PrP transgenic *Drosophila*

(a) $\beta$-actin-VRQ(cyt)

(b) Elav-VRQ(cyt)
Figure 6. PK digestion of prion-exposed cytosolic VRQ fly head homogenate

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<tr>
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31 -

5 days

20 days

40 days
Figure 7. Fly-to-fly transmission of prion-induced toxic phenotype

(a) VRQ(cyt)-derived

(b) 51D-derived
Figure 8. Primary transmission of atypical scrapie in $\beta$-actin-driven PrP transgenic Drosophila

(a) $\beta$-actin-AHQ(cyt)

(b) $\beta$-actin-AHQ(GPI)

(c) $\beta$-actin-ARQ(GPI)

(d) $\beta$-actin-51D
Figure 9: Lack of response by *Elav*-driven AHQ PrP transgenic *Drosophila* to atypical scrapie