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Research Article

Bioassay of prion-infected blood plasma in PrP transgenic *Drosophila*

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In pursuit of a tractable bioassay to assess blood prion infectivity, we have generated prion protein (PrP) transgenic *Drosophila*, which show a neurotoxic phenotype in adulthood after exposure to exogenous prions at the larval stage. Here, we determined the sensitivity of ovine PrP transgenic *Drosophila* to ovine prion infectivity by exposure of these flies to a dilution series of scrapie-infected sheep brain homogenate. Ovine PrP transgenic *Drosophila* showed a significant neurotoxic response to dilutions of 10^{-2} to 10^{-10} of the original scrapie-infected sheep brain homogenate. Significantly, we determined that this prion-induced neurotoxic response in ovine PrP transgenic *Drosophila* was transmissible to ovine PrP transgenic mice, which is indicative of authentic mammalian prion detection by these flies. As a consequence, we considered that PrP transgenic *Drosophila* were sufficiently sensitive to exogenous mammalian prions to be capable of detecting prion infectivity in the blood of scrapie-infected sheep. To test this hypothesis, we exposed ovine PrP transgenic *Drosophila* to scrapie-infected plasma, a blood fraction notoriously difficult to assess by conventional prion bioassays. Notably, pre-clinical plasma from scrapie-infected sheep induced neurotoxicity in PrP transgenic *Drosophila* and this effect was more pronounced after exposure to samples collected at the clinical phase of disease. The neurotoxic phenotype in ovine PrP transgenic *Drosophila* induced by plasma from scrapie-infected sheep was transmissible since head homogenate from these flies caused neurotoxicity in recipient flies during fly-to-fly transmission. Our data show that PrP transgenic *Drosophila* can be used successfully to bioassay prion infectivity in blood from a prion-diseased mammalian host.

Introduction

Prion diseases are fatal neurodegenerative conditions that affect humans and a variety of other vertebrate species [1]. These conditions, which include scrapie of sheep, bovine spongiform encephalopathy (BSE) of cattle and Creutzfeldt–Jakob disease (CJD) of humans, are associated with the misfolding of the normal host protein PrPC into a disease-specific conformer PrPSc (abnormal disease-specific conformation of PrP) [2]. The disease-associated form of prion protein (PrP) is enriched in β-sheet structure, and a proportion of the protein ensemble is partially resistant to proteolytic digestion [3,4]. The accumulation of misfolded PrP in the brain of prion-diseased individuals is central to the pathological process of these conditions [5,6], which typically manifests as synaptic loss and neuronal dysfunction, with resultant neurological signs and clinical symptoms. Prion diseases are unique among neurodegenerative conditions since they are transmissible, and PrPSc is considered to be the infectious prion agent [7].

Prion diseases are a significant risk to public health through the potential for zoonotic transmission of prions as evidenced by the BSE epizootic in U.K. cattle and subsequent emergence of variant CJD (vCJD) in humans [8,9]. The occurrence of vCJD prions in human lymphoid tissue, together with the detection of prion infectivity in the blood of animals with asymptomatic experimental prion disease

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[10,11], raised the possibility for the potential of blood-borne vCJD transmission. These concerns were realised with the emergence of cases of vCJD in individuals within the U.K. who had received red blood cell concentrates [12–15]. In addition, abnormal prion protein was detected in a post-mortem spleen sample from a haemophilic patient who had received purified Factor VIII prepared from plasma batches that included donations from individuals who later developed vCJD [12]. These particular cases of vCJD infection collectively provide strong support for the view that this prion disease can be transmitted by blood transfusion. Consequently, much attention has focused on understanding the biology of infectious prions in blood through the analysis of prion infectivity and PrPSc within different blood fractions from hosts with prion disease.

The only reliable method to detect prion infectivity is by bioassay in an appropriate indicator species. The bioassay of blood-borne prion infectivity has been performed for a variety of different species, such as rodents, sheep, cervids and primates including humans, undergoing, collectively, either experimental or natural prion disease [10,15–21]. These bioassays have typically involved intracerebral inoculation of blood, or blood fractions, into a recipient indicator species, usually rodents including mice with a PrP transgene autologous for the donor species. Collectively, these studies revealed that prion infectivity titres of blood samples harvested during the asymptomatic phase of prion disease were up to 10-fold less than those collected during the clinical phase [10,19,20,22]. Infectious prions were found to be associated with white and red blood cells, platelets and plasma, although interspecies variations existed with regard to the distribution of prion infectivity in these different blood fractions [10,15–21]. Studies in ruminants have analysed bioassays of autologous whole or fractionated blood by the intravenous route [19,20]. These experimental transfusion studies have confirmed that all blood fractions prepared by protocols similar to those used in transfusion medicine can transmit prion disease [23,24]. In addition, prion transmission by transfusion of cellular blood fractions was not solely dependent on their infectious titre since prion disease was more efficiently transmitted with viable rather than non-viable leukocytes [20]. While these studies have collectively been immensely informative in providing evidence that blood from prion-diseased hosts can harbour prion infectivity, the bioassays used to establish this are relatively cumbersome and time-consuming.

We have developed a novel invertebrate system to begin to establish a tractable bioassay to assess blood prion infectivity [25]. We have previously generated *Drosophila* transgenic for ovine PrP and have shown that after exposure to exogenous ovine prions at the larval stage these flies develop a neurotoxic phenotype in adulthood, evidenced by decreased locomotor ability and the accumulation of Proteinase K (PK)-resistant PrPSc [26–28]. Here, we determined the sensitivity of ovine PrP transgenic *Drosophila* to prion infectivity by exposure of these flies to a dilution series of scrapie-infected sheep brain homogenate. Dilutions of scrapie-infected sheep brain material in the range of 10^{-2}–10^{-10} were capable of inducing neurotoxicity in ovine PrP transgenic *Drosophila*. Furthermore, we have shown that ovine prion-induced neurotoxicity in ovine PrP transgenic *Drosophila* was transmissible to ovine PrP transgenic mice. These observations suggested that PrP transgenic *Drosophila* were sufficiently sensitive to detect the reportedly low level of prion infectivity in blood from prion-diseased hosts. To test this hypothesis, we exposed ovine PrP transgenic *Drosophila* to plasma from sheep with scrapie disease, a blood fraction notoriously difficult to assess by conventional prion bioassay in more sentient experimental animals. Pre-clinical plasma from scrapie-infected sheep induced a neurotoxic effect in PrP transgenic *Drosophila*, a response that was more pronounced after exposure to samples collected at the clinical phase of disease. The neurotoxic phenotype induced in ovine PrP transgenic *Drosophila* by scrapie-infected plasma was transmissible, since head homogenate from these *Drosophila* caused neurotoxicity in recipient flies during fly-to-fly transmission. Our data show that PrP transgenic *Drosophila* can be used successfully for the bioassay of prion infectivity in blood samples from a prion-diseased mammalian host.

Materials and methods

Fly stocks

The *UAS-PrP* fly line w; M{VRQ-PrP, 3xP3-RFP.attP}ZH-51D that is transgenic for ovine $V^{136}R^{154}Q^{171}$ PrP with an N-terminal leader peptide and a C-terminal glycosylphosphatidylinositol signal sequence [VRQ(GPI)] was generated as previously described [25]. The *UAS-PrP* fly line w; M{VRQcyt-PrP, 3xP3-RFP.attP}ZH-51D that is transgenic for cytosolic expression of ovine $V^{136}R^{154}Q^{171}$ [VRQ(cyt)] was generated as recently described [28]. *Cre*-mediated removal of the red fluorescent protein (RFP) gene from the VRQ(GPI) and VRQ(cyt) PrP fly genome was performed by conventional fly crosses. In some experiments, VRQ(cyt) PrP or the relevant control 51D flies that contained the RFP cassette were used as stated in Figures 3, 5 and 6 and Supplementary

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Data S3. The *Elav-GAL4* driver line (P{w[+mW.hs] = GawB}elav[^{C155}]) and control 51D (w; M{3xP3-RFP.attP} ZH-51D) fly line were obtained from the Department of Genetics, University of Cambridge, U.K. All fly lines were raised on standard cornmeal media [29] at 25°C and maintained at low-to-medium density, and pre-mated before experimental use.

Sheep brain material and plasma

Scrapie-infected sheep brain

This was either a classical scrapie PG127-infected VRQ/VRQ sheep brain stem sample that has been endpoint titrated by intracerebral inoculation in tg338 mice and has a titre of $10^{6.6}$ ID_{50}/g [30], or cerebral cortex brain material from a confirmed scrapie-positive VRQ/VRQ sheep (SE1848/0005) [31]. *Plasma samples from scrapie-infected sheep*: these were obtained from classical scrapie-positive VRQ/VRQ sheep. Plasma samples from sheep experimentally infected with classical scrapie were prepared from animals challenged by oral inoculation with PG127-infected sheep brain homogenate as previously described [19]. Plasma samples from sheep naturally infected with classical scrapie were prepared from animals born and maintained in a flock endemic with the disease [32] and that were confirmed positive for PrPSc through routine statutory surveillance (data not shown). *Control sheep brain material and plasma*: these were obtained from New Zealand-derived scrapie-free VRQ/VRQ sheep [33].

Inoculation of *Drosophila* with sheep brain material or plasma

Drosophila at the larval stage of development were exposed to ovine brain material or plasma from confirmed classical scrapie-positive or known scrapie-negative sheep. Two hundred and fifty microlitres of either 10% (v/v) plasma or 1% (v/v) sheep brain homogenate, or various dilutions of a 1/10 dilution series (v/v) of these samples prepared in PBS pH 7.4, were added to the top of the cornmeal that contained third instar *Drosophila* larvae in 3″ plastic vials. Following eclosion (i.e. hatching), flies were transferred to fresh non-treated vials.

Negative-geotaxis climbing assay

The locomotor ability of flies was assessed in a negative-geotaxis climbing assay [25] that was initiated with 45 ($3 \times n = 15$) age-matched, pre-mated female flies in each treatment group. *Drosophila* were placed in adapted plastic 25 ml pipettes that were used as vertical climbing columns and allowed to acclimatise for 30 min prior to assessment of their locomotor ability. Flies were tapped to the bottom of the pipette (using the same number and intensity of taps on each occasion) and then allowed to climb for 45 s. 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 and 25 ml mark were recorded. This procedure was performed three times at each time point. The performance index (PI) was calculated for each group of 15 flies (average of three trials) using the formula $PI = 0.5 \times (n\text{total} + n\text{top} - n\text{bottom})/n\text{total}$, where $n$total is the total number of flies, $n$top is the total number of flies at the top and $n$bottom is the total number of flies at the bottom, as described previously [25]. A PI value of 1 is recorded if all flies climb to the top of the tube whereas the value is 0 if no flies climb the tube past the 2 ml mark. The mean PI ± SD at individual time points for each treatment group was plotted, either as a fitted or regression line. In Figure 6, the PI endpoint was plotted and this represented the extrapolated time point for individual regression lines when the PI value was 0 (i.e. x-axis value when $y = 0$ in a regression line analysis).

Preparation of *Drosophila* head homogenate

Whole flies in an eppendorf tube were frozen in liquid nitrogen for 10 min and then vortexed for 2 min to cause decapitation. Individual fly heads were isolated and placed in clean eppendorf tubes using a fine paint brush. PBS pH 7.4 was added to give 1 μl/head and homogenates were prepared by manual grinding of the fly heads with sterilised plastic pestles.

Mouse prion bioassay

Mouse prion bioassays were carried out in tg338 mice, which are transgenic for ovine VRQ PrP and are highly efficient for the detection of sheep scrapie infectivity [34]. Mice ($n = 6$ per inoculum) were injected intracerebrally with 20 μl of diluted fly head homogenate (to give 2 fly head equivalents per mouse) and monitored daily until the occurrence of clinical signs of mouse prion disease. Inoculated mice were killed when they

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started to show locomotor disorders and any impairment in their capacity to feed, or at a pre-defined endpoint for the assay (165 days post-inoculation) [30]. Brain tissue (cerebral cortex) was collected from killed mice and frozen for PrPSc analysis by western blot (TeSeE, BioRad).

Fly-to-fly transmission of neurotoxicity induced by scrapie-infected sheep plasma

Drosophila head homogenates were prepared from 30-day-old flies that had been exposed at the larval stage to plasma samples from naturally infected scrapie-positive sheep. One hundred male and 100 female fly heads were homogenised in a hand-held plastic homogeniser in 50 μl of PBS and subsequently diluted to 2.5 ml in the same buffer. Two hundred and fifty microlitres of the fly head homogenate were added to the top of the cornmeal that contained third instar *Drosophila* larvae in 3″ plastic vials (20 fly head equivalents per vial). Following eclosion (i.e. hatching), flies were transferred to fresh non-treated vials and their locomotor ability assessed in a negative-geotaxis climbing assay as described above [25].

Statistical analysis

Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) together with Tukey's honestly significant difference (HSD) for *post hoc* analysis, or the Student's *t*-test, using Prism (GraphPad Software, Inc., San Diego, U.S.A.).

Results

Here, we have determined the ability of ovine PrP transgenic *Drosophila* to bioassay prion infectivity in blood plasma from scrapie-infected sheep. To achieve this, we have used flies that express either GPI-anchored ovine $V^{136}R^{154}Q^{171}$ [VRQ(GPI)] PrP [25,26] or cytosolic ovine $V^{136}R^{154}Q^{171}$ [VRQ(cyt)] PrP [28]. We have already established that both PrP transgenic fly lines are susceptible to ovine scrapie prion infectivity, which does not induce neurotoxicity in non-transgenic 51D *Drosophila* [25,26,28].

Sensitivity of PrP transgenic *Drosophila* to ovine prions

We first established the sensitivity of ovine PrP transgenic *Drosophila* to ovine scrapie prion infectivity. To do so, PrP transgenic *Drosophil*a at the larval stage were exposed to a dilution series of PG127 scrapie-infected sheep brain homogenate. The locomotor ability of prion-exposed *Drosophila* was assessed, after hatching from the larval stage, by a negative-geotaxis climbing assay and the data were expressed as a PI. Supplementary Data S1 shows that PrP transgenic *Drosophila* demonstrated an accelerated decline in locomotor ability after hatching following exposure to scrapie prions at the larval stage compared with the response seen after exposure to scrapie-free sheep brain material. This accelerated decline, which became apparent ∼10 days after hatching, diminished upon exposure to increasing dilution of scrapie-infected brain homogenate. This titration effect was evident over the duration of the assay and was most clearly shown during the first half of the time course when the climbing ability of flies was assessed. Accordingly, the data in Figure 1 show linear regression analysis of the PI from Supplementary Data S1 for each fly line from day 1 to day 22 after hatching. These data demonstrate that VRQ(GPI) (Figure 1A) and VRQ(cyt) (Figure 1B) PrP transgenic *Drosophila* both showed a similar sensitivity to all dilutions of scrapie-infected sheep brain material, although the magnitude of the response by the VRQ(cyt) fly line was somewhat greater than that of VRQ(GPI) flies. Supplementary Data S2 shows *P*-values for the statistical analysis of the PI for each fly line in response to dilutions of scrapie-infected sheep brain material compared with the response induced by the control sheep brain sample. A statistically significant decline in PrP transgenic *Drosophila* locomotor ability was induced in the VRQ(GPI) and VRQ(cyt) fly lines by dilutions of scrapie-infected sheep brain homogenate in the range of 10^{-2}–10^{-10}. The locomotor response seen for both fly lines after exposure to scrapie-free sheep brain homogenate was not significantly different from that seen after exposure to PBS ($P > 0.05$). The PG127 scrapie-infected sheep brain isolate used here has an endpoint titration of $10^{6.6}$ ID_{50}/g in tg338 mice [30]. The data presented here show that our *Drosophila* prion bioassay has a comparable, if not greater, sensitivity for ovine prions compared with a mammalian prion bioassay.

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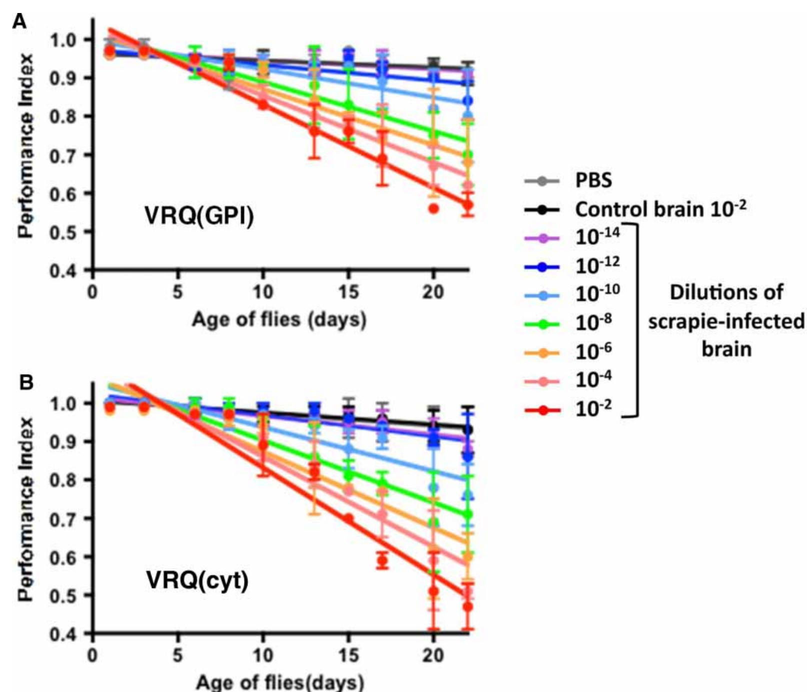

Figure 1. *Drosophila* bioassay of scrapie-infected sheep brain tissue.

Adult (**A**) VRQ(GPI) or (**B**) VRQ(cyt) PrP transgenic *Drosophila* were assessed for their locomotor ability by a negative-geotaxis climbing assay after exposure, at the larval stage, to various dilutions from a 1/10 (v/v) dilution series (as shown) of PG127 scrapie-infected or a 10^{-2} (v/v) scrapie-free sheep brain tissue. The data shown are linear regression plots of the mean PI ± SD for three groups of flies per time point calculated as described in the Materials and Methods section.

Prion infectivity in scrapie brain-exposed PrP transgenic *Drosophila*

Cardinal features of mammalian prion diseases are the accumulation of PK-resistant PrPSc in the brains of prion-infected individuals, concomitant with transmissible neurotoxicity [1,35]. We have previously shown that ovine PrP transgenic *Drosophila* accumulate disease-associated PrP after exposure to ovine prions [26]. PK-resistant PrPSc in the form of PrP27-30 was detected following protein misfolding cyclic amplification (PMCA) using head homogenate from ovine prion-exposed VRQ(GPI) PrP transgenic *Drosophila*. This was not due to carry over of PG127-infected brain material at the time of exposure of the flies to sheep scrapie since PrP27-30 was not detected using head homogenate from similarly treated non-transgenic 51D flies [26].

To determine whether the scrapie brain-induced neurotoxicity observed in ovine PrP transgenic *Drosophila* was transmissible, we performed a fly-to-mouse prion transmission study. Head homogenate from adult VRQ

Table 1 Transmission of neurotoxic fly phenotype to tg338 mice

Head homogenate from 30-day-old VRQ(GPI) PrP transgenic or control 51D *Drosophila*, exposed at the larval stage to scrapie-infected (SE1848/0005) or scrapie-free sheep brain homogenate, was inoculated into tg338 mice ($n = 6$ per sample). Mice were killed after the development of terminal signs of murine prion disease or at the assay endpoint of 165 days. The presence of PK-resistant PrPSc in the brains of killed mice was used to confirm prion disease.

		tg338 transmission		
Fly line	Prion exposure	Attack rate	PK-res PrPSc	Incubation time (days)
VRQ(GPI)	+	6/6	6/6	87 ± 2
51D	+	0/6	0/6	<165
VRQ(GPI)	–	0/6	0/6	<165
51D	–	0/6	0/6	<165

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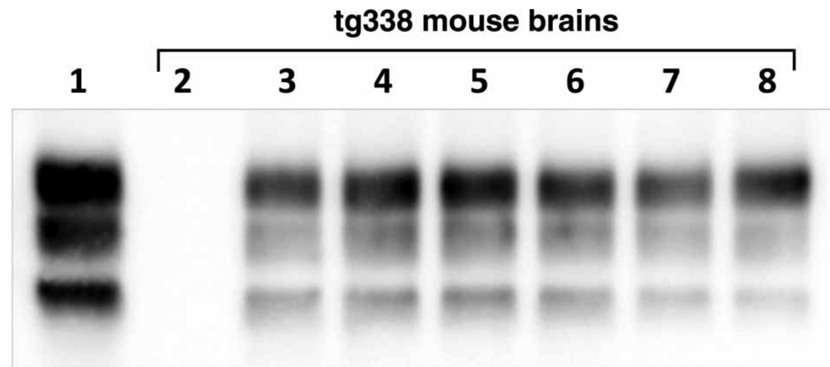

Figure 2. PrPSc detection in prion-diseased tg338 mouse brains.

Brain homogenates of tg338 mice previously inoculated with *Drosophila* head homogenate were digested with Proteinase K at 64 μg/ml and analysed by SDS–PAGE and western blot using anti-PrP monoclonal antibody Sha31 as described in the Materials and Methods section. Track 1: PG127-infected sheep brain homogenate (positive control); Track 2: non-prion-exposed tg338 mouse brain (negative control); Tracks 3–8: brain samples from prion disease-positive tg338 mice in Table 1.

(GPI) or VRQ(cyt) PrP transgenic *Drosophila*, exposed to scrapie-infected sheep brain homogenate at the larval stage, was inoculated into tg338 mice by an intracerebral injection. Inoculated tg338 mice that exhibited terminal clinical signs of mouse prion disease were killed and their brains examined for disease-associated PrP. Data given in Table 1 show that head homogenate from scrapie brain-exposed VRQ(GPI) PrP transgenic *Drosophila* induced terminal prion disease in tg338 mice with a 100% attack rate and a mean incubation time of 87 ± 2 days. Prion disease in the brains of terminal tg338 mice was verified by the presence of PK-resistant PrPSc as shown by the western blot data in Figure 2. The transmission of prion disease to tg338 mice was mediated by scrapie exposure of VRQ(GPI) *Drosophila* since head homogenate from similar flies exposed to scrapie-free sheep brain material failed to do so. In addition, prion disease in tg338 mice was not due to carry over of the original sheep inoculum from scrapie-exposed VRQ(GPI) *Drosophila* since similarly treated non-transgenic 51D flies did not induce prion disease in the tg338 mouse line. Clinical prion disease was not detected in tg338 mice inoculated with head homogenate from scrapie-exposed VRQ(cyt) *Drosophila* (data not shown). However, our previous studies have shown that head homogenate from scrapie brain-exposed VRQ (cyt) PrP transgenic *Drosophila* can induce neurotoxicity in recipient PrP transgenic flies during fly-to-fly transmission studies [26].

Bioassay of prion-infected blood plasma in PrP transgenic *Drosophila*

We reasoned that ovine PrP transgenic *Drosophila* showed a high sensitivity to ovine prions and that this rendered them sufficiently susceptible to be capable of detecting the apparent low titre of prion infectivity in the blood of scrapie-infected sheep. To test this hypothesis, we used ovine PrP transgenic *Drosophila* to bioassay blood samples from sheep experimentally infected with PG127 scrapie and that are known to contain prion infectivity [19]. We chose to bioassay sheep plasma as this blood fraction is reported to contain a low level of prion infectivity and is notoriously difficult to assess by conventional prion bioassay [19].

We first investigated the locomotor ability of VRQ(cyt) PrP transgenic *Drosophila* after exposure to blood plasma since this fly line typically showed the greatest magnitude of response to sheep scrapie prions in comparison with other PrP transgenic flies. The data given in Figure 3 show that adult VRQ(cyt) *Drosophila* displayed an accelerated decline in locomotor activity, after exposure at the larval stage, to plasma isolated from PG127-infected donor sheep compared with similar flies exposed to control scrapie-free sheep plasma. The decline in locomotor activity induced by plasma prepared from sheep 30 days post-PG127 challenge (Figure 3A) was less marked than that seen in response to plasma isolated from the same sheep at the clinical stage of scrapie disease (Figure 3B). The locomotor activity of VRQ(cyt) *Drosophila* was also assessed after exposure to plasma isolated from sheep at 60, 90 and 120 days post-PG127 challenge. Analyses of these additional time points provided further evidence that plasma isolated later in the time course of PG127-induced

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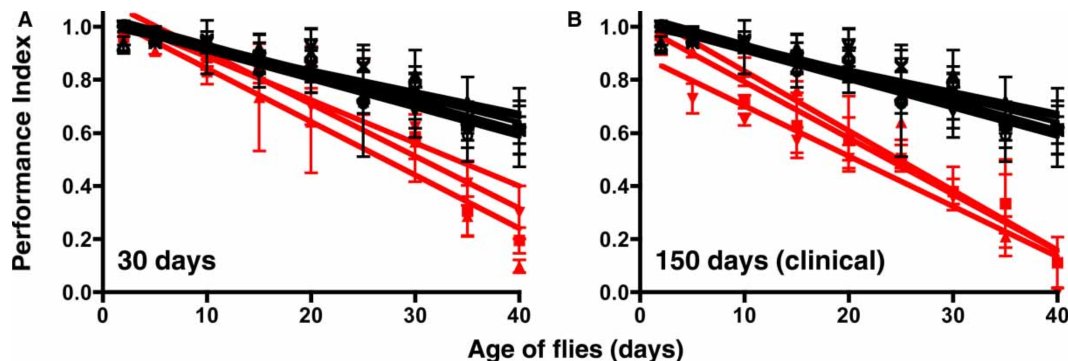

Figure 3. *Drosophila* bioassay of plasma from experimental scrapie-infected sheep.
Adult VRQ(cyt) PrP transgenic *Drosophila* (with RFP) were assessed for their locomotor ability by a negative-geotaxis climbing assay after exposure, at the larval stage, to a 10^{-1} (v/v) dilution of plasma isolated from VRQ/VRQ sheep (**A**) 30 days or (**B**) 150 days post-PG127 scrapie inoculation. Red lines: plasma from PG127 scrapie-infected donor sheep. Black lines: plasma from scrapie-free donor sheep. The data shown are linear regression plots of the mean PI ± SD for three groups of flies per time point calculated as described in the Materials and Methods section.

experimental scrapie of sheep caused a more marked decrease in the locomotor activity of VRQ(cyt) PrP transgenic *Drosophila* compared with plasma prepared at earlier time points (data not shown). Non-transgenic 51D *Drosophila* were used as the control fly line and were similarly exposed to plasma from PG127 scrapie-infected

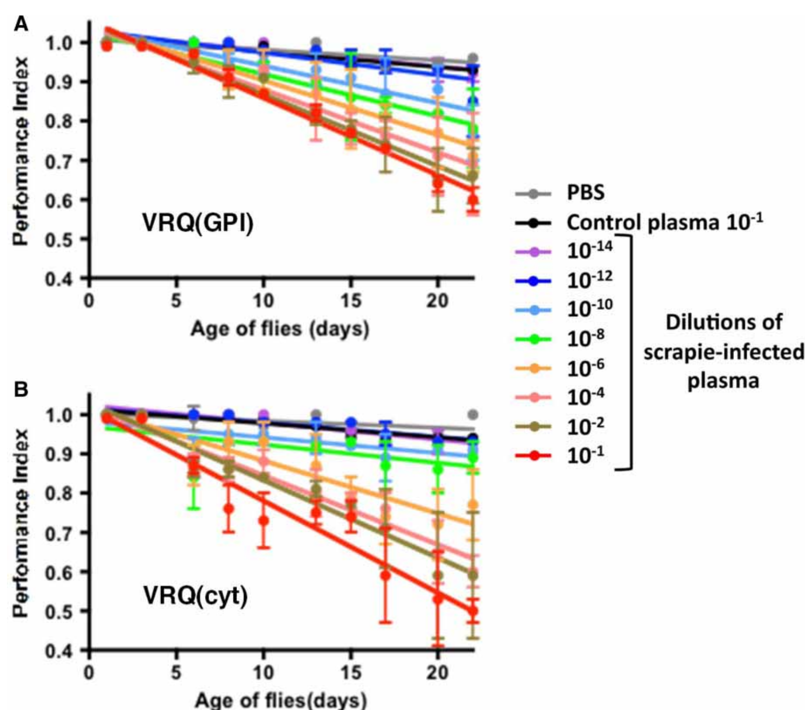

Figure 4. *Drosophila* bioassay of clinical plasma from experimental scrapie-infected sheep.
Adult PrP transgenic *Drosophila* were assessed for their locomotor ability by a negative-geotaxis climbing assay after exposure, at the larval stage, to various dilutions from a 1/10 (v/v) dilution series (as shown) of plasma from a clinically diseased PG127 scrapie-infected sheep or a 10^{-1} (v/v) dilution of scrapie-free sheep plasma. The data shown are linear regression plots of the mean PI ± SD for three groups of flies per time point calculated as described in the Materials and Methods section.

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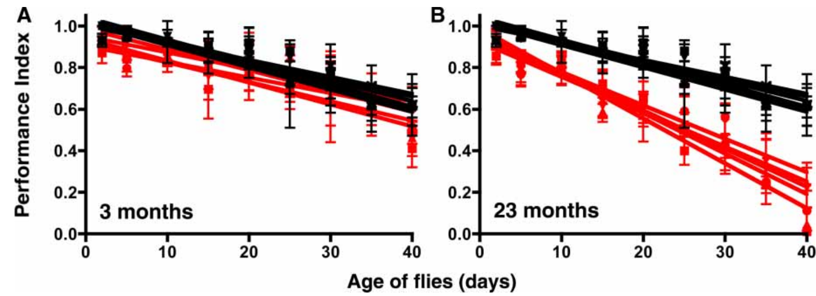

Figure 5. *Drosophila* bioassay of plasma from natural scrapie-infected sheep.
Adult VRQ(cyt) PrP transgenic *Drosophila* (with RFP) were assessed for their locomotor ability by a negative-geotaxis climbing assay after exposure, at the larval stage, to a 10^{-1} (v/v) dilution of plasma isolated from VRQ/VRQ sheep naturally infected with classical scrapie. Plasma samples were isolated from donor sheep at (**A**) 3 months or (**B**) 23 months of age. Red lines: plasma from natural scrapie-infected donor sheep. Black lines: plasma from scrapie-free donor sheep. The data shown are linear regression plots of the mean PI ± SD for three groups of flies per time point calculated as described in the Materials and Methods section.

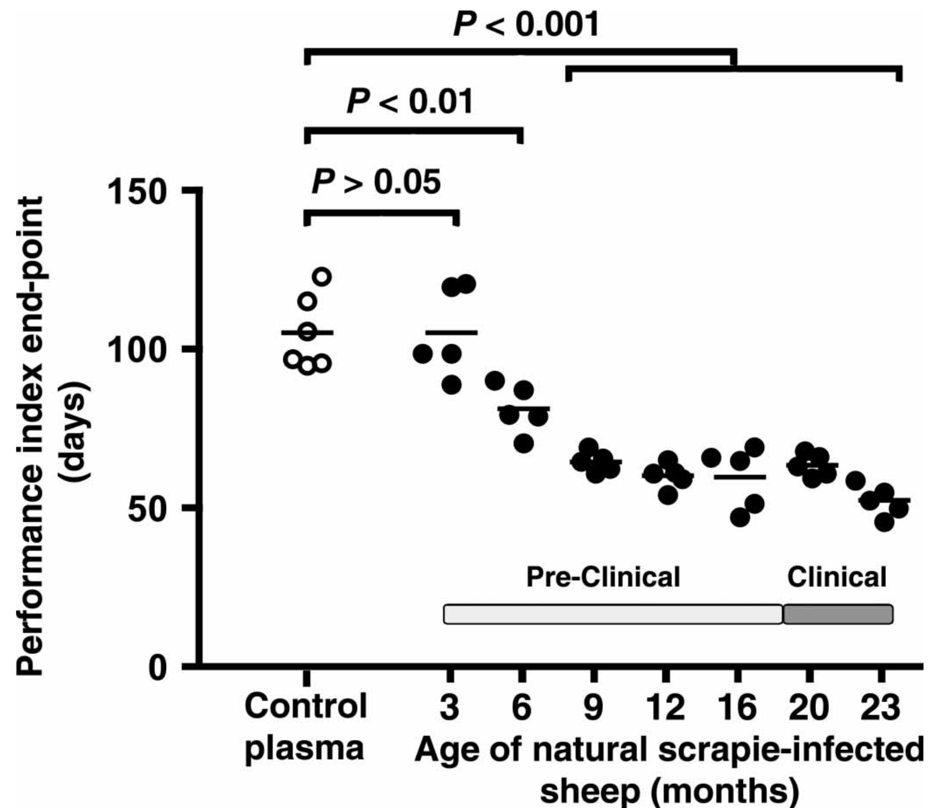

Figure 6. *Drosophila* bioassay of pre-clinical plasma from natural scrapie-infected sheep.
Adult VRQ(cyt) PrP transgenic *Drosophila* (with RFP) were assessed for their locomotor activity by a negative-geotaxis climbing assay after exposure, at the larval stage, to a 10^{-1} (v/v) dilution of plasma isolated from VRQ/VRQ sheep naturally infected with classical scrapie. The mean PI was calculated for three groups of flies per time point as described in the Materials and Methods section, and linear regression lines were plotted. The data shown are extrapolated PI endpoints (x-value when $y = 0$) for individual plasma sample treatment groups. Open circles: control scrapie-free plasma. Closed circles: plasma from natural scrapie-infected sheep. Horizontal bars represent mean PI endpoint for each group. Statistical analysis was performed by one-way ANOVA and *post hoc* Tukey's HSD.

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and scrapie-free sheep. Supplementary Data S3 shows that there was no difference in decline of locomotor activity in the 51D fly line after exposure to prion-infected or prion-free plasma.

We subsequently determined whether the neurotoxicity induced in ovine PrP transgenic *Drosophila* by plasma from PG127 scrapie-affected sheep could be titrated. Accordingly, ovine PrP transgenic *Drosophila* were exposed at the larval stage to a dilution series of plasma isolated from a sheep with PG127-induced clinical scrapie disease. The locomotor ability of plasma-exposed *Drosophila* was assessed, after hatching from the larval stage, by a negative-geotaxis climbing assay and the data were expressed as a PI. Supplementary Data S4 shows that PrP transgenic *Drosophila* demonstrated an accelerated decline in locomotor ability soon after hatching following exposure to PG127-infected sheep plasma at the larval stage compared with the response seen after exposure to scrapie-free sheep plasma. The rate of decline in locomotor ability diminished with exposure to increasing dilution of scrapie-infected plasma, indicative of titration of a particulate transmissible moiety, a characteristic feature of the infectious scrapie agent [36]. The accelerated decline in locomotor ability became apparent <10 days after hatching and was most evident during the first half of the time course when the fly climbing ability was assessed, in a similar manner to that seen in response to PG127 scrapie-infected sheep brain material. Accordingly, the data given in Figure 4 show linear regression analysis of the PI from Supplementary Data S4 for each fly line from day 1 to day 22 after hatching. These data demonstrate that VRQ (GPI) (Figure 4A) and VRQ(cyt) (Figure 4B) PrP transgenic *Drosophila* both showed a similar sensitivity to the dilution series of scrapie-infected sheep plasma, although the VRQ(cyt) fly line showed a somewhat greater magnitude of response compared with VRQ(GPI) flies. Supplementary Data S5 shows *P*-values for the statistical analysis of the PI for each fly line in response to dilutions of scrapie-infected sheep plasma compared with the response induced by the control plasma. These data show that the limit of detection in both VRQ(GPI) and VRQ(cyt) PrP transgenic *Drosophila* was a dilution of $\leq 10^{-6}$ of plasma from sheep with PG127-induced experimental clinical scrapie. The locomotor response seen for both fly lines after exposure to scrapie-free sheep plasma was not significantly different from that seen after exposure to PBS ($P > 0.05$).

We inoculated VRQ(GPI) PrP transgenic *Drosophila* at the larval stage with plasma from sheep with clinical experimental scrapie and probed head homogenate from these flies for PK-resistant PrPSc and prion infectivity. While head homogenate from VRQ(GPI) PrP transgenic *Drosophila* exposed to clinical scrapie plasma was a suitable substrate to allow the detection of PK-resistant PrPSc by PMCA, although not routinely, it did not induce clinical prion disease after intracerebral inoculation into tg338 mice (data not shown).

Detection of pre-clinical natural scrapie blood plasma by PrP transgenic *Drosophila*

We next investigated whether PrP transgenic *Drosophila* could detect plasma from sheep with natural scrapie. The donor animals were VRQ/VRQ sheep born and maintained in a flock endemic for natural classical scrapie [32]. Blood samples were collected from sheep shortly after birth and then at regular intervals until the animals showed clinical signs of terminal scrapie disease when they were killed. All of the killed blood donor sheep were shown to be positive for scrapie disease by routine testing for disease-associated PrP (data not shown), and the mean survival time of the animals was 695 ± 25 days. PrP transgenic *Drosophil*a, at the larval stage, were exposed to plasma from sheep with natural scrapie and the locomotor ability of adult flies was assessed by a negative-geotaxis climbing assay with the data expressed as a PI.

The data given in Figure 5 show the locomotor ability of adult VRQ(cyt) PrP transgenic *Drosophila* following exposure at the larval stage to plasma prepared from sheep with natural scrapie. Figure 5A shows that adult VRQ(cyt) PrP transgenic *Drosophila* showed no accelerated decline in locomotor activity after exposure at the larval stage to plasma prepared from natural scrapie-affected sheep aged 3 months compared with that seen following exposure to control plasma. In contrast, Figure 5B shows that an accelerated decline in locomotor activity was evident after exposure to plasma isolated from the same sheep at the terminal stage (23 months) of natural scrapie disease compared with the response seen to scrapie-free plasma. These observations show that plasma from sheep with natural scrapie can induce toxicity in PrP transgenic *Drosophila*. The locomotor ability of adult VRQ(cyt) PrP transgenic *Drosophila* was also assessed after exposure at the larval stage to plasma prepared at regular intervals during the time course of natural scrapie in sheep. The data given in Figure 6 show that plasma from sheep with natural scrapie aged ≥6 months induced a statistically accelerated decline in locomotor ability compared with that seen with control scrapie-free plasma. The decline in locomotor activity of VRQ(cyt) PrP transgenic *Drosophila* became more pronounced when exposed to plasma samples isolated

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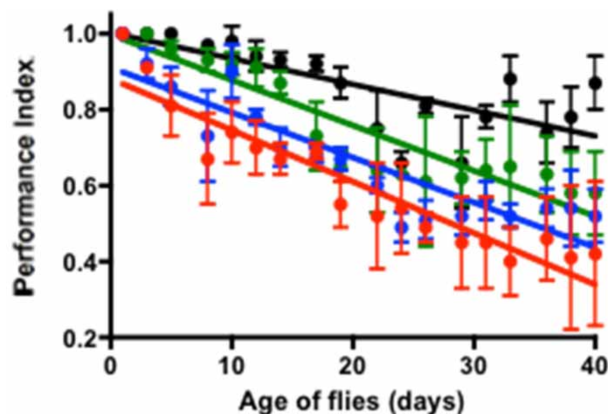

Figure 7. Transmissibility of neurotoxic fly phenotype induced by plasma from pre-clinical scrapie sheep.
Adult VRQ(cyt) PrP transgenic flies were assessed for their locomotor activity by a negative-geotaxis climbing assay following exposure at the larval stage to head homogenate from 30-day-old flies of the same genotype previously exposed to a 10^{-1} (v/v) dilution of plasma from sheep with natural scrapie at 6 months (green line); 12 months (blue line) or 23 months of age (red line). Control flies were adult VRQ(cyt) *Drosophila* exposed at the larval stage to a 10^{-1} (v/v) dilution of scrapie-free plasma (black line). The data shown are linear regression plots of the mean PI ± SD for three groups of flies per time point calculated as described in the Materials and Methods section. Statistical analysis was performed by the unpaired Student's *t*-test for each scrapie-infected treatment group of flies versus the control group of flies ($P \leq 0.025$ in all cases).

during the later stages of the time course for natural scrapie disease in sheep. These observations demonstrated the ability of PrP transgenic *Drosophila* to respond to plasma samples from asymptomatic scrapie-infected individuals since clinical signs of the disease were not evident until these sheep were ≥20 months of age.

We next investigated whether the neurotoxic fly phenotype induced by pre-clinical natural scrapie plasma was transmissible through fly-to-fly transmission. Accordingly, we prepared head homogenate from 30-day-old VRQ(cyt) PrP transgenic *Drosophila* that had been exposed at the larval stage to plasma isolated from sheep with pre-clinical or clinical natural scrapie. These head homogenates were subsequently used to inoculate fresh batches of recipient VRQ(cyt) PrP transgenic *Drosophila* larvae. After hatching, the locomotor ability of fly head homogenate-exposed *Drosophila* was assessed by a negative-geotaxis climbing assay. The PI data in Figure 7 show that head homogenate from natural scrapie plasma-exposed VRQ(cyt) PrP transgenic *Drosophila* induced a significantly accelerated decline in locomotor ability in recipient flies compared with similar flies exposed to control scrapie-free plasma. The magnitude of the decline in locomotor ability exhibited by recipient flies increased upon transmission of head homogenate prepared from flies exposed to plasma isolated increasingly later in the time course of natural scrapie in sheep. Collectively, these data are consistent with the accumulation of a transmissible moiety in the blood of sheep with natural scrapie, which can be detected at an early pre-clinical time point by bioassay in ovine PrP transgenic *Drosophila*.

Discussion

In our studies reported here, we have tested the ability of PrP transgenic *Drosophila* to act as a tractable bioassay for the detection of infectious prions in the blood from individuals with prion disease. We have shown that adult ovine PrP transgenic *Drosophila* exposed, at the larval stage, to scrapie-infected sheep plasma exhibited an accelerated decline in locomotor ability compared with the response seen after exposure to scrapie-free plasma. The neurotoxic phenotype in PrP transgenic *Drosophila* declined upon exposure to increasing dilutions of scrapie-infected plasma, indicative of titration of a particulate transmissible moiety, a characteristic feature of the infectious scrapie agent [36]. In addition, scrapie-infected sheep plasma does not induce toxicity in control non-PrP transgenic flies. These observations are compatible with a view that PrP transgenic *Drosophila* are sensitive to the neurotoxicity induced by a PrP-dependent transmissible moiety present in the blood of scrapie-affected sheep.

In mammalian hosts, prion-mediated neurotoxicity is coupled to prion replication, evidenced by the generation of prion infectivity, which may be accompanied by the accumulation of PK-resistant PrPSc, and these

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events only occur in hosts that express PrP [1,6,37–39]. Accordingly, it was important to determine whether any of these cardinal signs of mammalian prion infection were evident in PrP transgenic *Drosophila* exposed to prion-infected plasma to validate the specificity of our fly-based prion bioassay. For this purpose, we compared the response by PrP transgenic *Drosophila* to scrapie-infected plasma with that by these flies to a known source of infectious ovine prions, namely scrapie-infected sheep brain material. We have previously demonstrated that PK-resistant PrPSc was detected following PMCA using head homogenate from PrP transgenic *Drosophila* exposed to scrapie-infected sheep brain tissue [26] and as reported here, that the same fly head homogenate contained prion infectivity by successful transmission studies in tg338 ovine PrP transgenic mice. In comparison, our studies reported here have found that head homogenate from PrP transgenic *Drosophila* exposed to scrapie-infected plasma did not routinely show evidence of PK-resistant PrPSc by PMCA and did not induce clinical prion disease when inoculated into tg338 mice. However, ovine PrP transgenic *Drosophila* exposed to plasma from scrapie-infected sheep did show a transmissible neurotoxic phenotype, since head homogenate from these flies caused neurotoxicity when inoculated into recipient PrP transgenic flies during fly-to-fly transmission studies. We speculate that these observations indicate that scrapie-infected sheep brain tissue and blood plasma contain a common form of a PrP-dependent transmissible moiety, one that is responsible for induction of the neurotoxic locomotor defect in PrP transgenic *Drosophila*. We further speculate that scrapie-infected sheep brain tissue contains an additional form of PrP-dependent transmissible moiety, one capable of inducing bona fide prion-mediated effects, detected by transmission in a mammalian host, and that is absent or present at a sufficiently lower level in scrapie-infected sheep blood plasma to be undetectable by our fly bioassay. In support of these suggestions are the hypotheses that transmissible prions comprise an ensemble of PrPSc conformers and that the neurotoxic prion moiety may be distinct from PK-resistant PrPSc [2,37]. Therefore, despite the absence of a molecular definition of the neurotoxic agent present in plasma from blood of scrapie-infected sheep, this moiety would appear to bear the hallmarks of a transmissible prion, which can be efficiently detected by bioassay in PrP transgenic *Drosophila*.

Blood plasma from prion-infected hosts is notorious for its poor transmission in conventional prion infectivity bioassays using sentient experimental animals [19,21] and does not act as a seed for the initiation of *in vitro* PMCA [40]. Furthermore, plasma from scrapie-affected sheep was less efficient than either whole blood or white blood cells in transmission of the disease when recipient scrapie-free sheep were inoculated with these samples by the intravenous route [19]. This was evident from the extended scrapie disease incubation periods of sheep that received prion-infected plasma, which were considerably longer than those that received whole blood. Our titration studies carried out here in *Drosophila* show that the fly prion bioassay is extremely sensitive to a PrP-dependent transmissible moiety present in scrapie-infected sheep plasma. The observed diversity in the neurotoxic potential of scrapie-infected sheep plasma identified by our *Drosophila*-based bioassay and that seen during transfusion studies in the natural host may arise because of differences in the experimental systems used to analyse the samples on each occasion. Differences in experimental systems and protocols used to assess prion infectivity are evident in the analysis of blood products by animal bioassay [41]. For example, transfusion of whole blood from scrapie-affected sheep into scrapie-free recipients transmits disease more efficiently than does scrapie-infected sheep brain homogenate, although the latter has a significantly higher prion infectivity titre when measured by intracerebral inoculation in tg338 mice [19]. Our studies here support the view that the level of prion infectivity in blood from prion-diseased individuals may be underestimated when assessed by intracerebral inoculation of rodents in comparison with bioassay of similar material in other experimental systems. This is particularly pertinent to our assessment here of prion-infected plasma that could be diluted by several orders of magnitude and still trigger a phenotypic response in the PrP transgenic *Drosophila*, but appears to contain a low level of infectivity when assayed in other systems [10,15–21]. One possibility for the efficient detection of scrapie-infected sheep plasma by PrP transgenic *Drosophila* is that this invertebrate host does not normally express PrP and may therefore not have evolved suitable defence mechanisms that efficiently remove or sequester misfolded neurotoxic forms of this protein. The PrP transgenic *Drosophila* used here expressed either GPI-anchored or cytosolic ovine prion protein. While the response by VRQ(cyt) PrP transgenic *Drosophila* was usually of greater magnitude than that shown by VRQ(GPI) PrP flies when assessed phenotypically by the climbing assay, only the latter fly line showed evidence of PK-resistant PrPSc upon exposure to scrapie-infected brain material [26]. These variations in response to ovine prions may reflect differences in the topography and expression level of the PrP transgene in the different fly lines. Whichever the case, the fact that VRQ(cyt) transgenic *Drosophila* develop a neurotoxic phenotype in response to scrapie-infected

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sheep plasma and brain material indicates that the neurotoxic moiety present in these samples can interact with cytosolic PrP in the absence of a cell surface form of this protein.

A significant finding in our study reported here was that PrP transgenic *Drosophila* developed a neurotoxic phenotype in response to blood plasma isolated during the pre-clinical phase of experimental and natural scrapie disease in sheep. This response was enhanced when plasma obtained during the clinical phase of scrapie in sheep was bioassayed in the fly, which is consistent with the progressive accumulation of a neurotoxic moiety in the blood of the donor animals during the time course of the disease. Transfusion experiments in sheep have shown that whole blood isolated from asymptomatic ovine donors at ≥3 months of age can be used to detect scrapie-infected animals [19]. Our studies here demonstrated that prion disease could be detected in sheep ≥6 months of age when plasma from asymptomatic naturally infected donor sheep was bioassayed in PrP transgenic *Drosophila*. This was an early time point in the pre-clinical phase of natural scrapie in VRQ/VRQ sheep since these animals develop clinical signs at ∼20 months and terminal disease at ∼23 months of age. In this context, the PrP transgenic *Drosophila* model we describe here could be considered to be of comparable, if not greater, sensitivity than transfusion studies in the natural host since plasma from scrapie-affected sheep is reported to contain less prion infectivity than whole blood [19]. In addition, the length of time required to bioassay plasma in PrP transgenic *Drosophila* was considerably less than that required by transfusion studies in the natural host [23].

At the present time, vCJD imposes a significant burden on the human blood supply because of the risk of human prion infection through iatrogenic use of blood products [12–14]. However, the occurrence of vCJD cases caused by administration of prion-infected human blood has highlighted the realistic opportunity for the development of a blood-based diagnostic test for this condition. Recent developments have shown promise with the detection of vCJD-positive blood samples from clinical vCJD cases by immuno-biochemical selection of disease-associated PrP, without the use of PK [42,43], and by detection of PK-resistant PrPSc following PMCA [44]. Since transmissibility is a defining hallmark of prion diseases, it will be important to develop a reasonably rapid and versatile confirmatory prion infectivity bioassay to supplement these biochemical-based prion diagnostic assays. The relatively facile nature of our novel PrP transgenic *Drosophila*-based bioassay lends itself to a detailed analysis of blood fractions, including plasma, from prion-affected individuals in pursuit of a confirmatory blood test for prion infectivity. This ideal is enhanced by the relative ease of transgenesis in *Drosophila* that will allow the generation of flies that express different species forms of PrP in order to test blood samples from, for example, humans with vCJD or cattle with BSE. Such an approach would help determine the general applicability of this novel invertebrate prion bioassay. The apparent sensitivity of our novel system also suggests that PrP transgenic *Drosophila* are a suitable host to assess the reduction in prion infectivity mediated by proprietary devices aimed at causing its removal from blood products. Collectively, the data presented here highlight a role for the development of PrP transgenic *Drosophila* in the assessment of prion infectivity in blood from a prion-diseased mammalian host.

Abbreviations

ANOVA, analysis of variance; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; GPI, glycosylphosphatidylinositol; HSD, honestly significant difference; PI, performance index; PK, Proteinase K; PMCA, protein misfolding cyclic amplification; PrP, prion protein; PrPC, normal cellular PrP; PrPSc, abnormal disease-specific conformation of PrP; RFP, red fluorescent protein; UAS, upstream activating sequence; vCJD, variant Creutzfeldt–Jakob disease.

Author Contribution

A.M.T., O.A. and R.B. designed the experiments. A.M.T. and O.A. performed the experiments. A.M.T., O.A. and R.B. analysed the experimental data. A.M.T. and R.B. wrote the manuscript.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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