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Prion-induced toxicity in PrP transgenic *Drosophila*

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ABSTRACT

Prion diseases are fatal transmissible neurodegenerative diseases of humans and various vertebrate species. In their natural hosts these conditions are characterised by prolonged incubation times prior to the onset of clinical signs of terminal disease. Accordingly, tractable models of mammalian prion disease are required in order to better understand the mechanisms of prion replication and prion-induced neurotoxicity. Transmission of prion diseases can occur across a species barrier and this is facilitated in recipients transgenic for the same PrP gene as the individual from which the infectious prions are derived. Here we have tested the hypothesis that exogenous ovine prions can induce neurotoxicity in *Drosophila melanogaster* transgenic for ovine PrP. *Drosophila* that expressed ovine PrP pan neuronally and inoculated with ovine prions at the larval stage by oral exposure to scrapie-infected sheep brain homogenate showed markedly accelerated locomotor and survival defects. ARQ PrP transgenic *Drosophila* exposed to scrapie-infected brain homogenate showed a significant and progressive reduction in locomotor activity compared to similar flies exposed to normal sheep brain homogenate. The prion-induced locomotor defect was accompanied by the accumulation of potentially misfolded PrP in the brains of prion-inoculated flies. VRQ PrP transgenic *Drosophila*, which expressed less ovine PrP than ARQ flies, showed a reduced median survival compared to similar flies exposed to normal sheep brain homogenate. These prion-induced phenotypic effects were PrP-mediated since ovine prions were not toxic in non-PrP transgenic control flies. Our observations provide the basis of an invertebrate model of transmissible mammalian prion disease.

Introduction

Prion diseases, or transmissible spongiform encephalopathies, are chronic neurodegenerative central nervous system (CNS) disorders of mammals (Collinge, 2001). This group of invariably fatal conditions includes scrapie of sheep, bovine spongiform encephalopathy (BSE) of cattle, chronic wasting disease (CWD) of cervids and Creutzfeldt-Jakob disease (CJD) of humans. These diseases are characterised by the accumulation of PrPSc, an abnormal isomer of the host protein PrPC, in the brains of affected individuals (Aguzzi et al., 2008). During conversion of PrPC to PrPSc, a major refolding event occurs that results in a more extensive beta-sheet conformation. This conformational change would appear to be fundamental to prion propagation and substantial evidence suggests the infectious prion agent comprises principally, if not solely, of proteinaceous material in the form of an abnormal isomer of PrP (Castilla et al., 2005; Deleault et al., 2007; Legname et al., 2004, 2005; Prusiner, 1982; Saa et al., 2006; Wang et al., 2010; Weber et al., 2007). Different prion inocula, or strains, may be characterised by several criteria, including their biological properties, histopathology, and variations in the pattern of PrPSc deposition and the length of the disease incubation period following experimental inoculation (Collinge and Clarke, 2007).

Transmission of prion diseases can occur between individuals of the same species and between those of different species. The most efficient route for CNS prion propagation is by direct intracerebral inoculation. However, it is considered that the natural transmission of prion diseases occurs most commonly by peripheral inoculation (Glatzel and Aguzzi, 2000a). Accordingly, much attention has been focused on the oral route of inoculation, as this is believed to be the portal of entry of prions in BSE and vCJD (Collinge, 1999). Following oral inoculation of experimental mice with murine-adapted ovine scrapie, PrPSc and prion infectivity associate with cells of the lymphoreticular system, and accumulate on follicular dendritic cells (FDCs) within germinal centres of peripheral lymphoid tissue (Brown et al., 1999; Montrasio et al., 2000). Subsequent neuroinvasion of the CNS by prions is believed to occur via the sympathetic peripheral nervous system whose nerve terminals are in close proximity to germinal centres (Bencsik et al., 2001; Glatzel and Aguzzi, 2000b; Jeffrey et al., 2001). Since the molecular nature of the infectious prion agent is undefined, the only reliable method to measure prion infectivity in CNS and peripheral tissue sites of prion-

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infected individuals has been by bioassay in an appropriate indicator species, typically rodents. Mouse prion bioassays are cumbersome, time-consuming and are expensive in terms of the number of animals used.

Primary passage of ovine scrapie isolates in wild type mice results in relatively long incubation times prior to the onset of terminal prion disease that may be associated with low attack rates (Bruce, 1993; Dickinson, 1976). These protracted and sometimes in-efficient transmission properties are considered to arise as ovine prions cross the species barrier and propagate in the murine host (Prusiner, 2004). Facilitated transmission of ovine scrapie prions at primary passage in mice can be achieved in recipients transgenic for ovine PrP (Crozet et al., 2001; Thackray et al., 2008; Vilotte et al., 2001). Serial transmission studies of ovine prions into wild type and ovine PrP transgenic mice have provided valuable insights into how a single polypeptide chain may encipher multiple prion strains. However, despite a considerable amount of effort, the relationship between prion propagation in the CNS and the generation of the neurotoxic conformer of PrP remains undefined. An uncoupling of prion infectivity and toxicity is suggested by studies that demonstrate prion replication in the mammalian brain proceeds in two phases: the first, a clinically silent autocatalytic production of infectivity; the second, production of the neurotoxic conformer initiated when the prion infectivity titre reaches a maximal saturating level (Sandberg et al., 2011). Dissection of the biochemical pathways associated with ovine prion formation, clearance and neurotoxicity will be aided by the application of new tractable animal models of this condition. Furthermore, use of a genetically well-defined host will allow identification of genetic modifiers of ovine prion disease pathogenesis, providing the potential for development of new diagnostic markers and therapeutic targets for prion diseases in general.

The fruitfly *Drosophila melanogaster* has been used to model a number of human neurodegenerative diseases (Bilen and Bonini, 2005; Lu and Vogel, 2009). A major strength of *Drosophila* model systems is the ability to rapidly generate transgenic flies that express heterologous proteins in a tissue-specific manner (Ashburner et al., 2004; Greenspan, 2004). In addition, genetic pathways are extensively conserved between mammalian and *Drosophila* genomes (Hirth and Reichert, 1999; Moloney et al., 2010), which allows fly models of neurodegenerative disease to identify candidate genetic modifiers of the condition in the host species. Accordingly, we have investigated *Drosophila* as a new animal model of ovine scrapie in order to provide a more tractable model of transmissible mammalian prion disease. In pursuit of this objective we have already generated *Drosophila* transgenic for polymorphic variants of ovine PrP under expression control by the bi-partite *GAL4*/*UAS* system and have shown that ovine PrP is efficiently expressed pan neuronally in the fly (Thackray et al., 2011b). Here we have extended our studies to test the hypothesis that exogenous ovine prions can induce toxicity in *Drosophila* transgenic for ovine PrP. This is an important question to address since the identification of a prion-induced phenotype is required for the successful development of a *Drosophila* model of transmissible prion disease. Our studies reported here show for the first time that adult *Drosophila*, which express ovine prion protein pan neuronally and following oral exposure to ovine prions at the larval stage, show markedly accelerated locomotor and survival defects. The prion-induced locomotor defect was accompanied by the accumulation of potentially misfolded PrP in the brains of prion-inoculated flies. Our observations provide the basis for the development of a novel, rapid invertebrate model of transmissible mammalian prion disease.

Materials and methods

Fly stocks

The *UAS-PrP* fly lines w; M{ARQ-PrP, 3xP3-RFP.attP}ZH-51D and w; M{VRQ-PrP, 3xP3-RFP.attP}ZH-51D, that are transgenic for ovine $A^{136}R^{154}Q^{171}$ (ARQ) or $V^{136}R^{154}Q^{171}$ (VRQ) PrP, respectively, were generated by PhiC31 site-specific transformation as previously described (Thackray et al., 2011b). *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 (Lewis, 1960) at 25 °C, maintained at low to medium density. Flies were used in the assays described below or harvested at various time points and either frozen at −80 °C until required, or fixed in 10% formal saline.

Prion inoculation of Drosophila

Drosophila at the larval stage of development were exposed to brain homogenates of cerebral cortex tissue from confirmed scrapie-positive or known scrapie-negative sheep. The ovine scrapie isolates used were from scrapie-positive sheep that showed typical vacuolar pathology in the medulla oblongata of the brain stem and that were positive for disease-associated PrP as judged by immunohistochemistry or western blot. The scrapie-infected inocula were prepared from terminal scrapie-affected sheep identified by routine statutory surveillance (ARQ/ARQ isolate SE1848/0008 and VRQ/VRQ isolate SE1848/0005) (Thackray et al., 2008) or an experimentally inoculated VRQ/VRQ sheep (K1) killed at terminal scrapie disease (Thackray et al., 2005). New Zealand-derived ARQ/ARQ and VRQ/VRQ scrapie-free brain tissues were used as control material. Two hundred and fifty microlitres of a 1% brain homogenate prepared in PBS pH 7.4 was added to the top of the cornmeal that contained third instar *Drosophila* larvae in 3 inch plastic vials. Flies were transferred to fresh, non-treated vials following eclosion.

Preparation of fly head homogenates

Decapitation was achieved by freezing tubes of whole flies in liquid nitrogen for 10 min followed by 2 min of vortexing. Homogenates were prepared by manual grinding of fly heads in Eppendorf tubes with sterilised plastic pestles. Homogenates for immunodetection 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) benzenesulfonyl fluoride] followed by 10 min sonication on ice. Homogenates for conformational-dependent immunoassay (CDI) were prepared by processing 20 fly heads in 20 µl 8 M GdnHCl followed by a 1:10 dilution in assay buffer (Thackray et al., 2007).

Conformational-dependent immunoassay (CDI)

Triplicate 20 µl aliquots of fly head homogenate were diluted to 200 µl with assay buffer (Thackray et al., 2011a). PrP was quantified by CDI as described previously (Thackray et al., 2007) except that the capture reagent was anti-PrP monoclonal antibody 245 (Thackray et al., 2003) and the detector antibody was biotinylated SAF32 (Feraudet et al., 2005). The equivalent of 20 fly heads was assayed per well in triplicate. PrP expressed in fly heads was quantified against standard curves prepared using the relevant genotypes of ovine recombinant prion protein.

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. (2010). 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 min at 37 °C, 5 U of Benzonase was added and the sample was shaken at 225 rpm for a further 10 min at 37 °C. Sodium