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The acute inflammatory response in CNS following injection of prion brain homogenate or normal brain homogenate

S. Betmouni and V. H. Perry

CNS Inflammation Group, Department of Pharmacology, Oxford, UK

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The neuropathological hallmarks of end-stage prion disease are vacuolation, neuronal loss, astrocytosis and deposition of PrP^{Sc} amyloid. We have also shown that there is an inflammatory response in the brains of scrapie-affected mice from 8 weeks post-injection. In this study we have investigated the acute CNS response to the intracerebral injection of scrapie-affected brain homogenate. The ME7 strain of scrapie (Neuropathogenesis Unit, Edinburgh) was used, and control mice were injected with brain homogenate derived from normal C57BL/6 J mice. One microlitre of 10% w/v ME7 ($n=33$) and normal brain homogenate ($n=28$) was injected stereotaxically into the right dorsal hippocampus. Cryostat sections of brains taken at 1, 2, 5, 7, 14 and 28 days post-injection were examined histologically for neuronal loss, and immunocytochemically to study the inflammatory response. This study shows that ME7 is not acutely neurotoxic *in vivo*. There is also no difference (ANOVA) in the inflammatory response, which peaked between 2 and 5 days and resolved by 4 weeks after intracerebral injection of either ME7 or normal brain homogenate. The well circumscribed inflammatory response seen previously at 8 weeks is therefore a consequence of a disease process rather than a surgical artefact. This disease process may be related to a localized accumulation of PrP^{Sc} sufficient to stimulate an inflammatory response which in turn may contribute to neuronal loss. The role of the inflammatory response in chronic neurodegeneration can be usefully studied using this mouse model of prion disease, and this will undoubtedly shed light on the pathogenic mechanisms underlying other chronic neurodegenerative diseases.

Keywords: prion, mouse, inflammation, microglia, T-cells, CNS

Introduction

The prion diseases (or transmissible spongiform encephalopathies) represent a relatively rare group of chronic neurodegenerative diseases which can afflict both animals (scrapie in sheep, Bovine Spongiform Encephalopathy in cattle) and humans (Creutzfeldt-Jacob Disease, Gerstmann-Straussler–Scheinker Syndrome and Kuru). They can have a genetic, sporadic or infectious aetiology and the causative agent is currently believed to be a conformationally altered form (PrP^{Sc}) of a normal host protein (PrP^{C}) – the prion protein [28]. Typically the clinical course of these diseases is silent and prolonged, and the appearance of clinical signs usually heralds a rapid deterioration followed by death within months of the clinical onset of the disease. Although there are clinical indicators which would suggest a diagnosis of prion disease, at present definitive diagnosis can only be made at post-mortem.

The neuropathology of animal and human diseases is similar and includes spongiform changes (vacuolation), neuronal loss, astrocytosis and PrP^{Sc} amyloid deposition.

Correspondence: Dr S. Betmouni, CNS Inflammation Group, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, UK.

The deposition of PrP^{Sc} amyloid is a characteristic, albeit not an unequivocal feature [11] of the prion diseases. Although there is evidence to suggest that PrP^{Sc} is neurotoxic *in vitro* [9,20,24] there is only circumstantial evidence that PrP^{Sc} is neurotoxic *in vivo.* The latter derives mainly from the colocalization of PrP^{Sc} amyloid deposition and areas of vacuolation and neuronal loss. In the past these neuropathological changes have been said to occur in the absence of an inflammatory or immune response, but recent studies have shown otherwise.

In animal models, advanced stages of the disease are associated with a conspicuous atypical inflammatory response as seen by the presence of activated microglia in regions of the brain which also show vacuolation and PrP^{Sc} amyloid deposition [36]. In the human disease PrP^{Sc} amyloid plaque deposition has also been shown to be associated with the presence of activated microglia [3,16]. Although an inflammatory response is classically said to involve the recruitment and accumulation of leucocytes at the site of injury, it is established that the inflammatory response in the brain is unique being dominated by the recruitment of mononuclear cells and by the presence of activated microglia at the site of injury [25,26]. There is evidence from XX-XY chimeras that recruitment of blood monocytes to the brain does occur in mouse scrapie [37]. We previously reported that there is an inflammatory response in the brains of scrapie-affected mice from as early as 8 weeks post-injection, shown by the presence of activated microglia and also by the recruitment of CD8+T-lymphocytes to sites of pathology [5]. As we used a focal microinjection of scrapie-affected brain homogenate into the dorsal hippocampus it was possible to follow the development of pathology by the sequential appearance of activated microglia and CD8+T-lymphocytes at hippocampal projection targets including the contralateral hippocampus, the lateral septum and the vertical diagonal band of Broca. This occurrence of pathology in neuroanatomically connected regions of the brain is in keeping with the defined mode of transport of PrP^{Sc} along axons [32], and is similar to the proposed spread of pathology in Alzheimer's Disease [7,23].

As there have been no studies of the acute CNS response to scrapie-affected brain homogenate it has not been possible to determine whether the inflammatory response we observed at 8 weeks was initiated at the time of intracerebral injection, or whether it was related to an event(s) occurring at a later stage of the disease. In this study we have examined the acute CNS response following the focal microinjection of either normal brain homogenate (NBH) or the ME7 strain of scrapie.

Materials and methods

Surgery and perfusion

Male and female C57BL/6 J mice aged 2–3 months were used throughout. The mice were anaesthetized with Avertin (2,2,2 tribromoethanol in tertiary amyl alcohol) at a dose of 0.1 mL/5 g body weight. Once fully anaesthetized they were injected stereotaxically into the right dorsal hippocampus with 1 μL (10%, w/v) of either ME7 or normal brain homogenate (NBH) delivered via a pulled glass micropipette (co-ordinates: bregma −2.5, lateral −1.7 and depth −1.6 mm). The ME7 brain homogenate was derived from the brains of C57BL/6 J mice with advanced clinical signs of mouse scrapie, and the NBH was derived from the brains of age-matched normal C57BL/6 J mice. Following surgery the mice were allowed to recover under a warm lamp, and once recovered they were housed in a Scantainer Type D (Scanbur Ltd, Denmark) and observed daily.

Following terminal anaesthesia the mice were perfused with 0.9% heparinized saline via the transcardiac route, and the brains were removed rapidly and embedded in Tissue Tec (OCT Embedding Compound, Miles Inc.). The embedded brains were then frozen in isopentane on liquid nitrogen, and stored at −20 °C (Table 1).

Tissue processing and immunocytochemistry

Coronal brain sections 10 μm thick, were cut on a Brights cryostat and taken up onto 3-Aminopropyl-

Table 1. The number of mice used in an acute study of mouse scrapie

Time post-injection	*10% ME7 homogenate*	*10% normal homogenate*
1 day	7	5
2 days	6	6
5 days	5	6
7 days	7	5
2 weeks	4	3
4 weeks	4	3

Table 2. Primary antibodies

Antibody	*Antigen*
FA11	CD68, a predominately lysosomal antigen in macrophages [29]
KT3	CD3, a pan T-cell antigen [34]
YTS191	CD4, a helper T-cell marker [10]
YTS 169	CD8, a cytotoxic T-cell marker [10]
TIB120	MHC class II antigen [6]
M1/42	MHC class I antigen [33]
SER	Sialoadhesin receptor on macrophages [12]

triethoxysilane (APES) coated slides [22]. The sections were air dried, fixed in absolute alcohol for 10 min at 4 °C and processed for indirect immunocytochemistry. All incubations were carried out at room temperature. The sections were first incubated in 5% normal serum for 30 min to prevent non-specific Fc-receptor binding and this was followed by incubation with the primary antibody for 60–90 min (Table 2).

Negative control sections were incubated in the absence of the primary antibody. The sections were then incubated with the appropriate biotinylated IgG secondary antibody for 45 min. Non-specific peroxidase activity was eliminated by incubating the sections in 1% hydrogen peroxide in methanol for 30 min. The sections were then incubated with Avidin Biotin Complex (Vectastatin Elite ABC, Vector Laboratories, UK) for 45 min. The peroxidase was visualized using 0.05% diaminobenzidine hydrochloride (DAB) and 0.05% hydrogen peroxide as the substrate. The DAB staining was enhanced by reacting the sections for 20 s in 0.01% osmium tetroxide in 0.1 mmol/L phosphate buffer.

Quantification of results and statistical analysis

The area of activated microglia staining as seen with FA11 immunocytochemistry was calculated using a computerized image analysis package (Image Grabber 24.1 and Opti-Lab 242.1). The criterion for 'activated microglia' was enhanced CD68 expression in rounded cells with large nuclei and comparatively less cytoplasm and short, stout processes in distinction from resting microglia [21]. The number of MHC class II positive activated microglia and CD3 positive T-cells was counted throughout the injected hippocampus on coronal sections taken at the level of the injection site. Quantification of the results was made on one 10 μm brain section per mouse.

Group numerical data is presented as mean ± standard error of the mean (SE) for n observations. Statistical analysis was conducted using 'Instat' (Graphpad software, San Diego, USA). ANOVA with Bonferroni post-test was performed on the data and a P-value of <0.05 was taken to be statistically significant.

Results

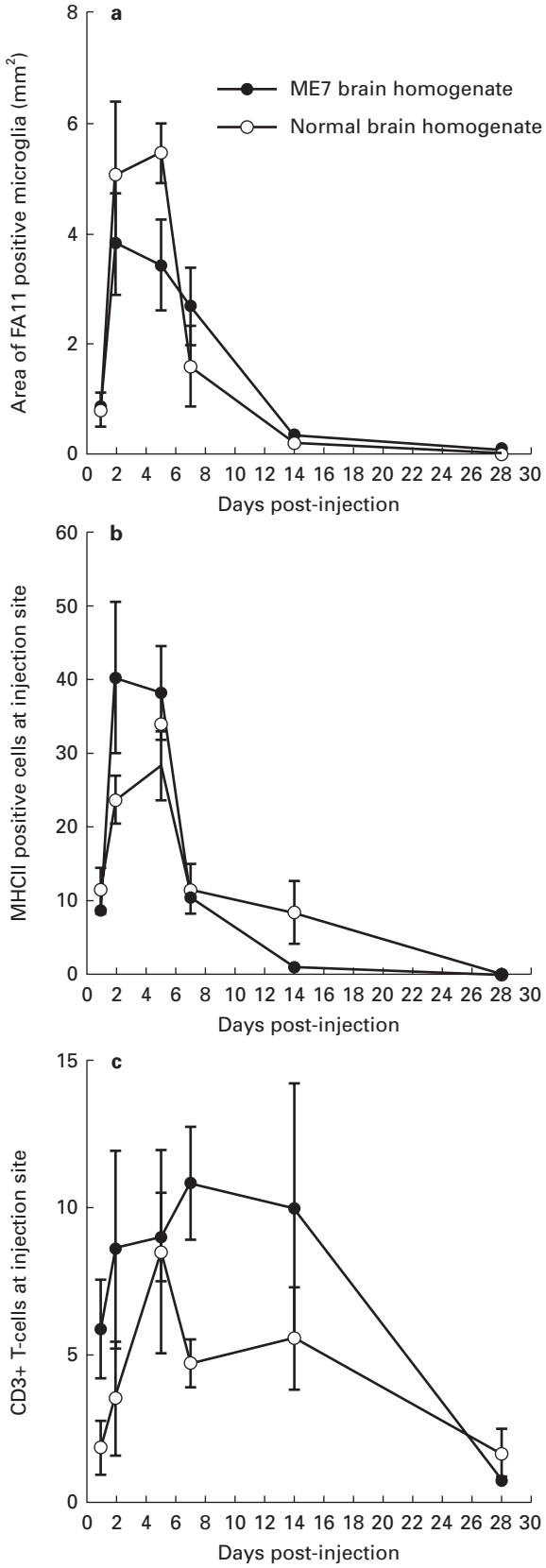
Clinical signs

Mice injected with NBH and ME7 brain homogenate made a complete recovery from the anaesthesia. Subsequently they remained well throughout the course of the experiment and could not be distinguished clinically from normal uninjected mice. The focal micro-injection of 1 μL of a 10% ME7 brain homogenate into the dorsal hippocampus has been reliably shown to produce the typical clinical signs of scrapie in mice by 23 weeks post-injection [5].

Microglial response

The profile of microglia staining, as seen with immuno-cytochemistry for FA11, was similar in brain sections taken from mice injected with ME7 and NBH. At 1 day activated microglia were distributed diffusely over the injected hippocampus covering an area of $0.87\ mm^2 \pm 0.16$ and $0.80\ mm^2 \pm 0.31$ in ME7 and NBH injected mice, respectively (mean ± SE). The area and intensity of this staining reached a peak between 2 and 5 days in both groups, and by 4 weeks only resting microglia could be seen (Figure 1**a**, Figure 2**a**,**b**). Activated microglia were not seen on the contralateral hippocampus.

Immunocytochemistry with the TIB120 antibody showed MHC class II positive staining predominantly on cells with a rounded morphology with comparatively little staining seen on cells with microglial morphology (Figure 2**c**). There was no difference in the pattern of MHC class II staining following intracerebral injection with either ME7 or NBH. The peak number of MHC class II positive cells was seen at 2 and 5 days, and at 5 days there were more cells with microglial morphology. At 1 week there was a reduction in the total number of cells, and by 2 and 4 weeks there were few MHC class



II positive cells in the brain parenchyma at the injection site (Figure 1**b**). Constitutive expression of MHC class II antigen on the macrophages of the choroid plexus was present at all time points in both NBH and ME7 injected mice.

Immunocytochemistry with the M1/42 antibody showed that the profile of MHC class I expression was the same in the brains of mice injected with either ME7 or NBH. There was expression of MHC class I antigen on parenchymal vessels and on the endothelium of the choroid plexus at all of the time points studied. At 1 day post-injection there were a few positively stained rounded cells in the parenchyma of the injected hippocampus (Figure 2**d**). Examination of coronal sections stained with an antibody against CD3, a pan T-cell marker, showed that the MHC class I positive cells were present in a similar distribution and number to T-cells (see below). By 2 days post-injection these positively staining round cells could be seen at the injection site, in close proximity to blood vessels. The number of positively staining parenchymal cells increased by 5 days and were distributed for a greater distance around the injection site both in the dorsal hippocampus and along the injection tract in the overlying cortex. The extent of the distribution of MHC class I antigen positive cells was similar to that observed for the CD68 positive activated microglia. There was a reduction of MHC class I positive cells by 7 days post-injection, at which time positively staining rounded cells were confined to the injection tract itself.

The sialoadhesin receptor is expressed on microglia following injury to the CNS that is associated with the breakdown of the BBB and the entry of plasma proteins, but is not present on resting microglia in the normal brain in areas where the BBB is intact [27]. Sialoadhesin receptor expression (SER-4) can be seen in normal brains on macrophages of the choroid plexus and meninges, and also in the brain parenchyma at sites in the CNS which are permeable to plasma proteins such as the circumventricular organs. Therefore, sialoadhesin receptor expression can act as a surrogate marker of BBB

Figure 1. Graph showing that there is no difference in the microglia and T-cell response in the CNS following the injection of either normal brain homogenate and ME7 brain homogenate. This is shown at each of the time points studied. **a**, The area of parenchyma at the injection site which contains activated microglia, as seen with immunocytochemistry for CD68. **b**, The number of MHC class II positive microglia at the injection site. **c**, The number of KT3 positive T-cells at the injection site.

breakdown. The profile of sialoadhesin expression on microglia followed the same course in mice injected with either ME7 or NBH (Figure 2**e**,**f**). It had reached its peak at the injection site by 5 days, and there was a reduction in microglial staining by 7 days after injection. By 2 and 4 weeks there were few SER-4 positive microglia remaining.

T-cell response

In the ME7-injected mice T-cells were seen in association with the injection site and their distribution was similar to the distribution of CD68 positive microglia in the injected hippocampus. T-cell numbers reached a peak between 2 and 7 days, and declined over the following time points such that by 4 weeks there were very few T-cells remaining at the injection site (Figure 1**c**). A similar T-cell response was seen in mice injected with NBH. In both ME7 and NBH injected mice the majority of CD3 + T-cells belonged to the CD8 + subset of T-lymphocytes.

Other markers of acute inflammation

Neutrophils were identified on the Cresyl violet stain by their typical multilobar nucleus. In both ME7 and NBH injected brains neutrophils were seen in association with the injection tract at 1 and 2 days, but were absent at all of the subsequent time points that were studied.

Neuronal response

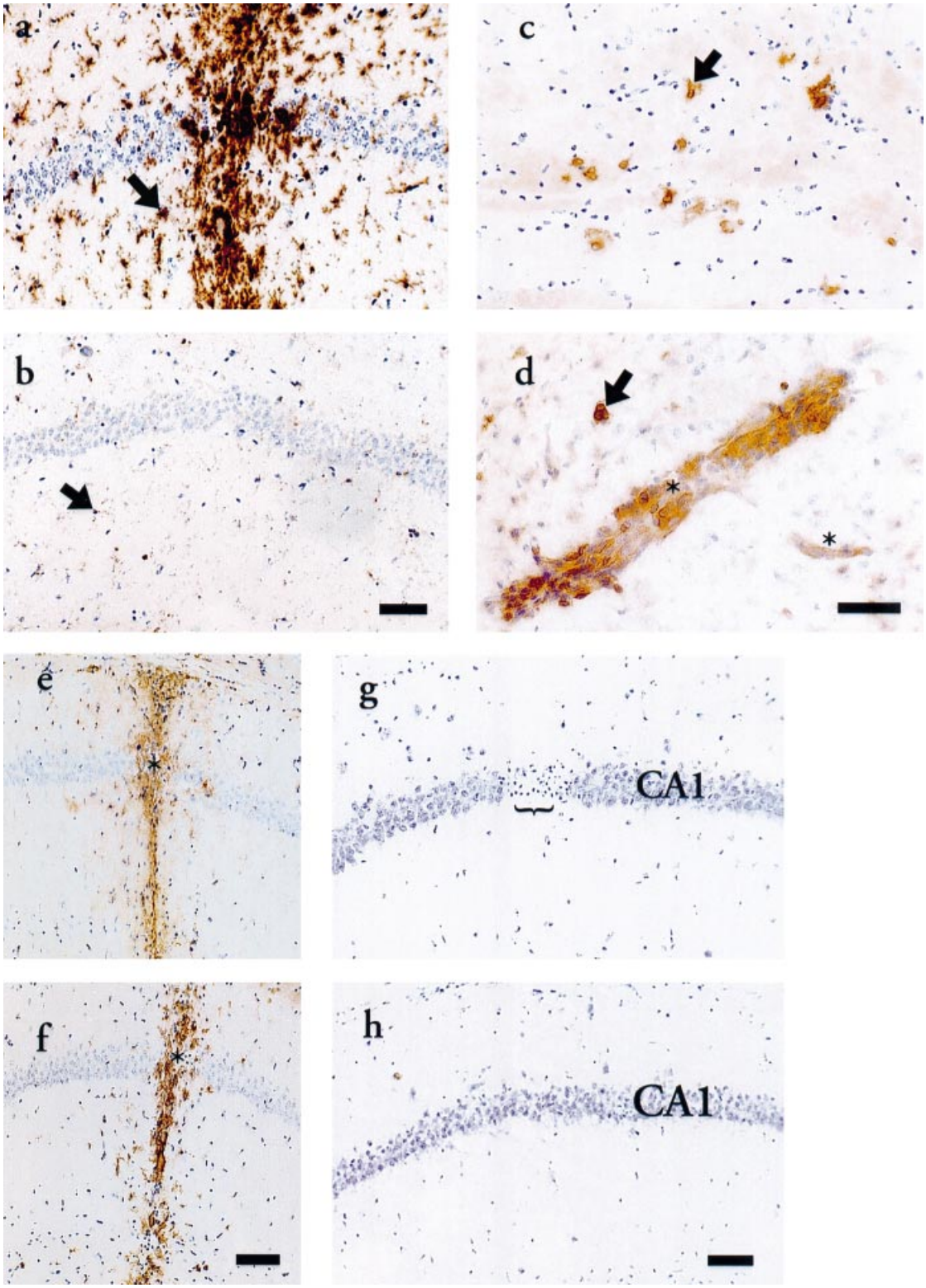
There was a discrete area of neuronal loss at the injection site in the CA1 region of the hippocampus in both the NBH and ME7 injected mice between 1 and 7 days. There were shrunken neurons with condensed clumped chromatin (Figure 2**g**,**h**). There was no observable difference in the degree of neuronal loss between NBH and ME7 injections. On occasion the injection had penetrated deeper into the dorsal hippocampus to involve the dentate gyrus in which case neuronal loss was also seen in this region. By 4 weeks the area of neuronal loss in the pyramidal layer of the hippocampus was no longer apparent presumably due to the shrinkage of the lesion.

Discussion

The acute inflammatory response in the CNS following the intracerebral injection of ME7 brain homogenate does not differ quantitatively or qualitatively from the brain's response to the injection of normal brain homogenate. There were very few neutrophils recruited to the site of injury, and when they were present they were closely related to the injection tract and did not penetrate for any distance into the brain parenchyma. The profile of the inflammatory response as seen by the presence of T-cells and activated microglia followed the same course over the 4-week period of study. There was a peak response at the injection site between 2 and 5 days which gradually declined so that by 4 weeks after intracerebral injection of either ME7 or normal brain homogenate the inflammatory response had completely resolved. The integrity of the blood–brain barrier, as assessed by microglial expression of the sialoadhesin receptor, was restored by 1 week after intracerebral injection of either ME7 or normal brain homogenate. The use of fresh cryostat sections in this study precluded the immunocytochemical localization of PrP^{Sc}, although we are now routinely able to detect PrP^{Sc} in our model on paraffin embedded tissue using the protocol described by Bell *et al.* [4].

The paucity of rapid neutrophil recruitment acutely to the site of injury, the delay and the predominance of a monocyte response are not typical of the classical inflammatory response described following injury to non-CNS tissue. These features of the acute inflammatory

Figure 2. Acute inflammatory response in mouse scrapie. **a,** Macrosialin (CD68) expression on activated microglia at the injection site in the dorsal hippocampus 5 days after the injection of ME7-affected brain homogenate. A typical activated microglia is indicated by arrow. **b,** Constitutive macrosialin (CD68) expression on resting microglia 4 weeks after the injection of ME7-affected brain homogenate. **c,** MHC class II expression on cells with rounded morphology (arrow) at 5 days post-injection with ME7 brain homogenate. **d,** MHC class I expression on blood vessels (*) and on cell with a rounded morphology (arrow) (Scale bar = 50 μm). The expression of sialoadhesin receptor on microglia (*) at the injection site at 5 days after intracerebral injection with **e.** normal brain homogenate, and **f,** ME7 brain homogenate [scale bar = 100 μm]. The acute neuronal response to the intracerebral injection of ME7. **g,** A discrete area of neuronal loss (parenthesis) in the dorsal hippocampus (CA1) 1 day following injection of ME7. This response is indistinguishable from that seen at the same time following intracerebral injection of normal brain homogenate. **h,** By 4 weeks after intracerebral injection of ME7 the area of neuronal loss is no longer apparent presumably due to the shrinkage of the original lesion.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response to brain homogenate are in keeping with the findings of Andersson and colleagues who were the first to draw attention to the unique nature of the acute inflammatory response in the CNS using a model of LPS and also kainate-induced CNS injury [1,2]. The profile of T-cell recruitment in response to ME7 or NBH in our experiment is similar to the response observed after acute spinal cord injury in mice [30]. In this model of acute spinal cord transection there is an acute recruitment of lymphocytes to the site of injury over the first 2–4 days after the lesion, and resolution of this infiltrate by 1 week after spinal cord transection. The recruitment of T-lymphocytes to an area of neuronal damage induced by middle cerebral artery occlusion in the rat has been shown by Shroeter *et al.* [31]. Therefore the acute response to the intracerebral injection of ME7 or normal brain homogenate has many similarities to the response to a number of other models of CNS injury, and as such probably represents the brain's response to the injection of brain homogenate rather than its response to the ME7 scrapie agent *per se.*

Furthermore, the degree of neuronal loss was not appreciably different in mice injected with ME7 compared with normal brain homogenate suggesting that ME7 is not acutely neurotoxic. Neuronal loss was seen at the injection site in both normal brain homogenate and ME7-injected mice up to 7 days post-injection. These findings indicate that the neuronal loss occurred as a consequence of the injury associated with the injection procedure itself, and not as a consequence of the ME7 scrapie agent. The relationship between PrP^{Sc} and neuronal loss remains to be fully defined. There is circumstantial evidence that PrP^{Sc} is neurotoxic *in vivo* because its accumulation in the brains of scrapie-affected mice is spatially related to areas of vacuolation [18] and astrocytosis [13] both of which are neuropathological hallmarks of the disease. In contrast, it has been shown that the typical neuropathology of the prion disease can be seen in the absence of PrP^{Sc} accumulation in atypical forms of human prion disease [11]. *In vitro* studies have shown that chronic exposure of rat hippocampal neurons to a fibrillogenic peptide fragment of PrP^{Sc} (PrP 106–126) causes neuronal loss by apoptosis in a dose dependent manner [14]. Therefore, studies of neurotoxicity in the prion diseases only provide evidence that a fibrillogenic peptide fragment of PrP^{Sc} is neurotoxic *in vitro*, but the case for PrP^{Sc} being directly neurotoxic remains unproven. Exposure of neurons to animal derived PrP^{Sc} has also been shown to be neurotoxic *in vitro* [17]. Our findings indicate that *in vivo* exposure of hippocampal neurons to ME7 is not in itself associated with neuronal loss, and that additional factor(s) could contribute to the neurodegeneration which is a marked feature of end-stage disease.

This study has highlighted an interesting feature of the natural history of the early stages of mouse scrapie in the CNS. We know from this study that the acute inflammatory response associated with the injection of either ME7 or normal brain homogenate resolves by 4 weeks. Our previous work has shown that at 8 weeks post-injection the inflammatory response is localized to the injection site in the dorsal hippocampus of ME7-injected mice only [5]. Furthermore, at 8 weeks post-injection there is no evidence of microglia activation or T-cell recruitment in the projection targets of the dorsal hippocampus at the injection site, including the contralateral CA3 region, the lateral septal nuclei and the vertical diagonal band of Broca. The circumscribed inflammatory response seen in the hippocampus at 8 weeks post-injection may be because the local levels of PrP^{Sc} had accumulated to a threshold level sufficient to stimulate a local inflammatory response. There is evidence to show that a peptide fragment of PrP^{Sc} (PrP106–126) can cause activation of microglia in culture [8,9]. Moreover, the *in vitro* neurotoxicity of PrP106–126 is dependent upon the presence of microglia in the culture system [20]. It has been suggested that PrP106–126 activates microglia to produce reactive oxygen species, and thereby cause neuronal loss via a mechanism of oxidative stress [15]. There is no further loss of pyramidal neurons at the injection site at 8 weeks post-injection, suggesting that neither the inflammatory response nor the accumulation of PrP^{Sc} is sufficient at this stage to cause neuronal loss. However, one cannot exclude the possibility that the neuron is responding either to the PrP^{Sc} or to the inflammatory reaction in a subtle way, and that with sustained exposure to this potentially damaging environment neuronal loss becomes an inevitable end-stage phenomenon [19,35]. Indeed overt neuronal loss, as seen on Nissl staining, is not a feature of the pathology in this model of scrapie until 20–23 weeks post-injection. We have recently shown that there are early behavioural changes in scrapie-affected mice, and that these occur when there is no overt neuronal loss, suggesting that an earlier neuronal dysfunction is a feature of mouse scrapie

(Betmouni *et al.* 1999, in press). The inflammatory response in mouse scrapie may signal very early neuronal impairment, and may indicate a period of time when neuronal dysfunction is still reversible.

Acknowledgements

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