Synaptic changes characterize early behavioural signs in the ME7 model of murine prion disease

C. Cunningham,1 R. Deacon,2 H. Wells,1 D. Boche,1 S. Waters,1 C. Piccano Diniz,3 H. Scott,1 J. N. P. Rawlins2 and V. H. Perry1
1CNS Inflammation Group, Southampton Neuroscience Group, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, UK
2Department of Experimental Psychology, University of Oxford, South Parks Road, Oxford OX1 3UD, UK
3University of Para, Brazil

Keywords: hippocampus, neurodegeneration, neuronal dysfunction, scrapie, synapse

Abstract

Prion diseases are fatal, chronic neurodegenerative diseases of mammals, characterized by amyloid deposition, astrogliosis, microglial activation, tissue vacuolation and neuronal loss. In the ME7 model of prion disease in the C57BL/6 J mouse, we have shown previously that these animals display behavioural changes that indicate the onset of neuronal dysfunction. The current study examines the neuropathological correlates of these early behavioural changes. After injection of ME7-infected homogenate into the dorsal hippocampus, we found statistically significant impairment of burrowing, nesting and glucose consumption, and increased open field activity at 13 weeks. At this time, microglia activation and PrPSc deposition was visible selectively throughout the limbic system, including the hippocampus, entorhinal cortex, medial and lateral septum, mamillary bodies, dorsal thalamus and, to a lesser degree, in regions of the brainstem. No increase in apoptosis or neuronal cell loss was detectable at this time, while in animals at 19 weeks postinjection there was 40% neuronal loss from CA1. There was a statistically significant reduction in synaptophysin staining in the stratum radiatum of the CA1 at 13 weeks indicating loss of presynaptic terminals. Damage to the dorsal hippocampus is known to disrupt burrowing and nesting behaviour. We have demonstrated a neuropathological correlate of an early behavioural deficit in prion disease and suggest that this should allow insights into the first steps of the neuropathogenesis of prion diseases.

Introduction

Despite the fact that neuronal death is a significant component of prion disease pathology, the events that lead to neuronal dysfunction and neurodegeneration remain poorly understood. In the ME7 murine model of prion disease, cell death is generally described to occur by apoptosis (Giese et al., 1995; Lucassen et al., 1995; Williams et al., 1997) but conspicuous cell death is not seen until late in the disease process. It is clear that there is a long separation between the first pathological changes of murine prion disease and the late events that characterize the clinical phase of the disease.

In the ME7 murine prion disease model in C57BL/6 J × VM/Dk mice that has a duration of 240 days, PrPSc deposition precedes neuronal death but there are synaptic and dendritic alterations that precede neuronal apoptosis by a considerable time (Jeffrey et al., 2000). Electron microscopy studies show a loss of synapses in the stratum radiatum of the hippocampus by 98 days (40% disease duration) in this ME7 model and this was coincident with PrPSc deposition in this area (Jeffrey et al., 2000). Dendritic abnormalities such as spheroids, varicosities and a decrease in dendritic spines are present in the terminal stages of this model (Belichenko et al., 2000) but have now been demonstrated as early as 126 days (53% of disease duration) (Jeffrey et al., 2001). However, the relationship of these pathological changes to neuronal dysfunction is unclear.

The usual criteria for the onset of clinical prion disease in mice have relied on detecting changes in locomotor activity and posture that appear within a few weeks of death (Hunter et al., 1986). The pathways that underlie these diverse clinical signs are complex and thus it is difficult to relate a particular neuropathological feature to a particular clinical symptom. We have shown that more subtle changes in behaviour can be detected reliably at or around 12 weeks (49% disease duration) after intra hippocampal injection of the ME7 scrapie agent into C57BL/6 J mice, a model with a disease duration of about 175 days (Betmouni et al., 1999; Deacon et al., 2001; Guenther et al., 2001). These studies demonstrated statistically significant decreases in burrowing and glucose consumption and a statistically significant increase in open field activity at 12 weeks in this model, although the deficits first appear at 10–11 weeks. Nest construction was also found to be significantly impaired at 20 weeks post injection in a previous study (Guenther et al., 2001) but was not tested at earlier time points. The identification of behavioural deficits allows us to make a noninvasive assessment of disease progression at early stages of the disease and furthermore the nature of behavioural changes can inform on regions of the brain that may be dysfunctional at the early stages. In a previous study, excitotoxic lesions of the dorsal hippocampus resulted in an impairment of burrowing behaviour, but did not alter open field activity or glucose consumption (Deacon et al., 2002), although hippocampal lesions do generally cause increased activity (O’Keefe & Nadel, 1978).

In this study we aimed to investigate what aspect of hippocampal pathology might correlate with the loss of burrowing. In addition, we
sought to discover whether there were pathological changes in other brain structures such as neuronal or synaptic loss that might correlate with the other behavioural deficits. To assess the areas of possible neuronal dysfunction, we have used immunocytochemistry to reveal regions of microglia activation. It is known that microglia activation is an early event in murine prion disease (Betmouni et al., 1996). Although the role of microglia in chronic neurodegeneration remains unclear, they are known to be very sensitive to disturbances in their environment (Kreutzberg, 1996) and thus it is likely that their activation in this model is indicative of some disturbance of neuronal homeostasis.

Materials and methods

Subjects

Female C57BL/6 J mice (Harlan, UK) were 8-weeks-old on arrival and were group-housed under a 12 h light : 12 h dark schedule throughout the study in plastic cages with woodchip bedding and a cardboard tube as environmental enrichment as described previously (Deacon et al., 2001; Guenther et al., 2001). Food and water were available ad libitum, except during the glucose test, when glucose solution was substituted for water.

Surgery

All procedures were carried out under a UK Home Office licence and in accordance with the Animals (Scientific Procedures) Act, 1986. Surgery was performed when the mice were 11–12-weeks-old, 17–22 g in weight. They were anaesthetized with Avertin (2,2,2-tribromoethanol solution) given at a dose of 0.25 mL/kg, intraperitoneally. They were mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), with the incisor bar set at −1 mm, to give an approximately level head. The scalp was incised and the skull exposed. A hole was drilled in the skull on each side of the midline to allow bilateral infusion of 1 μL of homogenate (10% w/v in sterile PBS) of either normal C57 mouse brain (NBH, n = 6) or of brain from a C57 mouse showing clinical signs of ME7 scrapie (scrapie, n = 17). Injections were made by a 5-μL Hamilton syringe adapted with a 34-gauge needle. Stereotaxic co-ordinates for hippocampal injections were ±1.3 mm from the interaural line, ±2.0 mm from the midline, and −2.0 mm from the skull surface measured at Bregma. The suspension was infused at 0.1 μL/min, after which the needle was left in place for 2 min before being withdrawn slowly. This injection protocol differs from the 20–30 μL volume currently used in most TSE transmission studies but reproducibly causes disease above liquid nitrogen and stored at −20 °C until used. Coronal sections (10 μm) of fresh frozen tissue were cut on a cryostat, dried, fixed in absolute alcohol for 10 min at 4 °C and processed for indirect immunohistochemistry. A number of animals were further perfused with 10% formal-saline and paraffin embedded (three NBH, three ME7 at 13 weeks, four ME7 at 19 weeks). Coronal sections (10 μm) of fixed tissue were cut on a microtome, dehydrated through alcohols. Primary antibodies against microglia (FA11) were obtained from Serotec (Oxford, UK), those for synaptophysin (SY38) from Chemicon (CA, USA), and those for PrP Sc (6H4) from Prionics (Zurich, Switzerland). Biotinylated secondary antibodies, normal sera, mouse-on-mouse blocking kit and avidin–biotin complex were from Vector Laboratories (Peterborough, UK). Avidin–horseradish peroxidase and primary antibodies against astroglia (GFAP) were obtained from DAKO (Cambridge, UK). TUNEL staining kits were obtained from Promega (Southampton, UK). Immunocytochemistry was carried out by the avidin–biotin-complex (ABC) method with minor mod-
ifications depending on the antibody used. Both hemispheres of the brain were examined and quantified, where relevant.

**FA11, GFAP, TUNEL**

Fresh frozen sections were fixed in absolute alcohol for 10 min at 4°C. Sections were washed in 0.1 M phosphate buffered saline (PBS, 2 × 10 min) and incubated for 30 min with 10% normal rabbit serum to block nonspecific Fc-receptor binding before incubation with FA11 (1:20), for 1 h. After washing, sections were incubated with biotinylated rabbit anti-rat IgG (1:100), washed and incubated with ABC. Peroxidase was visualized using 0.05% diaminobenzidine hydrochloride (DAB) with 0.05% hydrogen peroxide as substrate and this was enhanced using 0.01% osmium tetroxide in 0.1 M phosphate buffer for 30 s. Sections were counterstained with cresyl violet, dehydrated before mounting in Depex (BDH). Immunocytochemistry for GFAP was carried out as above with the following alterations. Prior to the addition of blocking serum (10% goat serum), 0.1% triton in PBS was added to the sections for 15 min at room temperature. Sections were incubated with rabbit anti-cow GFAP IgG (1:2000 in PBS containing 1% BSA) for 1 h followed by biotinylated goat anti-rabbit (1:100).

TUNEL staining was carried out on fresh tissue fixed in absolute ethanol at 4°C for 10 min and washed (PBS, 2 × 5 min). Sections were permeabilized in 0.1% Triton-X100 in PBS for 10 min, washed and incubated for 10 min at 37°C with equilibration buffer to prevent nonspecific binding of the nucleotide mix. TUNEL buffer (25 μL) containing equilibration buffer, nucleotide mix and TdT enzyme was added and incubated for 1 h and washed before blocking with 10% goat serum. Sections, including positive controls from kainic acid-injected mice (1 nmol, i.c.) were incubated with biotinylated goat anti-fluorescein IgG (1:200 in PBS) for 30 min. Positive cells were stained using the ABC method with DAB as substrate. The number of apoptotic cells was counted for each animal and the location of these cells noted.

**Synaptophysin**

Fixed paraffin sections were taken from three different groups of animals; NBH (n = 3), 13 weeks (n = 3) and 19 weeks post injection with the prion agent (n = 4). Sections were dewaxed, rehydrated and treated with 0.2 M boric acid, pH 9, 65°C for 30 min and cooled to room temperature. Non-specific peroxidase activity was eliminated by incubating sections in 1 mL H2O2/100 mL ethanol (1% H2O2) for 10 min. Sections were washed in PBS and blocked using 10% normal horse serum. Sections were incubated with SY38 (1:100), at 19°C, overnight before incubation with biotinylated horse anti-mouse secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200). The DAB reaction was carried out in the presence of ammonium nickel chloride (0.06% w/v) to intensify secondary IgG (1:200).

**PrPSc**

Fixed paraffin sections through the septum, hippocampus, thalamus and entorhinal cortex were rehydrated. Sections were autoclaved in distilled water for 15 min at 121°C to destroy PrPSc. After washing in PBS, sections were placed in 90% formic acid for 5 min and then washed in distilled water and PBS. To avoid nonspecific binding that may occur using a monoclonal mouse antibody, mouse-on-mouse blocking solution was added. The primary antibody, 6H4 (1:4000) was left overnight before incubation with biotinylated anti-mouse IgG (1:250). Incubation with ABC and DAB reaction were carried out as above. Sections were placed in 0.2% osmium tetroxide solution for 5 s to enhance the signal before dehydrating and coverslipping. Sections were haematoxylin counterstained.

**Cell counting**

Microglia were identified as CD68 positive cells (CD68+). In the normal brain, the microglia have a small heterochromatic nucleus and a small number of CD68+ lysosomes around the nucleus. It is rarely possible to see the processes of the resident microglia. The microglia were judged as ‘activated’ by their increased expression of CD68 that now revealed both the cell body and the processes of the cells. Only nuclei associated with clearly CD68+ staining were counted. The number of neurons in a 345-μm linear distance of the dorsal part of CA1 at ±2.5 AP were counted at ×400 magnification and compared between animal groups. Neurons were identified by the presence of Nissl substance in their cytoplasm and a clear nucleus and were counted only if the nucleus was clearly visible. Differential counting, due to possible differences in nuclear size was controlled for by measuring the nuclear diameter of a sample of the population and assessing whether there was a difference in average nuclear size between the experimental groups. There was no statistically significant difference in nuclear diameter between the groups.

**Statistical analysis**

Data from repeated behavioural tests would normally be analysed by repeated measures ANOVA, followed by post hoc tests with an appropriate correction (e.g. Bonferroni) for multiple comparisons. However, this approach was not entirely suitable for the present data. First, the data were often nonparametric, failing to fulfill the normality requirement of ANOVA. Second, the aim was to stop behavioural testing and process the brains for pathology at the very first clear indication of behavioural change. Thus, it was likely that an interaction of Group and Week would show up in an analysis while main effects would not. Moreover, conventional Bonferroni corrections would have been unduly conservative and would have failed to detect the first point when scrapie and control values differed. Therefore, planned comparisons at individual time points were by Mann–Whitney U-test. While acknowledging their limitations in the present circumstances, repeated measures ANOVAs are also presented to facilitate an overall view of the behavioural trend, with data transformed as noted to obtain the best fit to normality. The nesting scores were too nonparametric for repeated measures ANOVA so were analysed only by the U-test. The synaptophysin staining was determined by mono-
physin data are presented as the mean ± standard error of the mean (SEM) and these data have been compared by students t-test. Values of P < 0.05 were considered statistically significant.

Results

The behaviour of these groups of mice, ME7 prion infected and NBH controls, on open field activity, burrowing, and glucose preference was similar to that in our previous study (Guenther et al., 2001). We also found that nesting behaviour was a sensitive indicator of ongoing disease.

Open field

Rears

The control group scores decreased while those of the ME7 group increased towards week 13 (Fig. 1A). Repeated measures ANOVA of the scores (square root transformed to produce a normal distribution) showed no significant main effect of Group or Week (P > 0.1) but there was an interaction of Group and Week, F\(_{7,147}\) = 2.41, P = 0.023. At no point did the groups significantly differ by pairwise Mann–Whitey U-tests (lowest P-value, week 13, P = 0.06).

Squares crossed

The scores progressively diverged, with the ME7 group becoming hyperactive from week 12 (Fig. 1B). Some mice showed extreme hyperactivity (>200 squares per 3 min period). Repeated measures ANOVA of the scores (log transformed to produce the closest fit to normality) showed an effect of Week, F\(_{7,147}\) = 5.44, P < 0.0001, while the effect of group was not quite significant, F\(_{1,21}\) = 3.95, P = 0.060. There was, however, a significant interaction of Group and Week, F\(_{7,147}\) = 6.91, P < 0.0001 and Mann–Whitney tests showed that the groups significantly differed at weeks 12 and 13 (P < 0.05).

Burrowing

The ME7 group initially burrowed less than the controls (Fig. 1C; see also, Discussion). However, the weight of food pellets burrowed by the ME7 group declined progressively while that of the control group remained fairly constant apart from an unexplained dip at week 9 (Fig. 1C). Repeated measures ANOVA of the weight burrowed (untransformed, as transformations did not make the distribution more normal) showed an effect of Group, F\(_{1,21}\) = 22.40, P = 0.0001, an effect of Week, F\(_{7,147}\) = 5.98, P < 0.0001, and an interaction of Group and Week, F\(_{7,147}\) = 4.52, P = 0.0001. Mann–Whitney tests showed that the groups significantly differed at weeks 10, 11, 12 and 13 (P < 0.05).

Glucose consumption

Both groups drank considerably more glucose solution (>10 mL) than water (mice drink approximately 4 mL per night). At week 13, consumption started to decline in the ME7 group (Fig. 1D). Repeated measures of ANOVA showed no effect of Group, but there was an effect of week F\(_{7,147}\) = 4.88, P < 0.0001, and an interaction of Group and Week, F\(_{7,147}\) = 3.15, P = 0.004. Mann–Whitney tests showed that the groups differed significantly at weeks 10, 11, 12 and 13 (P < 0.05).

Nest construction

Almost all NBH mice built perfect nests from week 6 to week 13 (Fig. 1E) although as values are shown as medians this conceals the occasional mouse that scored less (these are revealed by error bars on weeks 10 and 11). The ME7 group also scored 5 until week 10, when scores started to decline. Scores for weeks 10–13 were 4, 3, 3 and 3.5, respectively; these were significantly different from control values (all P < 0.05, Mann–Whitney test). Thus alterations in nest building...
behaviour is the most sensitive indicator to date of the onset of prion related neurological disturbances.

Pathology

A statistically significant difference between the ME7- and NBH-injected groups on the battery of behavioural tasks was found in the thirteenth week postinjection. The mice were killed at this time and the brain tissue prepared for histopathological analysis.

Microglia

Microglia, revealed by immunocytochemistry for CD68, are not conspicuous in NBH-injected animals (Fig. 2A) and positively stained (CD68+) cells appear small with ramified processes only visible occasionally. In the brains of the ME7-injected mice, there was a very marked increase in both the number of CD68+ microglia and the levels of expression of CD68, particularly in limbic structures, with ramified processes more apparent than those in normal brain. The greatest increase in CD68 expression was observed in the hippocampus, particularly the stratum radiatum and stratum oriens (Fig. 2B). There was an approximately tenfold increase in the number of CD68+ cells in all regions of the hippocampus compared with the NBH-injected hippocampus (Table 1). Activated CD68+ microglia were also present in the medial and lateral septum, the dorsal thalamus, the entorhinal cortex and, to a lesser degree, the mamillary bodies (Fig. 2C–E). There was a modest increase in CD68 expression in regions of the brain stem proximal to the origin of projecting serotonergic and noradrenergic neurons, but the definition of this staining was insufficient to determine the precise cytoarchitectural location. Despite the presence of the activated microglia in structures known to be anatomically connected to the original injection site, the dorsal hippocampus, there was little microglial activation along the major fibre tracts.

Fig. 2. Microglial, astrocyte and TUNEL staining in animals at 13 weeks postinjection with ME7 or NBH. FA11 staining for the microglial antigen CD68 is shown in NBH-injected hippocampus (A) and ME7-injected hippocampus (B). This ME7 injection also induces microglial activation in the medial septum (C), lateral septum (D), entorhinal cortex (E) and areas of the brainstem (F). GFAP staining is shown in the CA1 area of ME7- (G) and NBH-injected (H) hippocampus and in the dorsal thalamus of the same animals (I and J, respectively). TUNEL staining for apoptotic cells in ME7-injected animal (K) and a kainic acid challenged animal (L). Scale bar, 200 μm (A–F); 70 μm (G–J) and 35 μm (K and L).
Astroglia

Glial fibrillary acid protein expressing (GFAP+) astroglia are present throughout the normal hippocampus, but are more conspicuous in the hippocampus of the ME7-injected mice (Fig. 2G and H). A similar increase in GFAP+ astroglia expression is present in the thalamus, entorhinal cortex and septal regions. There is a large increase in GFAP expression in the dorsal thalamus relative to normal animals (Fig. 2I and J), while the levels of expression in the brainstem appeared similar in ME7-injected and NBH-injected mice.

Cell death

There was no evidence of increased cell death in the brains of ME7-injected animals at 13 weeks post injection as judged by TUNEL staining. Although apoptotic cells were occasionally visible (Fig. 2K), no more than three were detected in any coronal section and numbers

<table>
<thead>
<tr>
<th>Table 1. Cell counts in NBH and prion-infected mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell type</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Microglia (CD68+) (per 10 μm hippocampal section at Bregma −2.5)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Apoptotic cells (TUNEL) (per 10 μm hippocampal section at Bregma −2.5)</td>
</tr>
<tr>
<td>Pyramidal cells (345 μm linear distance)</td>
</tr>
</tbody>
</table>

*Statistical significance (P < 0.05, students t-test).

Fig. 3. Hematoxylin, synaptophysin and PrPSc staining of NBH- and ME7-injected animals at 13 and 19 weeks postinjection. Hematoxylin stain of dorsal hippocampal CA1 of NBH (A); ME7 at 13 weeks (B) and ME7 at 19 weeks (C). Synaptophysin staining (SY38) of naive brain, showing the synaptic density of the entire hippocampal formation (D), and of the CA1 of NBH (E), ME7 at 13 weeks (F) and ME7 at 19 weeks (G). PrPSc staining (6H4) in the hippocampus (H), CA1 (I), dorsal thalamus (J) and lateral septum (K) of ME7-injected animals at 13 weeks postinjection. Scale bars, 70 μm (A–C and K), 200 μm (D and H), 100 μm (E–G) and 50 μm (I and J).
did not differ significantly between normal and prion-diseased animals. As a positive control for the staining method, we readily detected large numbers of TUNEL positive cells in sections from animals that had been given a kainic acid excitotoxic lesion (Fig. 2L). Apoptotic cells were observed in the ependymal layer of all animals and these were omitted from counting. TUNEL positive cell counts are shown in Table 1.

Previous studies have shown that in C57BL/6J × Vm/Dk mice injected with ME7 there is a loss of approximately 50% of neurons from the CA1 pyramidal layer of the hippocampus at 180 days into the disease (72%) (Jeffrey et al., 2000). We thus investigated whether there was a similar loss of pyramidal cells in the CA1 layer of the hippocampus of C57BL/6J mice. No significant difference in the number of pyramidal cells in dorsal hippocampus was observed between the NBH and ME7-injected animals at 13 weeks, while in animals at 19 weeks after ME7 injection there was a 40% reduction in CA1 pyramidal cells (Fig. 3A–C; Table 1).

**Synaptophysin**

As we were unable to detect any loss of neurons in the ME7-injected mice at 13 weeks, we investigated whether there was any loss of synaptic profiles from those regions of the brain where there was microglia activation. Immunocytochemistry for the synaptic protein, synaptophysin has been used previously to demonstrate loss of synapses in models of neurodegeneration (Toggas et al., 1996; Jeffrey et al., 2000). Synaptophysin staining gives a clear picture of synaptic densities within the hippocampus of the normal brain, demonstrating a high density of synapses along the mossy fibre projection to CA3 and in the stratum radiatum and stratum oriens of CA1, where the CA1 dendrites receive their innervation from CA3 (Fig. 3D). In ME7-injected animals at 13 weeks post injection, there is a visible disruption of synaptophysin staining in the stratum radiatum when compared with NBH-injected animals. The synaptophysin staining in the region of the proximal dendrites of the CA1 pyramidal cells is reduced at 13 weeks and this has further deteriorated by 19 weeks (Fig. 3E–G). Even at 19 weeks, a relatively late stage of the disease, there is no discernable loss of synaptophysin staining at the mossy fibre terminals. Quantification of the changes in synaptophysin staining intensity was carried out using pixel density analysis of digitally captured images. The ratio of staining intensity in the stratum radiatum relative to the stratum lacunosum-moleculare is statistically significantly decreased at 13 weeks ($P < 0.00002$; Fig. 4) and further reduced at 19 weeks. There was no clear evidence of synaptic loss in other structures where microglial activation was observed, although subtle changes elsewhere are difficult to detect as the laminar structure of the hippocampus made this area particularly convenient for assessment.

**PrPSc**

Sections from animals at 13 and 19 weeks postinjection were stained to reveal PrPSc deposition. In all prion diseased animals, there were deposits of PrPSc, 6H4 positive granular staining was observed throughout the hippocampus but was particularly marked in the hilus and along the mossy fibres (Fig. 3H). There were larger plaques in the dorsal thalamus (Fig. 3I), granular deposits in the lateral and medial septal regions (Fig. 3K) and in the entorhinal cortex (not shown). In prion-diseased animals at 19 weeks, PrPSc plaque material was more widespread, particularly through the thalamus and in the cortex, where large plaques were occasionally observed.

**Discussion**

In this study we have investigated the neuropathological substrates that underlie the early behavioural deficits in the ME7 model of murine prion disease in C57BL/6J mice. In accord with our previous work, we have found that at 13 weeks following initiation of the disease there are significant impairments in burrowing, glucose consumption and open field activity. Disruption of nest building was also found to be a sensitive indicator of disease onset. At 13 weeks, there are subtle pathological alterations to be found in limbic structures but not other regions of the brain. Microglia throughout the limbic circuit are activated and there were also deposits of PrPSc in these structures. Using the TUNEL method to reveal apoptotic cells we did not detect any significant increase in cell death in the brains of the ME7-injected animals and there was no significant neuronal cell loss from the hippocampal CA1 field at this time. We did, however, find that there was significant loss of synaptophysin staining in the stratum radiatum. Our results suggest that the synapse is the primary target for prion induced neuropathology and that synaptic loss or dysfunction underlies the early behavioural deficits we describe.

**Behaviour**

Central to our understanding of the pathogenesis of prion diseases is to establish the relationship between the presence of the infectious agent and neuronal dysfunction or degeneration. Until recent years, the vast majority of the studies on prion disease neuropathogenesis have focused on the terminal stages of the disease. However, the complexity of the spectrum of clinical symptoms and the complexity of the neuropathology at terminal disease has made it difficult to draw any conclusions about this relationship and in turn the mechanisms of pathogenesis. We reasoned that if the earliest signs of neuronal dysfunction could be established, and these early signs could be related to neuropathological features in particular brain regions, this would in turn facilitate studies of the earliest cellular and molecular components of the pathogenesis.

We have shown in this, and previous studies (Betmouni et al., 1999; Deacon et al., 2001; Guenther et al., 2001) that many weeks prior to the onset of the so-called ‘clinical signs’ (often used to define the incubation period of different prion strains), the ME7-injected mice show subtle but reproducible and robust changes in a number of behaviours. One of the first deficits to appear is the change in burrowing behaviour. We know that this task requires an intact dorsal hippocampus as excitotoxic lesions of this structure abolish burrowing (Deacon et al., 2002). Nest building, a behaviour that also requires an intact dorsal hippocampus, is significantly disturbed at week 13. The
other limbic structures and neuroanatomical circuits that underlie this behaviour have not yet been established. The apparent lower tendency of the ME7 group to burrow from the outset in the current study reflects an initial bias between the two groups and is not an early effect of scrapie inoculation. The burrowing test has only recently been developed and we now know that mice vary in the amount they burrow and it is best to take 2 h and overnight measurements. The 2 h-test is very sensitive but with this comes high variability. In ongoing studies, we have also observed scrapie groups to burrow more than control groups in the 2 h session while almost all mice burrow 200 g overnight. It is now our practice to balance the groups before with a baseline burrowing test.

The preference that mice show for glucose is disrupted by week 13 of the disease but this task is not affected by hippocampal lesions (Deacon et al., 2002). Unfortunately the circuitry that underlies glucose preference is poorly defined. It is known that lesions of the lateral parabrachial nucleus (Reilly & Trifunovic, 2000) and lesions of the gustatory thalamus, the parvocellular region of the ventroposteromedial nucleus, lead to a loss of sucrose preference. However, there was no evidence of microglia activation or PrPSc accumulation in these regions. It is not clear whether the loss of glucose consumption in our mice involves changes in taste preference or in later stages of the rewarding experience of drinking glucose.

It was important to measure open field activity in the ME7- and NBH-injected groups as changes in burrowing activity, nesting and glucose preference could all be affected by a loss of mobility or a locomotor deficit. However, at week 13, in accord with our previous studies (Guenther et al., 2001), the animals with prion disease show an increase in both open field activity and rearing indicating that they are not suffering from a simple locomotor deficit. Dell’Omo et al., 2002 have also observed increased open field activity in prion-diseased mice but conversely have also reported decreases in spontaneous activity in the home cage monitored throughout the incubation period (Dell’Omo et al., 2002). This may reflect a different aspect of locomotion to that observed in the open field.

Neuropathology

To identify those areas of the brain that might be involved in the early behavioural changes, we have mapped the areas of microglia activation in the brain at 13 weeks after the initiation of the disease. It is well established that microglia are exquisitely sensitive to changes in neuronal homeostasis, they rapidly up-regulate, or express de novo, a number of cell surface antigens and alter their morphology (Perry & Gordon, 1988; Kreutzberg, 1996). Subtle changes in the brain microenvironment, including spreading depression (Gehrmann et al., 1993), the retrograde cell body reaction (Streit & Graeber, 1993) and Wallerian degeneration (Rao & Lund, 1989) are sufficient to activate these cells. At 13 weeks following disease initiation, microglia were activated selectively throughout the limbic system, including regions connected to the dorsal hippocampus, the site of injection of the prion agent. Activated microglia expressing elevated levels of CD68 were found in the hippocampus, septum, mammillary bodies, anterior thalamus and entorhinal cortex. The distribution of the activated microglia, and thus potential sites of pathology, is consistent with the notion that the disease spreads along anatomical pathways in both a retrograde and anterograde direction (Scott et al., 1992; Taraboulos et al., 1992).

We do not know what is the stimulus for the microglial activation. One obvious possibility is the presence of PrPSc, which is also present throughout the limbic structures. However, there is not a very precise relationship between the degree of microglial activation and the density of PrPSc deposition. This may reflect the fact that numerous changes downstream of PrPSc deposition may activate the microglia, including alterations in the astrocytes and neurons. We thus investigated whether the microglia were activated by the degeneration of neurons. A convenient tool for the detection of cells undergoing apoptosis is the TUNEL method. It has been shown previously that neurons degenerate by apoptosis in models of murine prion disease (Giese et al., 1995; Lucassen et al., 1995; Williams et al., 1997). We found no evidence of an increase in apoptotic cells in the diseased brains at 13 weeks consistent with previous studies showing that this is only significant at later stages. Neuronal cell loss of hippocampal CA1 pyramidal cells has been described in the ME7 model in C57BL/6J × Vm/Dk mice at 180 days into the disease (72%) (Jeffrey et al., 2000). In our model, there was conspicuous CA1 pyramidal cell loss at 19 weeks (76%) and no significant loss at 13 weeks (52%).

In the absence of significant cell death or neuronal loss, we investigated whether there were detectable changes in synaptic density in areas with activated microglia. Synaptophysin immunocytochemistry has been used in a number of studies to detect changes in synaptic density at the light microscope level (Togga et al., 1996; Jeffrey et al., 2000). We observed a clear reduction in synaptophysin staining in the stratum radiatum at 13 weeks (52% of the disease time course) and this could be further quantified as a change in the relative density ratio between the synapse-rich stratum radiatum and the relatively synapse-poor stratum lacunosum. The change in the ratio of the density of staining between the stratum radiatum and stratum lacunosum molecule most probably reflects a loss of synapses from the stratum radiatum, although we cannot rule out the possibility that there are also changes in the stratum lacunosum. It is also possible that this decrease represents a decrease in synaptophysin expression, which has been reported to be activity dependent (Li et al., 2002). The current study was designed such that a clear behavioural phenotype was most likely to produce measurable pathological features. However, as ME7-diseased mice were impaired on some tasks up to 2 weeks before termination, it is also possible that pathological features would already be apparent at this time. The loss of synaptophysin staining was dramatic and widespread in the hippocampus by 19 weeks postinjection of ME7, although CA3–mossy fibre staining was reasonably well preserved. Early signs of pathology in the hippocampus have also been reported in the ME7 in C57BL/6J × Vm/Dk mouse model: PrPSc accumulation is present as early as 60–70 days (Jeffrey et al., 2001), loss of synapses in the hippocampus by 84 days (34%) (Jeffrey et al., 2000) and dendritic abnormalities by 109 days (44%) (Brown et al., 2001). The same group have shown that maintenance of long-term potentiation (LTP), a test reflecting synaptic plasticity, is impaired in hippocampal slice preparations made from brains 100 days (40%) postinjection with the same strain, but not from animals at 80–85 days (Johnston et al., 1998). In our model, impairments on learning and memory tasks are not significant until 14 weeks postinjection (56%) on a passive avoidance task (Bettouni et al., 1999) and at 18 weeks (72%) on a spontaneous alternation task (Guenther et al., 2001). It is unfortunate that we cannot make a precise comparison of the timing of different events between the different models given the differences in mouse strains, injection protocols and other parameters. However, it is clear that alterations at the synapse precede neuronal loss and we suggest that these changes in synaptic density and/or function are sufficient to account for the behavioural symptoms. A more detailed study of synapse-associated proteins may reveal the structural basis of behavioural dysfunction even prior to synaptic loss. A recent study has demonstrated that the expression of several synaptic proteins are reduced or absent at the late stage of RML-induced murine prion disease (Siso et al., 2002). It would be of interest to know which proteins are lost in earlier stages of disease evolution.
A further aspect of the pathology that has yet to be considered in our model is which pathways or cells lose their synapses early on in the disease. There is evidence for a severe and selective loss of GABA-ergic cells in CJD, experimental CJD and scrapie (Guentchev et al., 1998; Guentchev et al., 1999). It will be important to establish whether there is any early neurochemical selectivity in the early synaptic loss.

Conclusions

We have demonstrated that the earliest behavioural changes in this model of murine prion disease are associated with microglia activation, astrocyte activation, and deposition of PrPSc throughout the limbic system. A probable component for the behavioural deficits found in burrowing and nestling behaviour is the loss of synapses from the stratum radiatum of the dorsal hippocampus. The loss of these synapses cannot account for all of the behavioural changes described but this study serves to focus our attention on the synaptic changes that precede neuronal loss in prion disease. These results suggest that the synaptic dysfunction and loss is an early and pivotal step in the neuropathogenesis of prion disease. Furthermore, it is now important to establish whether the many strains differentiated on the basis of different vacuolation patterns and incubation times show similar synaptic alterations of hippocampal pyramidal neurones in scrapie-infected mice.

Acknowledgements

This research was supported by the Wellcome Trust. C. Picano-Diniz was supported by CNPq, Brazil.

Abbreviations

ABC, avidin–biotin–complex; BSA, bovine serum albumin; CD68, cluster differentiation 68, macroysin; DAB, diaminobenzidine; GFAP, glial fibrillary acidic protein; NBH, normal brain homogenate; OCT, optimal cutting temperature; PBS, phosphate buffered saline; PrPc/Sc, prion protein, cellular/scrapie form; TUNEL, TdT mediated, dUTP nick end labelling.

References


© 2003 Federation of European Neuroscience Societies, European Journal of Neuroscience, 17, 2147–2155