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Overexpression of IL-1β by adenoviral-mediated gene transfer in the rat brain causes a prolonged hepatic chemokine response, axonal injury and the suppression of spontaneous behaviour

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Acute brain injury induces early and transient hepatic expression of chemokines, which amplify the injury response and give rise to movement of leukocytes into the blood and subsequently the brain and liver. Here, we sought to determine whether an ongoing injury stimulus within the brain would continue to drive the hepatic chemokine response and how it impacts on behaviour and CNS integrity. We generated chronic IL-1β expression in rat brain by adenoviral-mediated gene transfer, which resulted in chronic leukocyte recruitment, axonal injury and prolonged depression of spontaneous behaviour. IL-1β could not be detected in circulating blood, but a chronic systemic response was established, including extended production of hepatic and circulating chemokines, leukocytosis, liver damage, weight loss, decreased serum albumin and marked liver leukocyte recruitment. Thus, hepatic chemokine synthesis is a feature of active chronic CNS disease and provides an accessible target for the suppression of CNS inflammation.

Keywords: Adenovirus; Rat; IL-1β; Brain; Acute phase response; Behaviour and injury

Introduction

Interleukin-1 (IL-1β) contributes to a broad spectrum of central nervous system (CNS) pathologies, where it is known to modify behaviour and exacerbate neuronal loss (for a review, see Lucas et al., 2006) via IL-1 receptors present on neurons, glia and endothelial cells. We have previously shown that adenoviral-mediated gene transfer to overexpress IL-1β (AdIL-1β) in the brain leads to the prolonged recruitment of neutrophils to the brain parenchyma (Ferrari et al., 2004). While the presence of IL-1β generated by AdIL-1β was not found to be directly toxic to neurons, the chronic expression of IL-1β induced extensive striatal demyelination, which was followed by remyelination. The ultrastructural characteristics resemble those observed in a model of demyelination using Theiler virus (Sathornsumetee et al., 2000). IL-1β *per se* has been shown to be cytotoxic to adult oligodendrocytes *in vitro* (Merrill, 1991; Brogi et al., 1997), and others have also shown that substantial demyelination results from the focal inflammatory lesion caused by the injection of lipopolysaccharide directly into the rat dorsal funiculus (Felts et al., 2005). In acute multiple sclerosis (MS) lesions and in the active border of less acute lesions, axonal damage is closely associated with active inflammation and demyelination (Ferguson et al., 1997; Trapp et al., 1998). We have also shown that axonal injury is associated with bystander tissue destruction in an immune-mediated lesion (Newman et al., 2001), but it is not known whether the myelin damage generated by an innate inflammatory response, such as that induced by AdIL-1β, can also result in axonal injury. Axonal injury is known to be a feature of many immune and non-immune CNS pathologies where it is a predictor of outcome (Medana and Esiri, 2003), but the mechanisms that give rise to the damage remain unclear.

In our studies, the magnitude and the specificity of the recruitment of neutrophils into the brain following the injection of the IL-1β-expressing adenovirus was striking. No other populations of leukocytes, except for a few monocytes or macrophages in or around vessels, were observed (Ferrari et al., 2004). In other studies, in which the same adenoviral vector AdIL-1β was introduced into the peritoneum or into the lung, neutrophils also dominated the inflammatory response (Kolb et al., 2001; Margetts et al., 2002). Neutrophil recruitment to the CNS is preceded by the hepatic release of the chemokine CXCL-1, which generates a granulocytosis and occurs before there is any evidence of an inflammatory response at the injury site (Wilcockson et al., 2002; Campbell et al.,

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2003, 2005). CCL-2, another liver-produced chemokine, is responsible for the appearance of monocytes in the blood (Campbell et al., 2005). Hepatic chemokine release amplifies the local injury response by priming immune cells and increasing the number circulating in the blood. These cells migrate not only into the injured site where they contribute to damage in organs such as the brain, but also into the liver where they cause injury (Campbell et al., 2003). After an acute injury, the hepatic chemokine response is very short lived (Campbell et al., 2003, 2005) and appears to be actively suppressed by the induction of NF-κB inhibitors (S. Campbell, personal communication). However, where leukocytes are chronically recruited to the brain it is not known whether the hepatic chemokine response is extended. Should chronic leukocyte recruitment to the brain also be dependent on hepatic chemokine production, the inhibition of liver chemokine production would become a useful therapeutic objective in many different neuropathologies where the presence of an intact blood–brain barrier (BBB) is often an obstacle to effective therapy.

While IL-1β does not seem to be neurotoxic *per se* (Allan et al., 2000), it is known that many populations of neurons express IL-1 receptors and are thus are capable of responding to a pro-inflammatory environment. In our initial studies (Ferrari et al., 2004), we did note that the animals exhibited some behavioural changes that were not formally tested. Single-bolus injections of IL-1β into the periphery and into the cerebrospinal fluid of rodents have been shown to result in a reduction in general activity and grooming (Kent et al., 1992), loss of social contacts (Dantzer and Kelley, 1989), anorexia (Sonti et al., 1996), adipsia and hypersomnia (Dantzer and Kelley, 1989). However, the behavioural consequences of chronic focal inflammatory disease in the CNS parenchyma have not been so well studied.

Thus, in this study, we have used the AdIL-1β adenovirus to generate an active chronic inflammatory lesion in the brain to determine whether chronic IL-1β expression within the brain continues to drive the hepatic chemokine response and how it impacts on behaviour and CNS integrity. Our findings reveal that for as long as there is production of central cytokines, the hepatic chemokine response remains active and continues to amplify and promote leukocyte recruitment to the liver and to the CNS, where it is associated with axonal injury and a long-term decrease in spontaneous behaviour.

Methods

Animals

Adult male Hooded Lister rats (approximately 300 g) (Harlan, UK) were used throughout and were housed in groups of 3–6 animals, under a controlled temperature (22 °C ± 2 °C), in a 12-h light/dark cycle, supplied with water and food *ad libitum*.

Reagents

Rat recombinant IL-1β was obtained from the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK). The cytokine was dissolved in endotoxin-free saline (vehicle). The IL-1β contains 100 IU endotoxin/mg (corresponding to 10 ppm by weight), which, in view of previous studies (Andersson et al., 1992), was considered negligible in the context of these experiments. All other reagents were obtained from Sigma-Aldrich, Poole, UK, unless otherwise stated.

Adenoviral vectors

The construction of the adenovirus vectors AdIL-1β and dl70-3 (control) has been previously described (Ferrari et al., 2004). Adenovirus preparations were purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography (Amersham Pharmacia, Baie d'Urfe, Canada) and plaque-titered on 293 cells as previously described. Stocks had less than 1 ng/ml of endotoxin, assayed with E-TOXATE® Reagents. Viral stocks were free of auto-replicative particles as assessed by PCR and transduction of non-transcomplementary cells (HeLa, ATCC) (Lochmuller et al., 1994).

Surgery

Rats were anaesthetised with Avertin (1.25 g/l 2,2,2-tribromoethanol; 1 ml/100 g) for the duration of the surgical procedures. Stereotaxic surgery was performed essentially as described previously described for mice (Cunningham et al., 2003). Briefly, anaesthetised rats were held in a stereotaxic frame, a burr hole was drilled in the skull and the adenovirus or control was administered through a microinjector (34 guage) of an adapted Hamilton syringe, into the left ventricle (bregma, −1.8 mm; lateral, ±1.3 mm; ventral, −4 mm from the skull at bregma). The viruses were diluted in sterile PBS (pH 7.4) and administered at doses of 5×10^9, 1×10^8 or 1×10^7 plaque-forming units (pfu) in a 1-μl volume. All surgical procedures took place in the morning to avoid effects of circadian variations in cytokine expression. In a separate set of experiments the adenovirus was heat-inactivated by several freeze/thaw cycles before microinjection at the highest dose used in these experiments. Animals were killed at 8 days post-surgery. All procedures were carried out with ethical committee approval under a UK Home Office Licence (30/2008).

Intraperitoneal injection of cytokine

A single bolus injection of rrIL-1β (4 μg/kg) in a volume of 1 ml/kg of sterile endotoxin-free saline was administered by intraperitoneal injection. Doses of rrIL-1β were based on previous experiments from our laboratory (Campbell et al., 2003). Burrowing experiments were initiated 90 min post-challenge.

Tissue and serum collection

After appropriate survival times, rats were deeply anaesthetised with sodium pentobarbitone. Blood was collected and allowed to clot for 2 h at room temperature and then serum was collected by centrifugation. Trans-cardiac perfusions were performed using heparinized saline. Tissue was removed and either frozen in liquid nitrogen or embedded in Tissue-tek (Sakura Finetek, Zoeterwoude, NL) and frozen for histology. In a separate set of animals, tissue for histology was perfusion-fixed with 10% buffered formalin fixative immediately after perfusion with heparinized saline. Fixed tissue was cryopreserved for 48 h and then embedded in Tissue-Tek and frozen for histology as described above. A separate set of experimental animals was generated for mRNA analysis. Animals were killed and saline-perfused as described above before a 3 mm × 2 mm × 2 mm region of brain tissue around the ventricles was microdissected and used for mRNA analysis (Campbell et al., 2002, 2003, 2005).

Analysis of serum albumin

Analysis of serum albumin was carried out at 30 °C using commercial assay reagents according to manufacturers' instructions (Raichem, San Diego, CA, USA). Assay control serums with known levels (normal and elevated) of albumin were used as standards for each assay.

Analysis of circulating leukocyte numbers

Whole blood was collected in the presence of 5 mmol/l EDTA, pH 8.0, and leukocyte numbers were assessed in a Cell-Dyn 1600 haematology analyzer (Unipath, Mountain View, CA, US) and verified from immunohistochemically labelled blood films. This analyzer is able to discriminate neutrophils from other leukocytes.

Immunohistochemistry

To identify leukocyte populations, frozen, 10-μm-thick serial coronal sections were cut from tissue blocks, through the injection site in the brain or through a representative lobe of liver. Using immunohistochemistry under standard conditions, neutrophils were identified using anti-neutrophil serum (Anthony et al., 1998a,b) and activated macrophages and recruited monocytes were identified using the anti-ED-1 serum (Serotec, Oxford, UK). For each tissue section, 4 representative fields were chosen for quantitation, and the average number of positive cells was calculated and expressed as number of cells per mm^2. ED-1-immunopositive labelling was quantitated only when it was associated with a cell nucleus. For markers of liver injury, frozen, 10-μm-thick sections were cut from fresh liver blocks and fixed for 10 min in dried acetone (−20 °C). Cells expressing activated caspase-3 (Abcam Ltd, Cambridge, UK) or Gadd45 (Santa Cruz Biotechnology, Heidelberg, Germany) were identified using rabbit-specific polyclonal antibodies. For matrix proteins, coronal 10-μm-thick sections from formalin-fixed (laminin) or fresh frozen (collagen 1, GFAP) brain at bregma +1 mm were immunostained. Fresh sections were fixed for 5 min in acetone before labelling. Sections were incubated overnight in rabbit anti-laminin, 0.3 μg/ml (Sigma-Aldrich, Dorset, UK), mouse anti-collagen-1, 1 μg/ml (Abcam, Oxford, UK) or rabbit anti-GFAP, 2 μg/ml (Abcam, Oxford, UK). For all immunohistochemistry, primary antibodies were detected with the relevant biotinylated IgG, amplified using standard ABC and immunopositivity was revealed with DAB (Vector Laboratories, Peterborough, UK). Tissue sections were counterstained with cresyl violet (brain) or haematoxylin (liver).

Assessment of blood–brain barrier (BBB) breakdown

Coronal sections throughout the brain were assayed for BBB breakdown. Sections were fixed in cold ethanol for 20 min and then subsequently incubated for 2 h in 10% normal rabbit serum then overnight with biotinylated anti-rat immunoglobulin (IgG) antibody (Vector Laboratories, Peterborough, UK), at a dilution of 1 μg/ml at 4 °C. Positivity was identified using standard ABC (Vector Laboratories, Peterborough, UK), and immunoreactivity was revealed with DAB. All sections were processed, immunolabelled and assessed for BBB breakdown in the same batch. Sections were not counterstained.

mRNA extraction and RT-PCR

RNA extraction and quantitative RT-PCR assays were performed essentially as previously described (Campbell et al., 2002, 2003, 2005). Standard curves were generated from serially diluted cDNA from IL-1β-challenged rat liver. The use of rat CCL-2 (Behr et al., 2000; Campbell et al., 2005), rat CXCL-1 (Campbell et al., 2002) and rat IL-1β (Docagne et al., 2005) primers and probes has been reported by us elsewhere. Results are expressed in arbitrary units corrected to the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) and show values above those observed in naïve rat brain.

Measurement of cytokine production

ELISA for the measurement of rat IL-1β (R and D Systems, Abingdon, UK) was carried out essentially as described in manufacturers' instructions (sensitive to 3.5 pg/ml). Rat CXCL-1 ELISA (NIBSC, Potter bar, UK) (Campbell et al., 2002, 2003, 2005) and CCL-2 (Campbell et al., 2005) were carried out essentially as we have previously described (sensitive to 3 pg/ml). ELISA data are expressed as pg/ml of serum. Recombinant proteins for standard curves were diluted in naïve serum at concentrations identical to those used in test samples.

Burrowing test

Burrowing tests were carried out in a similar fashion to those described previously in mice except that food pellets were substituted for pea shingle (small stones) (Deacon et al., 2002a,b). Rats were given baseline burrowing tests for 2 days before surgery. For chronic IL-1β studies, rats were tested for burrowing beginning on the first day after the operation, whereas for the acute IL-1β studies, rats were tested 90 min post-surgery. At approximately 4.30 pm, 2.5 h before the start of the dark phase at 7 pm, rats were placed in individual cages with a black plastic tube (burrow) sealed at one end filled with 2.5 kg of pea shingle and elevated 5 cm at the other end. Measurements were taken of the weight of material displaced after the first 2 h and overnight (12 h).

Locomotor activity test

A locomotor activity test was carried out essentially as we have described previously (Deacon et al., 2001a,b). Locomotor activity was assessed at 10 am daily, excluding the post-operative day. The rats were placed individually into a set of 24 hanging wire cages (39 × 24 × 24 cm; Modular Systems and Developments Co. Ltd, London, UK) in which two horizontal photocell beams were located along the long axis of each cage (2 cm above the floor and 13 cm apart). Activity data were collected by a computer (Acorn Archimedes RISC PC 600) with specialised software (Arachnid Activity Monitor, Paul Fray Ltd, Cambridge, UK). The total number of beam breaks and the number of crossovers (when the front and the back beam were broken consecutively) made by each animal were recorded in 5-min time intervals.

Statistical analysis

The data were presented as mean ± SEM at each time point. When statistical analysis was employed, data were analysed relative to the saline vehicle control values at the identical time point by a

one-way ANOVA. For behavioural analysis, pairwise comparisons between control and IL-1β-treated groups were made with either the Mann–Whitney *U* test (for burrowing, data were non-parametric) or Student's *t*-test (activity and body weights data). Results were considered significant when $p<0.05$. Animal numbers are displayed in figure legends.

Results

We have previously found that we can achieve chronic focal IL-1β expression using a replication-deficient recombinant IL-1β-expressing adenovirus (AdIL-1β) in the rat brain (Ferrari et al., 2004). In this study, we were interested to determine whether a widespread and prolonged inflammatory lesion in the brain would give rise to chronic sickness behaviour, an extended hepatic acute phase response and/or axonal injury. To this end, we microinjected AdIL-1β or control intracerebroventricularly (ICV) at a range of doses (1×10^7 pfu, 1×10^8 pfu, 5×10^8 pfu) and assessed spontaneous behaviours, CNS integrity and the systemic APR.

AdIL-1β-mediated chronic IL-1β overexpression in the brain gives rise to decreased spontaneous behaviours in a dose-dependent manner

We first sought to identify a behavioural test that would allow us to assess changes in spontaneous activity. In mice, food-pellet burrowing has been previously used as a relatively simple model of spontaneous behaviour that can be performed daily without evidence of habituation (Deacon et al., 2002a,b). In our previous studies, control rats presented with containers of food in their home cages did not empty them in the way that mice do (Deacon et al., 2002a,b). However, we have recently discovered that rats do indeed burrow if given 10 mm pea shingle (small stones). To assess whether rats burrow following an acute IL-1β challenge, we injected rat recombinant IL-1β (4 μg/kg) or vehicle intraperitoneally and tested the burrowing activity for a 2-h period, immediately followed by an overnight (12 h) duration on sequential days (Figs. 1A and B). We find that the IL-1β-treated rats displayed a significant depression in burrowing activity over the initial 2-h test period ($p=0.017$) but displayed a return to baseline levels, established over the previous two days, over the corresponding period on the next day. No change was observed in the overnight burrowing activities after the injection of IL-1β compared to the baselines values.

After demonstrating that a bolus injection of IL-1β induces changes in short-term burrowing behaviour, we then sought to establish the effects of chronic widespread IL-1β expression in the brain on spontaneous activity. Changes in burrowing following administration of the range of adenoviral doses were assessed each day from day 1 post-surgery, for a 2-h period, immediately followed by an overnight (12 h) period, on sequential days (Figs. 1C–F). The 5×10^8 pfu dose proved to be unusable as the animals became overtly unwell and immobile, and thus we were unable to complete the experiment (results not shown). No significant differences in burrowing activity were observed between rats receiving vehicle control or inactivated virus (Fig. 1C). At the lowest dose of AdIL-1β (1×10^7 pfu), there was a transient depression of burrowing for 3 days, reaching a trough on day 2 post-treatment, which was not statistically significant for the 2-h duration test (Fig. 1D). By day 4 the AdIL-1β group exhibited burrowing behaviour at control levels. The 1×10^8 pfu dose produced a profound and enduring depression of burrowing activity (Figs. 1E and F), indicating that the burrowing test was sufficiently sensitive to detect dose-dependent changes in cytokine expression. Locomotor activity at the 1×10^8 pfu dose was also similarly depressed (Fig. 2A) and thus burrowing correlates well with established locomotor tests used in rats. All subsequent analysis was performed on animals receiving the 1×10^8 pfu dose of AdIL-1β.

AdIL-1β-mediated chronic IL-1β expression in the brain is associated with cachexia

We noted, as have others (Lucas et al., 2006), that chronic IL-1β expression gives rise to a marked reduction in body weight (Fig. 2B). The failure to perform in the behavioural experiments was unlikely to be as a consequence of the weight loss since the animals performed most poorly on the first days after the AdIL-1β challenge, when weight loss was only just beginning. Interestingly, the animals appeared to continue to eat and drink normally throughout the experimental period (data not shown).

AdIL-1β-mediated chronic IL-1β expression is associated with a loss of CNS integrity

For up to 8 days, after the injection of AdIL-1β we could detect elevated levels of rat IL-1β ($p<0.04$) in the brain, a finding that was not observed in control-virus-injected brains (Fig. 3A). After 8 days we observe persistently elevated levels of IL-1β and long-term behavioural changes, and we were interested to determine whether there were any concomitant long-term changes in CNS integrity. To this end, BBB breakdown, leukocyte recruitment and axonal damage were assessed by immunohistochemistry. Widespread recruitment of large numbers of both neutrophils (Fig. 3B) and ED-1-positive mononuclear cells (Fig. 3C) was observed in the AdIL-1β-challenged brains spread along the anteroposterior axis. The highest densities were detected around the injected ventricle and in the meninges, although recruited leukocytes were also visible in large numbers throughout the entire ipsilateral hemisphere including the cortex, striatum, medial septum, hippocampus and thalamus. Marked widespread vasodilatation was observed, with vessels filled with marginated leukocytes, although large numbers of leukocytes had diapedesed into the brain parenchyma. As expected, no recruited leukocytes were observed in vehicle-injected brains or in the control vector-injected brain at the 8-day time point (results not shown). The BBB was damaged in the AdIL-1β-injected brains (results not shown) and axonal damage was also clearly evident, as illustrated by the accumulation of axonal precursor protein (APP) in damaged fibres in regions of marked leukocyte infiltration (Fig. 4). As before, no overt neuronal loss was observed in the brain (results not shown). The pathology of the vehicle-injected brains was comparable to that observed in a naïve animal of a similar age (results not shown).

AdIL-1β-mediated chronic IL-1β expression is associated with differential changes in the extracellular matrix

Our previous experiments with AdIL-1β in the lung and peritoneum (Kolb et al., 2001; Margetts et al., 2002) led us to expect evidence of fibrosis or the deposition of abnormal matrix in the brain by 8 days. Surprisingly, the chronic expression of IL-1β did not give rise to a fibrotic response. Laminin was observed as continuous labelling around vessels, and increased deposition was

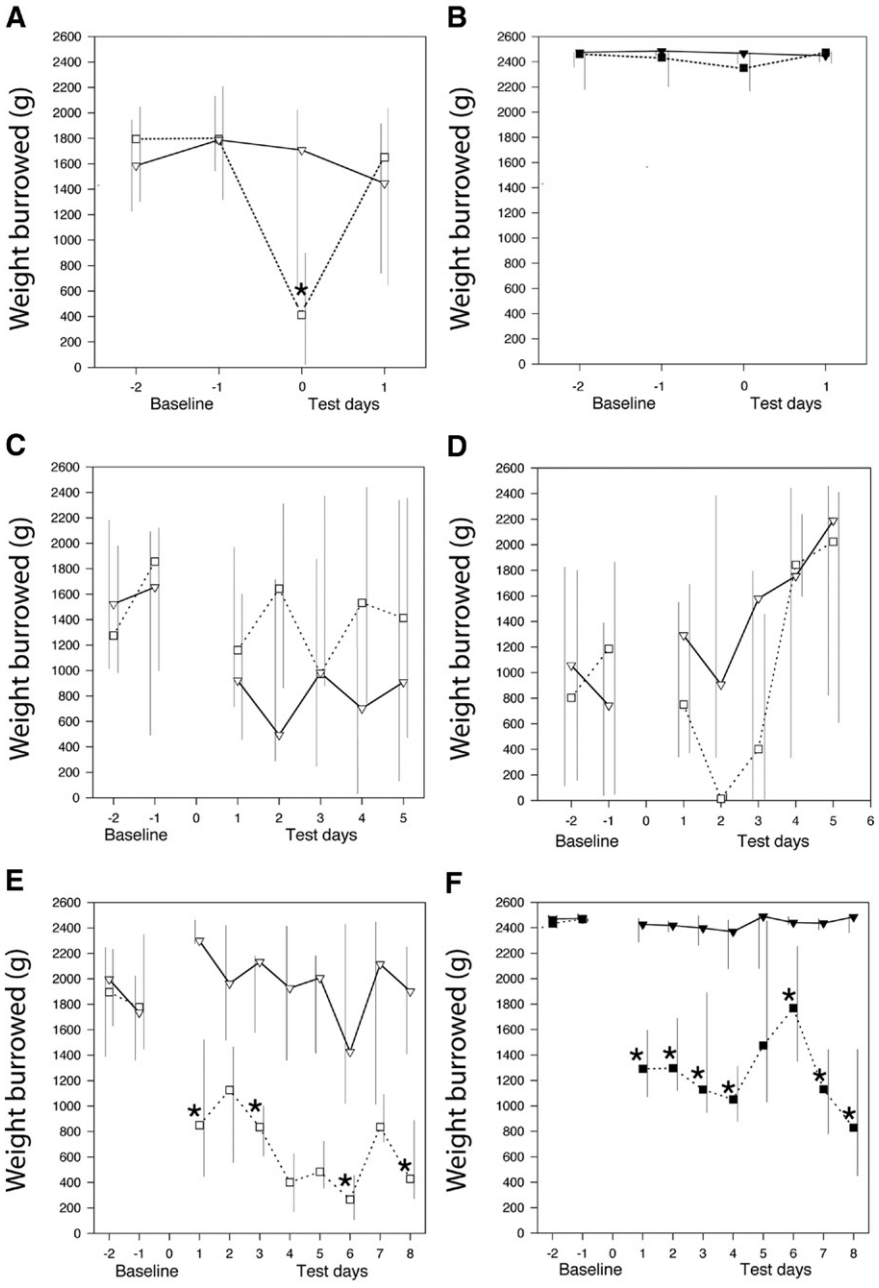

Fig. 1. The presence of IL-1β in the brain reduces spontaneous behaviour. Control values are shown as triangle symbols, and AdIL-1β or recombinant rat IL-1β protein values are shown as square symbols. Unfilled symbols show the weight burrowed in a 2-h period and symbols filled in black show the weight burrowed overnight. The injections were given on day 0. (A, B) Effects of recombinant rat IL-1β (1 μg) on rat burrowing measured each day (A) after a 2-h period and (B) overnight, $n=12$ per group. (C) The effect of denatured AdIL-1β on burrowing, $n=6$ per group. No comparisons (control versus test) reached statistical significance. (D) Effect of a low dose (1×10^7 pfu) of AdIL-1β on rat burrowing, $n=6$ per group. (E, F) Effect of a moderate dose (1×10^8 pfu) of AdIL-1β on rat burrowing measured after 2 h (E) and overnight (F), $n=5$ for control and $n=6$ for AdIL-1β. Note that 1×10^8 pfu significantly inhibits spontaneous behaviour over the 8-day test period. Values are medians and interquartile ranges. *$p<0.05$ compared to corresponding control group (Mann–Whitney U test).

observed in cuffs of leukocytes close to sites of diapedesis (Fig. 3D). In contrast to the vessel-associated increased laminin expression, collagen type 1 was lost entirely from the brain vasculature in vessels actively participating in leukocyte recruitment [compare Fig. 3E (control) and 3F (AdIL-1β)]. Glial fibrillary acidic protein (GFAP), a marker of gliosis, was observed at similar levels in both the control (Fig. 3G) and AdIL-1β-treated (Fig. 3H) brains, although around the vessels there was evidence of gliosis (Fig. 3H).

De novo hepatic CXC and CC chemokine expression is chronically elevated and results in subsequent leukocytosis and recruitment of neutrophils to the liver

To discover whether a chronic inflammatory response in the brain gives rise to a chronic activation of the hepatic chemokine response, we analysed the livers of AdIL-1β-challenged animals and controls for CXCL-1 (Fig. 5A) and CCL-2 chemokine (Fig. 5B)

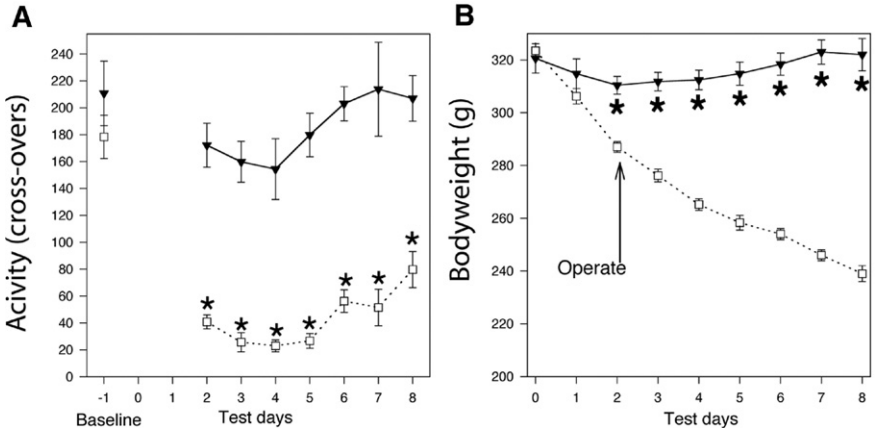

Fig. 2. Chronic IL-1β expression in the brain generates weight loss and decreased open field activity. Effect of 1×10^8 pfu AdIL-1β (unfilled squares) or control dl70-3 adenovirus (black triangles) on (A) the number of beam breaks over a 5-min period and (B) body weight. *$p < 0.05$ compared to corresponding control group (Student's *t*-test). Values are means and bars are SEMs; $n = 5$ controls, 6 AdIL-1β. (In B, the SEMs are sometimes too small to be visible.)

mRNAs by quantitative RT-PCR. Hepatic expressions of CXCL-1 mRNA ($p < 0.04$) and CCL-2 mRNA ($p < 0.003$) were significantly elevated compared to controls at this late time point.

AdIL-1β-mediated chronic IL-1β expression in the brain is associated with a prolonged leukocytosis

To determine whether a persistent leukocytosis associated with an ongoing acute phase response was present at the 8-day time point, we examined the numbers of leukocytes (Fig. 5C) and neutrophils (Fig. 5D) in the blood of AdIL-1β-injected and control animals. Significant elevations in total circulating leukocytes ($p < 0.01$) and neutrophils ($p < 0.005$) were observed in AdIL-1β-treated rats as compared to controls. No similar elevation was however observed in the numbers of circulating blood monocytes (data not shown).

Recruited leukocytes cause damage to the liver when IL-1β expression is prolonged in the brain

To determine whether the persistently elevated hepatic chemokine levels resulted in the enhanced recruitment of leukocytes to the liver, we examined the numbers of neutrophils by immunohistochemistry at the 8-day time point. Compared to controls, increased numbers of neutrophils were observed in the liver at the 8-day time point (Figs. 5D and E; $p < 0.0003$). We have previously shown that leukocyte recruitment to the liver after acute brain injury generates hepatocellular damage (Campbell et al., 2003), and we were interested to discover whether chronic central AdIL-1β expression was also associated with liver damage. We used immunohistochemistry to assess the number of cells in the liver expressing the cell damage response indicator Gadd45 (growth arrest and DNA damage) or activated caspase-3. Cells positive for Gadd45 (Fig. 6A) and activated caspase-3 (Fig. 6B) were widely distributed in the livers of rats 8 days after the microinjection of AdIL-1β, but not in the livers of the control animals. To evaluate whether other classical markers of the acute phase response accompany the extended hepatic chemokine response, we tested for the presence of the negative acute phase protein, serum protein albumin, in vehicle-injected controls and in dl70-3-injected and AdIL-1β-injected animals after 8 days (Fig. 6C). We found that the serum levels of albumin were decreased in AdIL-1β-injected rats, indicative of an ongoing acute phase response.

Discussion

In our present experiments, we have investigated the effects of chronic CNS expression of IL-1β on parameters that have been previously unexplored. In particular, we have examined the behavioural consequences of widespread chronic IL-1β expression in the brain, and whether prolonged IL-1β expression is associated with injury to axons. The extended expression of IL-1β over 8 days generated chronic leukocyte recruitment to the CNS and a prolonged depression in spontaneous behaviour as assessed by locomotor activity and burrowing. In addition to demyelination and BBB breakdown, axon injury also occurs as a consequence of chronic IL-1β expression in the brain. Furthermore, we have shown that for as long as there is an inflammatory response in the CNS, a systemic acute phase response is maintained. The systemic response to central IL-1β resulted in sustained weight loss, elevated levels of hepatic and circulating CXCL-1 and CCL-2 chemokines, leukocytosis and a significantly elevated number of leukocytes recruited to the liver. IL-1β could not be detected in the circulating blood. The cellular indicators of hepatocellular damage, activated caspase-3 (Wanner et al., 1999) and Gadd45 (Ito et al., 2006), were significantly elevated in response to chronic central IL-1β expression. Thus, our findings reveal that for as long as there is production of central cytokines in response to injury or disease, the hepatic chemokine response remains active and continues to amplify and promote leukocyte recruitment to the CNS and to the liver. Each of these findings will be discussed in turn below.

AdIL-1β-mediated chronic IL-1β expression in the brain gives rise to decreased spontaneous behaviours in a dose-dependent manner

The chief characteristic of animals or humans that are sick is a reduction and reprioritisation of their behaviours. Although this is mostly shown simply as a reduction in activity, decreases in specific behaviours such as eating and social interaction may also be noted (Dunn et al., 2005). An important component of the present study was to develop and characterise a simple robust behavioural task that would be suitable for use in a non-specialised laboratory to

study the behavioural response to injury and disease in rats. Burrowing is a spontaneous behaviour not previously modelled in rats, which, as we have found, enables repeated testing with little habituation to the task. The burrowing paradigm was first used to detect, in mice, early signs of scrapie disease (Deacon et al., 2001a,b). It was also shown to be sensitive to cytotoxic hippocampal lesions (Deacon et al., 2002a,b). However, studies in rats failed, as rats, unlike mice, are unwilling to burrow food pellets in their home cage. Fortunately, it was discovered that rats would burrow provided that the substrate did not consist of food pellets; earth-like materials (pea-shingle) were readily displaced. We used a single bolus injection of IL-1β to validate the burrowing technique prior to the assessment of the effects of chronic adenoviral-mediated IL-1β expression on the spontaneous burrowing behaviour. There was no evidence that burrowing was a more sensitive measure of IL-1β behavioural depression than an assessment of locomotor activity. Notably, however, rats appear to enjoy burrowing and it may therefore have advantages in situations such as sickness behaviours where hedonistic activities (such as consumption of sweet solutions (Willner et al., 1996) are particularly disturbed. Furthermore, burrowing tests are simple to perform and the experiments require very little prior experience with behavioural tests on the part of the scientist.

The behavioural experiments were ended after 8 days as there was evidence of recovery; we were interested in relating the behavioural data to our molecular and histological outcome measures. Over the 8-day period, there was little evidence of tachyphylaxis to the continued expression of IL-1β. In the present study, the decrease in locomotor activity and burrowing activity was not directly related to the loss of body weight in the rats as they were apparent soon after the operation when relatively little weight loss had occurred. Moreover, while the body weights progressively declined over the course of the experiment, the behaviour pattern did not parallel this decline. The IL-1β-treated rats appeared quieter and less active, but there were no clinical signs of malaise such as piloerection, hunched posture or prolonged immobility; this may be because the weight loss, although notable towards the end of the experiment, was gradual, with most rats appearing to eat normally every day.

AdIL-1β-mediated chronic IL-1β expression in the brain is associated with cachexia

It is now well established that IL-1β plays an important role in cachexia in rodents and humans (Roubenoff et al., 1994; Langen et al., 2001). Recent experiments have shown that mice gene-deleted for IL-1β and IL-6 gain weight and that the administration of IL-1β either centrally or peripherally causes weight loss (Lawrence and Rothwell, 2001). Other experiments have shown that the chronic ICV delivery of pathophysiological doses of several cytokines (IL-6, IL-8 and TNF-α) revealed that IL-1β was the most potent

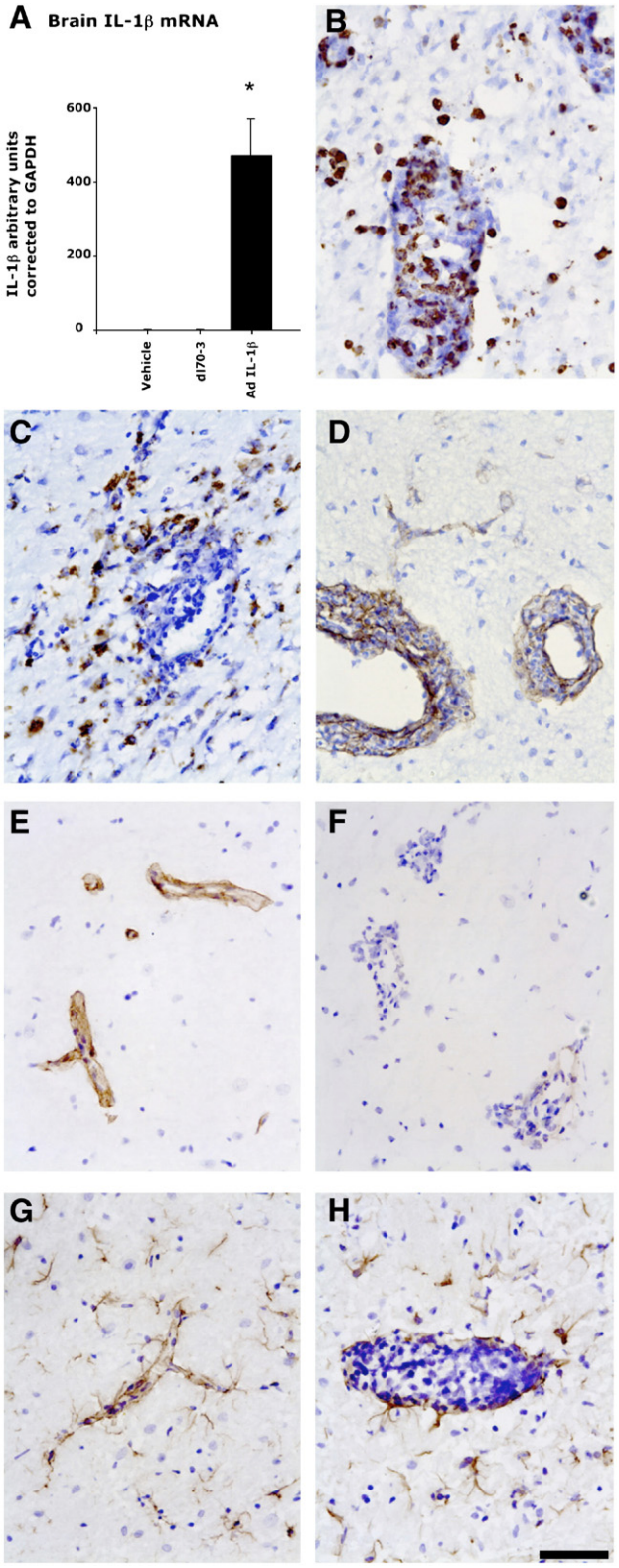

Fig. 3. IL-1β expression in the brain after the intracerebroventricular microinjection of AdIL-1β. (A) The amount of IL-1β mRNA was determined by quantitative RT-PCR 8 days after the ICV microinjection of vehicle, dl70-3 (1×10^8 pfu, control virus) or AdIL-1β (1×10^8 pfu) into the brain. Note the elevated levels of IL-1β mRNA in response to AdIL-1β, but not after the control virus or saline vehicle. (B–G) Photomicrographs of sections adjacent to the injection site showing the effect of AdIL-1β on CNS integrity. Neutrophils (B) and ED-1-positive mononuclear cells (C) are recruited to the brain. The deposition of laminin (D) around the vessel is a striking feature of the pathology. Vessel-associated collagen-type-I staining in control (E) and in AdIL-1β-injected brain (F) reveals the loss of collagen-I from the basement membrane of vessels with cuffs of leukocytes. No differences were observed in GFAP immunoreactivity in control (G) and AdIL-1β-injected (H) brains. Scale bar represents 100 μm. *$p<0.05$ compared to corresponding control group.

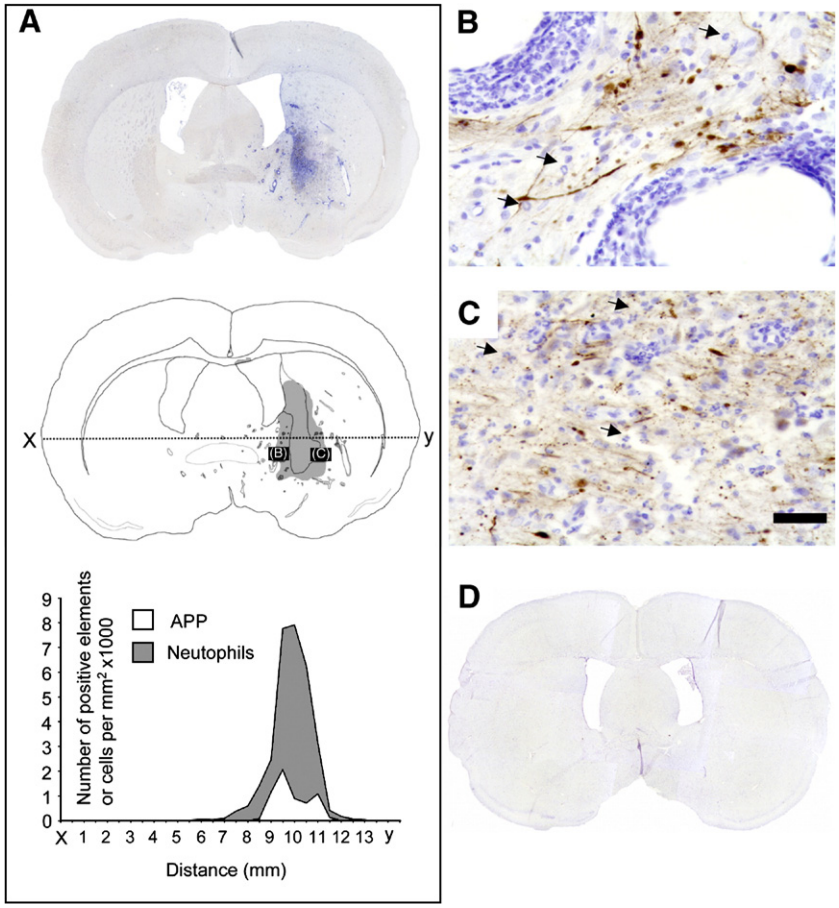

Fig. 4. Chronic AdIL-1β expression in the brain results in axonal damage. (A) Photomicrograph and accompanying schematic of a coronal brain section 8 days after the microinjection of AdIL-1β: the region shaded in grey on the schematic indicates the area of hypercellularity which can be seen as an intense purple region in the cresyl-violet-stained section. The number of neutrophils and APP-positive elements on the transect (*X–Y*, dotted line) have been quantified to reveal the association between neutrophil recruitment and axonal injury. (B, C) High-power photomicrographs show representative regions of APP-immunopositive endbulbs at the locations detailed in the schematic. Note the immunolocalization of APP adjacent to vessels associated with leukocyte recruitment (B) and on the border of the hypercellular region (C). Black arrows indicate the characteristic multi-lobed cresyl-violet-counterstained nuclei of neutrophils present in large numbers in the hypercellular regions. (D) Low-power photomicrograph of a section through the brain adjacent to the injection site of a control virus (dl70-3)-injected brain. Note the absence of any hypercellularity. Scale bar represents 50 μm.

anorectic of the cytokines tested (Plata-Salaman, 1998). Interestingly, in contrast to previous studies we recorded extended weight loss, with little evidence of a reduction in water or food intake. A recent study has shown that in MyD88-deficient mice anorexia and weight loss can be dissociated following treatment of the animals with either LPS or IL-1β, which indicates that separate mechanisms underlie these phenomena (Ogimoto et al., 2006). The mechanism by which IL-1β contributes to anorexia is becoming clearer after experiments that suggest that the cytokine and melanocortin systems may be linked. IL-1β-induced anorexia can be reversed in rats by co-administration of melanocortin-3/4 receptor antagonist SHU9119 (Lawrence and Rothwell, 2001). However, despite advances, it remains unclear how neuroinflammatory pathways within the brain communicate signals to the periphery that can result in metabolic wasting. There is some evidence that IL-1β expression may increase in neurons in some areas within the brain that are responsible for regulating energy homeostasis, but the present results are equivocal (King et al., 2000; Guijarro et al., 2006). Compared to the practical difficulties associated with the implantation of Alzet minipumps or the cost of the recombinant cytokines, the single ICV injection of AdIL-1β represents a useful model to study the mechanisms that underlie cachexia.

AdIL-1β-mediated chronic IL-1β expression is associated with a loss of CNS integrity

In this study we used amyloid precursor protein (APP) immunocytochemistry to investigate whether chronic IL-1β expression in the brain might be associated with axon injury. The detection of APP accumulation in axon end bulbs is now used extensively as a method for detecting axonal injury (Sherriff et al., 1994). Closed head injury and spinal cord trauma in man (Ahlgren et al., 1996; McKenzie et al., 1996) and traumatic brain injury in rats (Bramlett et al., 1997) are all examples of settings in which APP immunocytochemistry has been used to identify the presence of injured axons. The application of APP immunocytochemistry to multiple sclerosis (MS) lesions has shown that axon injury occurs early in the evolution of an MS lesion and the extent of axon injury is closely correlated with the number of inflammatory cells (Ferguson et al., 1997; Trapp et al., 1998). Interestingly, in immune-mediated

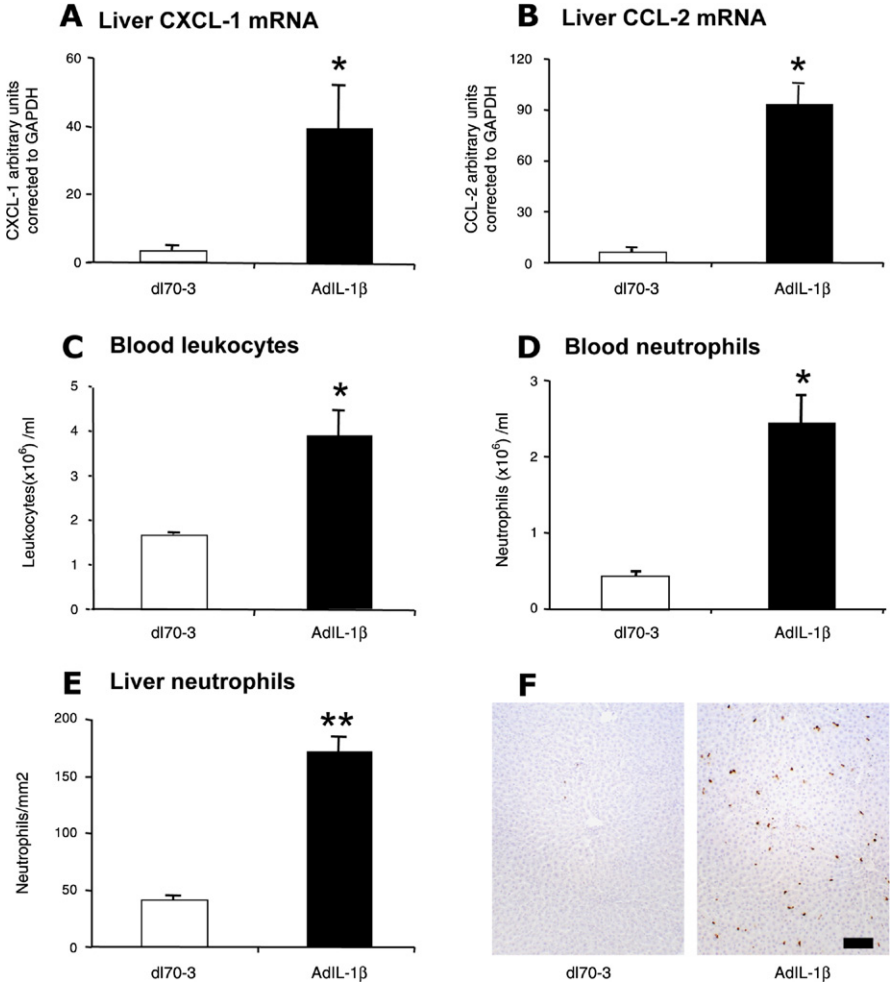

Fig. 5. A prolonged hepatic APR is associated with chronically elevated IL-1β in the brain. Liver was evaluated for markers of the APR 8 days after the microinjection of control virus or AdIL-1β (1×10^8 pfu) into the brain. Hepatic CXCL-1 mRNA (A) and CCL-2 mRNA (B) were assessed by quantitative RT-PCR. Total circulating leukocytes (C) and circulating neutrophils (D) were found to be increased using a haematology analyzer, and marked neutrophil recruitment to the liver (E) accompanied the microinjection of AdIL-1β into the brain. (F) Representative photographs of immunopositive neutrophils (brown) in the liver following dl70-3 control virus or AdIL-1β. The sections are counterstained with Mayer's haematoxylin. Scale bar is 100 μm. *$p < 0.05$ compared to the corresponding control virus group.

lesions, axonal injury, such as that seen in MS, does not depend upon an immune response directed against a particular CNS antigen. An immunologically non-specific response can give rise to 'bystander' axonal injury and is thought likely to be mediated via molecules secreted by macrophages in the presence of T-cells. In the present study, we show that axonal injury is also a feature of an innate inflammatory response in the absence of any trauma. It is now often asserted that leukocyte recruitment to the spinal cord after injury can have a beneficial effect, and that macrophages and CNS-resident microglia in particular can be protective (Schwartz, 2003). While we accept that granulocytes may facilitate endogenous repair processes such as axonal sprouting and remyelination, it is clear that their presence in a CNS lesion can lead to axonal injury. Thus, therapies targeting the destructive effects of neutrophils and macrophages are likely to preserve CNS integrity in the first instance. It may be possible with adjunct therapies to mimic some of the reparative functions attributed to granulocytes later in the pathogenesis.

In comparison to an intraparenchymal injection of AdIL-1β, where recruitment was limited to neutrophils, we found that the leukocyte recruitment profile in response to IL-1β is less restricted after an ICV injection of the adenovirus. This is consistent with previous studies, which have shown that if the meninges or choroid plexus are involved the inflammatory response is often more reminiscent of a peripheral inflammatory response (Andersson et al., 1992). However, no lymphocytes could be detected in our AdIL-1β-induced lesions. In our previous studies, in which the same adenoviral vector AdIL-1β was introduced into the peritoneum or into the lung, neutrophils dominated the inflammatory response, but the mononuclear cells were also present (Kolb et al., 2001). A similar pattern was observed in the brain after the ICV injection of AdIL-1β. Both neutrophils and macrophages are able to release matrix metalloproteinases (MMPs). We have shown that the injection of MMPs into the subcortical white matter leads to the formation of large numbers of APP-positive end bulbs and that axons in this tract are differentially sensitive to certain members of the MMP family (Anthony et al., 1998a,b). MMP-9 caused more axon injury than MMP-2 and in turn MMP-2 was more damaging than MMP-7. Neutrophils express MMP-9 constitutively, and this is likely be an important contributor to the axonal injury observed in

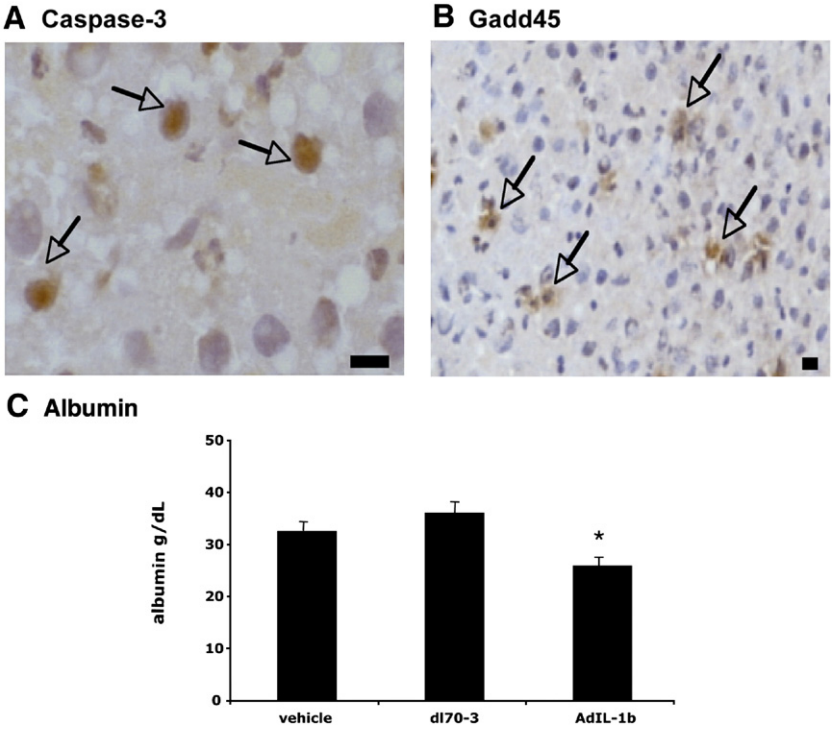

Fig. 6. Chronic IL-1β expression in the brain causes hepatocellular damage and an extended acute phase response. Liver damage was assessed by immunohistochemistry 8 days after the microinjection of vehicle, dl70-3 (1×10^8 pfu, control virus) or AdIL-1β (1×10^8 pfu) into the brain. (A, B) Immunoreactive caspase-3 (A) or Gadd45 (B), markers of liver cell damage, in response to AdIL-1β (brown stain). Nuclei are counterstained with Mayer's haematoxylin (blue). Scale bar represents 10 μm. The levels of serum albumin were assessed by quantitative spectrophotometry in the same samples (C). Note the reduction in serum albumin in response to AdIL-1β, which is indicative of an on-going acute phase response. *$p<0.05$ compared to the corresponding control virus group.

our AdIL-1β-induced lesions. The precise mechanism by which an MMP leads to axon injury remains unclear, but the MMPs have been shown to influence the morphology of mature dendritic spines and have also been implicated in apoptotic cell death in other organs through modulation of the extracellular matrix (Ishizuya-Oka et al., 2000). Neutrophils are also the source of a number of other toxic metabolites, such as oxygen free radicals, that may also damage axonal integrity.

AdIL-1β-mediated chronic IL-1β expression is associated with differential changes in the extracellular matrix

The transient overexpression of IL-1β in the epithelial cells of rodent lung following AdIL-1b induces acute inflammation with alveolar tissue destruction, resulting in progressive interstitial fibrosis (Kolb et al., 2001). IL-1β is a potent inducer of TGF-β, and it is suggested that at least a part of its pro-fibrotic effect is mediated through this growth factor. We were interested to discover whether IL-1β would have a similar effect in the brain, where TGF-β is constitutively expressed. The extracellular matrix of the brain is unusual and is comprised predominantly of lecticans, which contain a lectin domain and a hyaluronic acid-binding domain, and two extracellular matrix components – tenascins and hyaluronic acid – to which the lecticans bind (Dityatev and Schachner, 2003). In contrast to other tissues, the brain contains relatively few fibrous matrix proteins, such as collagens, fibronectin, vitronectin and laminin. In contrast to the effect of IL-1β in the lung, there was no evidence of fibrosis in the brain at 8 days. It is possible that fibrotic changes may have followed, but we have also examined tissue from the brains of animals killed 30 days after the focal intraparenchymal injection of AdIL-1β and still have observed no evidence of fibrosis. However, the change in the composition of the basement membrane around vessels was striking. There was a loss of collagen and an increase in laminin staining. These vessels also exhibited BBB breakdown. Thus, remodelling, rather than destruction, of vessels is a feature of active chronic inflammation in the brain.

De novo hepatic CXC and CC chemokine expression is chronically elevated and results in subsequent leukocytosis and recruitment of neutrophils to the liver

Overexpression of IL-1β in the lung results in signs of respiratory distress and systemic involvement, such as fever and weight loss, which are accompanied by increased serum levels of the acute phase protein α1-cysteine-protease inhibitor during this period. It is well known that IL-1β induces a hepatic acute phase reaction with upregulated acute phase proteins (Wilcockson et al., 2002; Campbell et al., 2005), but it was not clear until now whether a chronic inflammatory response in the brain would generate an extended acute phase response and whether chemokines, produced acutely in response to injury, would be expressed for a longer period.

In our present studies, IL-1β mRNA was still detectable in the brain 8 days after the intracerebroventricular injection of AdIL-1β.

In our previous studies, IL-1β was still detectable up to 30 days after the intraparenchymal injection of the adenovirus. We were concerned that introduction of the virus into a more permissive environment, in terms of the inflammatory response, might cause the virus to be more rapidly cleared and that some IL-1β might be transported into the blood. This was not the case; expression was maintained, and we could not detect the presence of IL-1β in the circulation by a highly sensitive ELISA. Following a single bolus injection of IL-1β, others have shown that some escapes from the CSF into the peripheral circulation (Banks et al., 1991; De Simoni et al., 1993), but the production of IL-1β by the virus is likely be at a level that can be cleared by endogenous mechanisms. Similar findings have been reported in other models. Traumatic brain injury in the rat results in the local production of IL-1β, but no IL-1β can be detected in the plasma of these animals after the injury (Kamm et al., 2006). Thus, the mechanism(s) by which injury signals are conveyed to the liver to generate an extended acute phase response remain unclear.

Following acute brain injury, and, most likely, other injuries, we hypothesise that the absolute level of circulating CXC and CC chemokines is a pivotal factor governing the process of leukocyte recruitment (Campbell et al., 2005). The microinjection of recombinant IL-1β into the brain is associated with the hepatic expression of CXCL1, which appears to control the timing and magnitude of neutrophil mobilisation and subsequent recruitment to the brain and probably other injuries. No tachyphylaxis was observed following intracerebroventricular injection of AdIL-1β, and the production of chemokines by the liver remained high over the 8-day period. Thus, the accepted model of leukocyte recruitment along a chemokine gradient, which proposes that local chemokines at the site of injury attract a circulating population of phagocytes from the blood up a concentration gradient appears to be an oversimplification. Our new studies now raise further problems: high levels of chemokines are present in the blood throughout the period when local chemokines are being produced, thus confounding the establishment of the necessary chemokine gradients. We had previously shown that the hepatic synthesis of chemokines was transient, which suggested that the gradient might reverse to permit entry (Campbell et al., 2003). We now know that this is not the case, but that leukocyte recruitment to the brain and to the liver remains active. Interestingly, others have noted that experimental allergic encephalomyelitis in mice is associated with hepatic chemokine expression, which suggests that hepatic chemokine synthesis may also be a feature of immune-mediated disease in the CNS (Glabinski et al., 1995). It is clear that the classical model of leukocyte recruitment does not account for the extended production of chemokines by the liver in response to injury.

Recruited leukocytes damage the liver when IL-1β expression is prolonged in the brain

Peripheral injections of lipopolysaccharide (Tkalcevic et al., 2000) or the adenoviral-mediated expression of CINC in the liver (Maher et al., 1997) are associated with increases in the number of neutrophils in the liver and elevations in markers of hepatocellular damage. We were surprised to discover that after focal acute lesions to the brain the neutrophil recruitment to the liver gives rise to hepatocellular injury. Now over an extended period we have found that the number of leukocytes recruited to the liver remains elevated, and that indicators of hepatocellular damage are still present at 8 days. This secondary organ damage may be similar to the pathology that often occurs in a number of organs distant from the brain in patients with acute brain injury and may be a factor determining clinical outcome.

Thus, the findings in this study imply that the hepatic production of chemokines may represent a useful target for acute and chronic brain injury and that such therapies need not necessarily cross the BBB, as has been previously thought. Furthermore, the inhibition of systemic chemokine production may also guard against the systemic sequelae of acute brain injury.

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