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RESEARCH ARTICLE

Genetic regulation of microglia activation, complement expression, and neurodegeneration in a rat model of traumatic brain injury

Bo-Michael Bellander · Olle Lidman · Marcus Ohlsson · Britt Meijer · Fredrik Piehl · Mikael Svensson

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Abstract Secondary brain damage following traumatic brain injury in part depends on neuroinflammation, a process where genetic factors may play an important role. We examined the response to a standardized cortical contusion in two different inbred rat strains, *Dark Agouti* (*DA*) and *Piebald Virol Glaxo* (*PVG*). Both are well characterized in models of autoimmune neuroinflammation, where *DA* is susceptible and *PVG* resistant. We found that infiltration of polymorphonuclear granulocytes (PMN) at 3-day postinjury was more pronounced in *PVG*. *DA* was more infiltrated by T cells at 3-day postinjury, showed an enhanced glial activation at 7-day postinjury and higher expression of C3 complement at 7-day postinjury. Neurodegeneration, assessed by Fluoro-Jade, was also more pronounced in the DA strain at 30-day postinjury. These results demonstrate differences in the response to cortical contusion injury attributable to genetic influences and suggest a link between injury-induced inflammation and neurodegeneration. Genetic factors that regulate inflammation elicited by brain trauma may be important for the development of secondary brain damage.

Keywords Traumatic brain injury · Inflammation · Complement · Neurodegeneration · Strain dependent · T cells · Polymorphonuclear cells

B.-M. Bellander (✉) · M. Ohlsson · B. Meijer · M. Svensson
Department of Clinical Neuroscience, Section for Neurosurgery, Karolinska University Hospital Solna, R3.02, S-171 76, Stockholm, Sweden
e-mail: bo-michael.bellander@karolinska.se

O. Lidman · F. Piehl
Department of Clinical Neuroscience, Section for Clinical CNS Research, Karolinska University Hospital Solna, S-171 76, Stockholm, Sweden

Abbreviations

cDNA	Complementary DNA
CNS	Central nervous system
DA	*Dark Agouti*
DAB	3,3′-diaminobenzidine
DDT	Dithiothreitol
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalitis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
IR	Immunoreactivity
MAC	Membrane attack complex
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
PBS	Phosphate Buffer solution
PCR	Polymerase chain reaction
PMN	Polymorphonuclear granulocytes
PVG	*Piebald Virol Glaxo*
RNA	Ribonucleic acid
ROI	Region of interest
RT-PCR	Reverse transcriptase-polymerase chain reaction
TBI	Traumatic brain injury

Introduction

Traumatic brain injury (TBI) is a common cause of disability among young adults. In addition to the direct loss of tissue caused by the trauma, secondary mechanisms leading to additional tissue injury are important for outcome and therefore constitute important therapeutic targets.

Interest has been focused on neuroinflammation as a mechanism involved in the development of secondary brain damage. Immune cells, like activated T cells (Wekerle et al. 1987; Hickey et al. 1991), monocytes, granulocytes and lymphocytes have been shown to accumulate in the traumatized cerebral parenchyma (Holmin et al. 1995; Soares et al. 1995). These cells secrete lysosomal enzymes, release free radicals (Kontos and Wei 1986; Ikeda and Long 1990) and cytokines (Giulian and Ingeman 1988; Suzumura et al. 1990), as well as induce microvascular occlusion with subsequent increased vascular permeability and edema formation (Harlan 1985; Lucchesi and Mullane 1986; Kochanek and Hallenbeck 1992).

Accumulation of blood borne immune cells at the site of the lesion is paralleled by activation of CNS-resident astrocytes and microglia, where the latter transform into phagocytic macrophages (Katayama et al. 1995; Koshinaga et al. 2000). Activated microglial cells secrete toxic compounds, such as oxygen intermediates (Théry et al. 1991), nitric oxide (Boje and Arora 1992) and glutaminergic agonists (Olney et al. 1971; Campbell et al. 1981; Faden et al. 1989; Piani et al. 1991; Vaca and Wendt 1992; Giulian et al. 1993). Microglia are involved in the activation of the complement cascade following peripheral nerve lesions (Svensson and Aldskogius 1992), experimental optic nerve lesions (Ohlsson et al. 2003), brain contusions in the rat (Bellander et al. 1996, 2004) and in the human brain (Bellander et al. 2001). A growing body of evidence indicates an important role of complement in the development of secondary brain damage (Stahel et al. 1998). However, the concept of inflammation as an altogether harmful event following TBI has been challenged (Morganti-Kossman et al. 1997; Rapalino et al. 1998; Schwartz 2003).

The variability in clinical outcome of TBI may depend on genetic factors (Teasdale et al. 1997). This may involve functional polymorphisms in genes that regulate neuronal or glial susceptibility, as well as innate and systemic immune responses. In line with this, the E4 allele of apolipoprotein E has been associated with a more severe outcome after TBI (Teasdale et al. 1997; Friedman et al. 1999). Experiments performed in various animal models underscore the impact of genetic heterogeneity on the result of standardized nerve injuries (Popovich et al. 1997; Schauwecker and Steward 1997; Inman et al. 2002; Lidman et al. 2002), including functional outcome measures (Royle et al. 1999; Steward et al. 1999). Contradictory findings have been reported concerning experimental TBI in mice following fluid percussion injury, comparing C57BL/6 J and C57BL/10 J (Witgen et al. 2006), as well as between C57BL/6, FVB/N and 129/SvEMS mouse strains following controlled cortical impact (Fox et al. 1999), where hippocampal loss was found to be more dependent on factors related to mechanics and secondary consequences of the injury instead of genetic variation.

Furthermore, mice lacking the genes for tumor necrosis factor (TNF), lymphotoxin-χ (LT-χ) and interleukin-6 (IL-6) subjected to experimental closed head injury did not differ in degree of blood–brain barrier dysfunction or amount of infiltrating polymorphonuclear neutrophils in the injured hemisphere compared to wild littermates, still a significantly higher mortality was found in the TNF/LT-χ -/- mice compared to wild littermates (Stahel et al. 2000).

The aim of the present study was to determine to what degree the response to experimental TBI, with emphasis on the inflammatory phenotype, is subject to regulation by genetic factors. In the study, we choose to use *DA* and *PVG* rats. The *DA* rat is known to be susceptible in many autoimmune disease protocols but also mounts a more severe inflammatory response following peripheral nerve injury (Lorentzen et al. 1997; Weissert et al. 1998; Lundberg et al. 2001), while the *PVG* strain is relatively resistant in these disease models.

Quantification of the inflammatory response, the extent of complement activation and subsequent cellular damage also provide important clues about the role of inflammation in the development of secondary brain damage.

Materials and methods

The experiment was reviewed and approved by the local ethic committee at the Karolinska institutet (N73/02). Seventy adult male rats, 34 *DA* (21 for immunohistochemistry, 2 sham, 6 for in situ hybridization and 5 for PCR), and 36 *PVG* (21 for immunohistochemistry, 3 sham, 6 for in situ hybridization and 6 for PCR), weighing between 200 and 250 g, were deeply anesthetized with intraperitoneal injections of 2.7 ml of a solution containing 1.0 ml Hypnorm®, 1 ml Dormicum® and 2 ml H_2O. Three rats (1 DA and 2 PVG) died in the postoperative period and were excluded from the study. Spontaneous ventilation in air was monitored by using pulse oximetry to detect hypoxia. Hypoxia was defined as persistent $SpO_2 < 90\%$. If hypoxia occurred, the animals immediately received oxygen. Body temperature, 37–38°C, was controlled using a thermistor-regulated electric blanket (LSI, Barcelona, Spain). The rats were placed in a stereotactic frame, and 1 ml of 1% Xylocain was used as local anesthetic in the skin prior to craniotomy performed 3 mm posterior to bregma and 2 mm lateral to the midline on the left side. A standardized, reproducible, parietal contusion, modified from Feeney (Feeney et al. 1981; Holmin et al. 1995; Bellander et al. 1996), was created by dropping a 24-gram weight from the height of 4 cm onto a piston 1.8 mm in

diameter, which rested on the dura and performed a compression in the cortex of approximately 2 mm.

The rats processed for immunohistochemistry were re-anesthetized and perfused with saline at body temperature followed by cold 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.3–7.4) at 3-, 7- and 30-day postinjury. Postfixation of the brain was performed for 1–2 h in the same fixative and was followed by wash over night at 4°C in 0.15 M phosphate buffer with 10% (w/v) sucrose. Fourteen-micrometer-thick sections of the brain containing the entire lesion area were serially cut on a cryostat. The slides were mounted for immunohistochemistry and Fluoro-Jade staining.

Rats to be processed for in situ hybridization or reverse transcriptase-polymerase chain reaction (RT–PCR) were re-anesthetized and perfused with saline at body temperature at 7-day postinjury. The brains were frozen in liquid methyl butane with dry ice.

Immunohistochemistry

Immunohistochemistry was performed using the ABC method (Vectastain Elite ABC peroxidase kit, Vector laboratories Inc. Burlingame, CA) as previously described (Bellander et al. 1996). Briefly, sections were rehydrated in phosphate buffer solution (PBS) and then incubated for 1 h in bovine serum albumin rinsed in PBS and incubated overnight at 4°C with primary antibody diluted in PBS with 1% bovine serum albumin and 0.3% triton-X. After washing in PBS, sections were incubated for 1 h with the appropriate biotinylated secondary antibody, washed again with PBS and incubated with avidin–biotin complex for one hour (Vectastain Elite ABC kit; Vector). Immunoreactivity was developed with 3,3'-diaminobenzidine (DAB, Vector SK-4100) for 4 min, and the reaction was stopped by rinsing in distilled water. Subsequently, sections were counterstained with hematoxylin and mounted in a non-aqueous DPX mounting medium (BDH Laboratory supplies, Poole, England).

The following antibodies were used: Mouse anti-rat CD43 (w3/13; T cells and PMN) (Barclay 1981; Dallman et al. 1984), mouse anti-rat OX42 (microglia) (Robinson et al. 1986); and mouse anti-rat ED1 (phagocyting macrophages) (Damoiseaux et al. 1994). All antibodies used in this paper are listed in Table 1.

Fluoro-Jade (FJ; Histochem Inc. Jefferson, AR.), a marker of neuronal degeneration (Schmued et al. 1997), was diluted to a working solution of 0.00002% in 0.1% acetic acid, and sections were incubated for 30 min. Sections were rinsed in distilled water and dehydrated through a series of graded ethanol, air-dried and dipped in xylene and then mounted in DPX medium.

In situ hybridization

Fresh frozen tissue was sectioned using a cryostat (14 μm) and mounted on slides (SuperFrost*/Plus, Menzel-Gläser Braunsweig, Germany). The sections were dehydrated in a graded ethanol series and air-dried. The oligonucleotides used were a 48-mer (antisense) oligonucleotide probe complementary to nucleotide 3,301 to 3,348 of complement C3 (de Bruijn and Fey 1985; Svensson and Aldskogius 1992) and a 48-mer sense probe (Scandinavian Gene Synthesis AB, Sweden). These were labeled at the 3′ end with [alpha ^{35}S]-dATP (du Medical NEN, Bruxelles, Belgium) using terminal deoxyribonucleotidyl transferase (TdT, Takara, Amersham Pharmacia Biotech, Uppsala, Sweden) at 37°C for 1 h and purified using mini Quick Spin Oligo Columns (Roche) to a specific activity of approximately 8×10^8 cpm/μg.

Hybridization was performed using a modified protocol from Svensson et al. (1995). The hybridization cocktail contained 50% formamide, 4 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1 × Denhardt′s solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0,02% bovine serum albumin; Sigma), 10% dextran sulfate (Amersham), 0.05 mg/ml heat-denaturated salmon sperm DNA (Sigma), 1% sacrosyl (N-lauroylsarcosine), 0.02 M $NaPO_4$ (pH 7,0), 50 mM dithiothreitol (DDT, Amersham, OK.) and 5×10^6 cpm/ml of probe. Sections were incubated with hybridization cocktail (100 μl/slide) overnight at 42° in a sealed chamber humidified with 4 × SSC and 50% (v/v) formamide. After hybridization, the slides were removed from the chamber and washed in 1 × SSC for 1 h at 55°C and 1 min at room temperature, followed by a brief rinse in dH_2O, and dehydration in a graded ethanol series, air-dried prior to mounting for 1 to 3 weeks in an X-ray cassette and exposed on beta-max hyperfilm, after which the sections were dipped in Kodak NTB-2 photo emulsion diluted 1:1 in water. The sections were exposed for 1 to 4 weeks at

Table 1 Antibodies used in the present study

Antibodies		Species	Conc.	Source
w3/13	Monoclonal	Mouse	1/1000	Sera-lab Sussex, UK
OX42	Monoclonal	Mouse	1/1.600	Harlan Sera-lab MAS 370b, Leicestershire, UK
ED1	Monoclonal	Mouse	1/4.000	Serotec MCA 341, Oxford, UK

4°C, developed in Kodak D19, dehydrated in a graded ethanol series to xylene and mounted in DPX medium.

Reverse transcriptase-polymerase chain reaction (RT–PCR)

The cortical area that had been subjected to contusion and the surrounding pericontusional zone (approximately 3 × 3 × 3 mm) from the brains of five *DA* and six PVG rats were sampled by micro dissection under RNAse-free conditions. The tissue samples were mechanically homogenized, and total RNA was extracted (Qiagen total RNA extraction kit, Hilden, Germany). Reverse transcription was performed with 10 µl of total RNA, random hexamer primers (0.1 µg; Gibco, BRL) and Superscript Reverse Transcriptase (200U; Gibco, BRL). Amplification was performed on an ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Norwalk, CT) by using the 5′ nuclease method (TaqMan) with a two-step PCR protocol (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). The following primers and probes, which all were designed in the Primer Express software (Perkin Elmer), were used in the PCRs: rat C3 (forward primer 5′ GGGAGCCCCATGTACTCCAT; reverse primer 5′ GGGACGTCACCCTGAGCAT; and probe 5′CAAT GTCCTGCGGCTGGAGAGTGAAG) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5′ TCAACTACATGGTCTACATGTTCCAG; reverse primer 5′ TCCCATTCTCAGCCTTGACTG; and probe 5′ TGACTCTACCCACGGCAAGTTCAACG).

Amplification/detection of contaminating genomic DNA was avoided by placing one of the primers over an exon/intron boundary. The probes were labeled with FAM as reporter dye and TAMRA as quencher dye. Relative quantification of mRNA levels in each sample was performed by using the standard curve method, with amplification of C3 mRNA and GAPDH mRNA in separate tubes. Standard curves for C3 and GAPDH were constructed by using serial dilutions of cDNA from spinal cord tissue obtained from a rat suffering from experimental allergic encephalitis (EAE). Samples without added cDNA served as negative controls. All samples were analyzed in duplicate.

Quantification of immunohistochemistry and Fluoro-Jade

Sections were analyzed using a Leica DMRB microscope equipped with a Nikon N90 camera and Kodak DCS 420 digital unit, and the NIH image 1.60 (NIH Bethesda, Maryland) software as previously described (Bellander et al. 2001; Ohlsson et al. 2003). The vicinity of the contusion area in immunostained sections was photographed (×40) and digitized. Images were obtained from all immunostained sections.

The region of interest (ROI) was defined and delimited medially by the interhemispheric fissure, basally by the corpus callosum and laterally by a border of corresponding size as the medial part of the ROI, including the pericontusional area and mainly cortical tissue. The central necrotic part of the contusion, including parts with presence of erythrocytes, was omitted from the ROI.

The ROI was outlined in each slide, and the total number of pixels in this area was measured. An observer blinded to the identity of the images set the density slice threshold level. Within these levels, the immunoreactivity was included. Pixels within the density slice range were counted within the ROI. The quotient between the total number of pixels obtained within the ROI and the number of pixels obtained within the density slice range was calculated using Microsoft Excel® software. Immunoreactivity in in situ hybridization as well as sham-operated controls was quantified in parallel.

The w3/13-positive cells were counted manually in each ROI and optically classified as polymorphonuclear cells or as T cells.

Statistical analysis

The non-parametrical Mann–Whitney U-test was used for statistical analysis of the quantification of immunohistochemistry, in situ hybridization as well as RT–PCR. Asterisks indicate the level of significance (Mann–Whitney U-test).

Results

Immunohistochemistry

Immunostainings for various glial markers, macrophages and T cells were performed at three different postoperative survival time points (3, 7 and 30 days) in order to assess the inflammatory response in *DA* and PVG rats.

Immunohistochemistry was performed using w3/13, a known antibody for CD43 located on T cells and PMN cells (Williams et al. 1977; Reynolds et al. 1981), which revealed a significant increased influx of PMN cells in the PVG strain ($P < 0.01$), with an increasing amount of T cells over time and presenting a significant higher share of T cells in the *DA* rat compared to PVG rat at 3-day postinjury ($P < 0.01$; Figs 1 and 5a and b).

A weak positive OX42 labeling of resting microglial cells was present in all studied sections, including those from sham-operated rats. A slight increase in the number and shape of OX42-positive cells, indicative of microglial

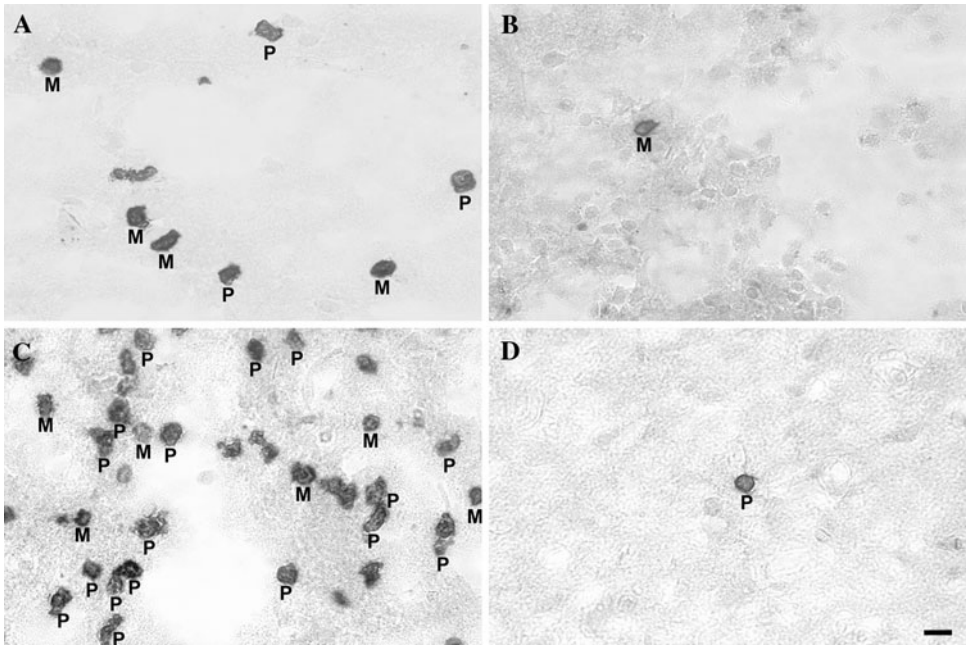

Fig. 1 Immunohistochemistry for w3/13 labeling infiltrating immune cells, including polymorphonuclear cells and T cells. **a** w3/13-IR in DA rat pericontusional zone: Infiltration of w3/13-positive cells in the pericontusional zone of DA rats is evident at 3-day postinjury. There were relatively more w3/13-IR positive T cells in the DA rat compared to the PVG rat. **b** w3/13-IR in DA sham: Corresponding sham-operated DA animal. **c** w3/13-IR in PVG rat pericontusional zone: A more dense infiltration of w3/13-positive cells is present in the pericontusional zone of PVG rats at 3-day postinjury. **d** w3/13-IR in PVG sham: Corresponding sham-operated PVG animal. (Bar = 25 μm; P = Polymorphonuclear granulocytes, M = Monocytes/T cells)

activation, was evident in the pericontusional area 3-day postinjury. The labeling intensity was drastically increased at 7-day postinjury and then decreased by 30-day postinjury (Fig. 2). Semi-quantitative assessment of OX42-IR showed that it was significantly enhanced compared to control at 7-day postinjury in both strains. An inter-strain comparison at the same time point showed that the increase in OX42-IR was more pronounced in *DA* rats compared to PVG rats ($P < 0.01$). OX42 staining was somewhat stronger in *DA* rats at 30-day postinjury, but this difference did not reach statistical significance (Fig. 5c).

The labeling pattern for ED1, a marker for accumulation of macrophages in the periphery of the contused area, was largely similar to that of OX42 (Fig. 3). However, ED1-IR did not statistically differ between strains at any of the studied time points (Fig. 5d).

In situ hybridization

The immunohistochemical studies were complemented with determination of the expression patterns for GFAP and C3 complement protein with in situ hybridization at 7-day postinjury. The expression of GFAP mRNA in the pericontusional area was significantly increased in both *DA* and PVG compared to the respective sham-operated controls. In addition, *DA* rats displayed increased labeling compared to PVG rats at the corresponding time point ($P < 0.001$) (not shown). The mRNA hybridization labeling for C3 demonstrated a strong induction of expression in the pericontusional area that was more pronounced in *DA* rats compared to PVG rats ($P < 0.001$) (Figs. 4 and 5f). In both cases, the expression was increased compared to sham-operated controls.

RT–PCR

Levels of C3 mRNA in the contusion area were determined by semi-quantitative RT–PCR at 7-day postinjury. This demonstrated a robust increase in both strains compared to the respective sham-operated control (not shown). However, C3 mRNA levels were more than twice as high in *DA* rats compared to PVG rats (Fig. 5e), and the difference between strains was significant ($P < 0.01$). These findings, together with the difference obtained by in situ hybridization, strengthen the notion of increased expression of a central component of the complement cascade in the *DA* strain compared to the PVG strain.

Semi-quantification of neurodegeneration

Fluoro-Jade labeling was performed in order to assess the extent of neurodegeneration. There was a prominent

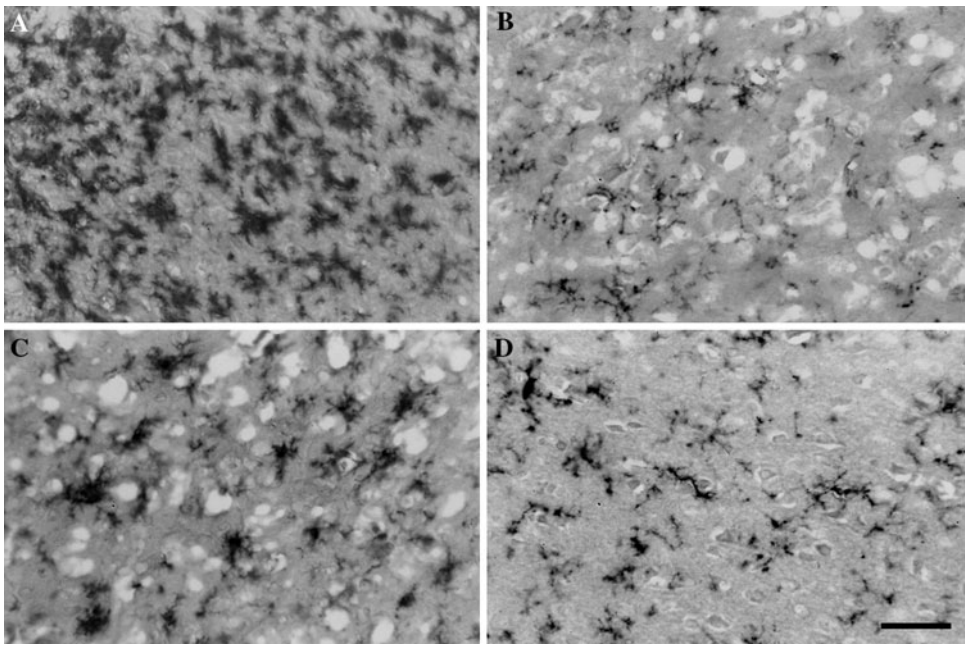

Fig. 2 Immunohistochemistry for OX42 labeling microglial cells. **a** OX42-IR in DA pericontusional zone (7-day postinjury): A pronounced OX42 labeling is present in the pericontusional zone of DA rats at 7-day postinjury. **b** OX42-IR in DA control: Corresponding sham-operated DA animal. **c** OX42-IR in PVG pericontusional zone (7-day postinjury): OX42 labeling is increased in the pericontusional zone of PVG rats at 7-day postinjury, but less than in the DA strain. **d** OX42-IR in PVG control: Corresponding sham-operated PVG animal. (Bar = 50 μm)

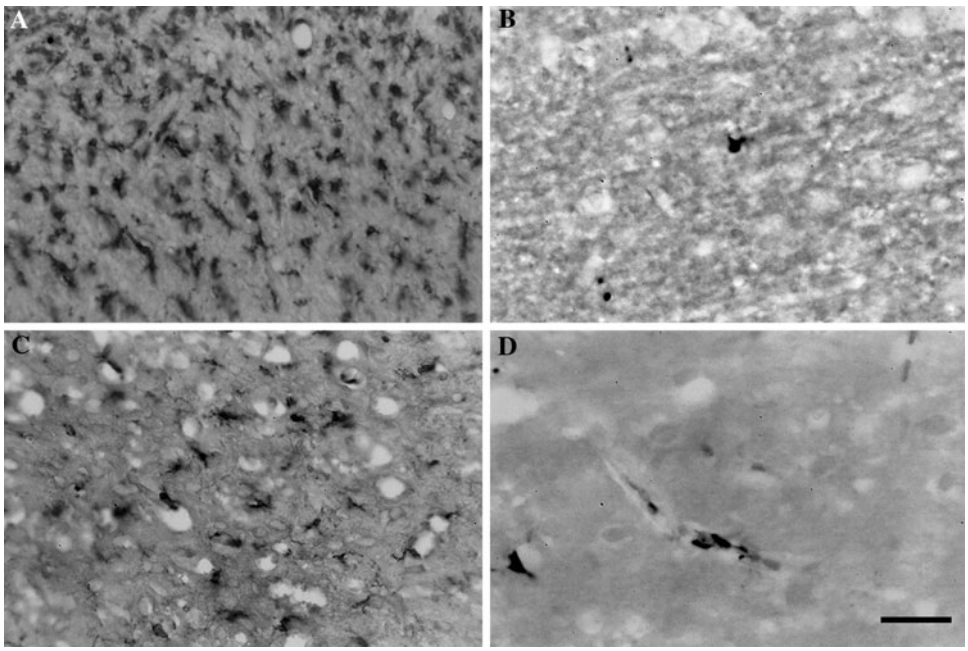

Fig. 3 Immunohistochemistry for phagocyting macrophages (ED1). **a** ED1-IR in *DA* pericontusional zone (7-day postinjury): An increase in ED1-IR is found in the pericontusional zone of the *DA* rat compared to controls, but not statistically different to the *PVG*-rats. **b** ED1-IR in *DA* control: Corresponding sham-operated *DA* animal. **c** ED1-IR in *PVG* pericontusional zone (7-day postinjury): ED1-IR is increased in the pericontusional zone of the *PVG* rat compared to controls. **d** ED1-IR in *PVG* control: Corresponding sham-operated *PVG* animal. (Bar = 50 μm)

increase in Fluoro-Jade-IR in the pericontusional zone of the contusions in both strains initially (Figs. 5g and 6a–d). Variability between animals was relatively large at the first two time points, and there was only a trend toward stronger labeling in *DA* rats at 7-day postinjury. ($P = 0.064$) (Fig. 5g). At the last studied time point, the *DA* strain displayed significantly increased staining compared to PVG strain ($P < 0.01$), but there was no significant difference in volume of the tissue loss at 30 dpi between DA and PVG.

Discussion

The inflammatory response in TBI has gained an increasing interest in recent years (Holmin et al. 1995; Stahel et al. 1998; Bellander et al. 2001). However, it is still unclear to what degree, inflammation may influence outcome. A main issue is to differentiate the inflammatory response from other biochemical processes in the development of secondary brain injuries and to explore what pathways in the inflammatory response that mediate detrimental and/or

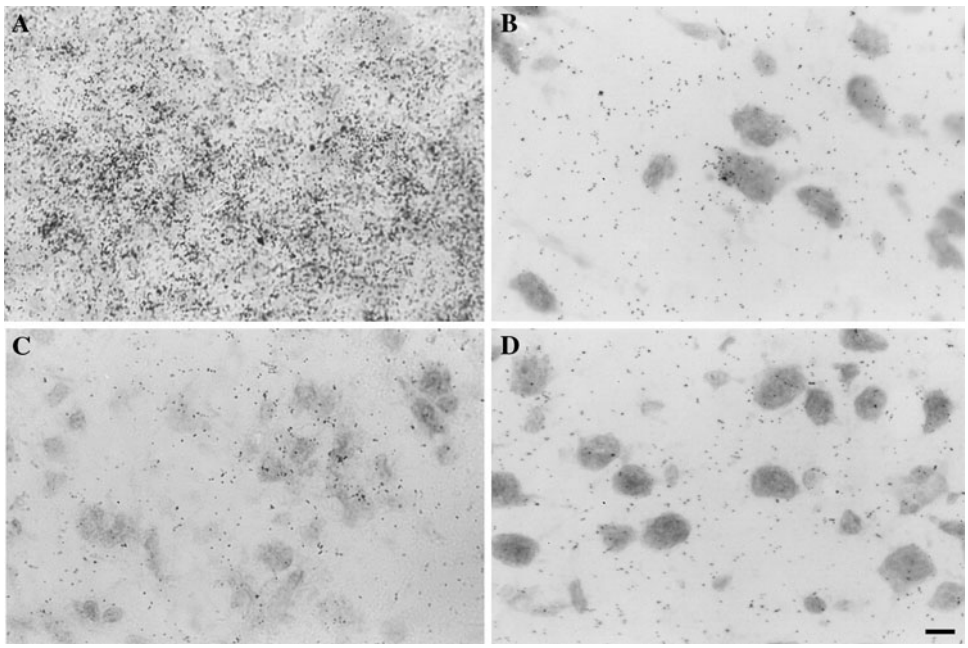

Fig. 4 In situ hybridization for complement C3 mRNA expression. **a** C3-mRNA in DA pericontusional zone (7-day postinjury): A strong induction of C3 mRNA labeling is evident in the pericontusional zone of DA rats at 7-day postinjury. **b** C3-mRNA in DA control: Corresponding sham-operated DA animal. **c** C3-mRNA in PVG pericontusional zone (7-day postinjury): A modest increase in C3 mRNA labeling is present in the pericontusional zone of PVG rats at 7-day postinjury. **d** C3-mRNA in PVG control: Corresponding sham-operated PVG animal. (Bar = 50 μm)

protective effects following TBI. As one approach to address these questions we have here explored the genetic influence on inflammation in a rat TBI model. Characterization of the genetic regulation of this type of reaction is important for at least two reasons. First, the mere existence of any degree of genetic polymorphisms affecting the TBI response is conceptually interesting, since it implies a level at which susceptibility for neuroinflammation/neurodegeneration is regulated. Second, since TBI-induced inflammation and nerve cell death are likely to be determined by several interconnected events that depend on many different genes, the chances of identifying important regulatory mechanisms using purely mechanistic approaches, even if based on educated guesses, are limited. Instead, careful comparison of responses between strains that display differences in phenotype may identify candidate pathways that can be associated with disease outcome. Phenotypic differences also allow for screening studies for genetic susceptibility loci using linkage studies in intercrosses between susceptible and resistant strains, where phenotypic features are linked to specific genome regions identified with genetic markers. This type of approach is currently used as a strategy in the search of regulatory genes in complex traits diseases, such as arthritis, multiple sclerosis and diabetes, with the final goal of exactly defining disease-promoting genes. We have previously used a similar approach on the response to peripheral nerve trauma. In an initial screening of a panel of inbred rat strains, the *DA* strain was identified as the most susceptible to nerve injury-induced neurodegeneration and inflammation, while the PVG strain was the most resistant (Lundberg et al. 2001). These observations were subsequently extended by demonstrating in a F2(*DA*xPVG) intercross discrete gene regions regulating nerve cell loss, lymphocyte infiltration and MHC class II expression on microglia (Lidman et al. 2003). Finally, strain differences in the expression of MHC class II were positioned to the *Mhc2ta* gene (Swanberg et al. 2005).

We here wanted to explore whether and to what degree, experimental brain contusion is subject to genetic variability, with special focus on the inflammatory response. The *DA* rat is known to be susceptible in many autoimmune disease protocols, such as experimental arthritis and EAE but also mounts a more severe inflammatory response upon a primarily non-immunological trigger such as a peripheral nerve injury (Lorentzen et al. 1997; Weissert et al. 1998; Lundberg et al. 2001). In contrast, the PVG strain is relatively resistant in these disease models. The rats were subjected to a reproducible, standardized brain contusion model modified from Feeney previously described (Feeney et al. 1981; Holmin et al. 1995; Bellander et al. 1996) leading to similar contusions in the both strains. Our results demonstrate a conspicuous difference in the inflammatory response between the *DA* and *PVG* strains that include elements of the adaptive and innate arms of the immune system. In the present study, the *PVG* displays a greater infiltration of w13/3-positive cells in the border zone of the contusions at 3-day postinjury compared to the *DA* ($P < 0.01$), but the relative amount of w3/13-positive T cells compared to PMNs was significantly higher in the DA strain this time point ($P < 0.01$). In parallel, microglia activation was also greater in the *DA* strain, suggesting that this strain not only mounts a more intense T-cell response, but also enhanced microglial activation. It is possible that

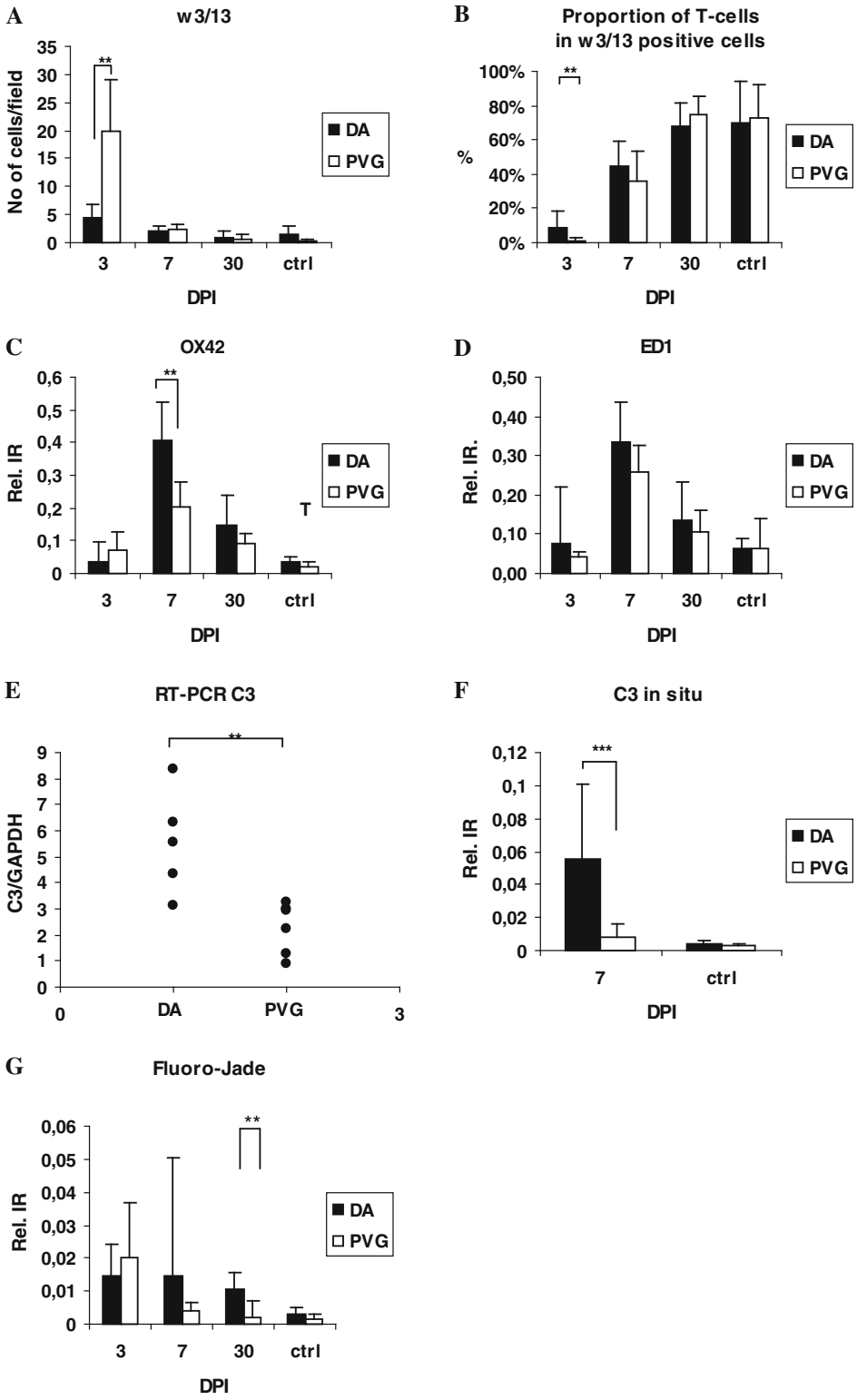

Fig. 5 Summary of semi-quantitative measures of immunohistochemical staining (**a**–**d**), RT–PCR (**e**), in situ hybridization signal (**f**) and Fluoro-Jade labeling (**g**) in the pericontusional zone of DA and PVG rats. **a** w3/13-IR: w3/13 labeling of infiltrating leukocytes reveals a relatively stronger response in *PVG* rats compared to *DA* rats at 3-day postinjury. **b** T cells vs polymorphonuclear cells: The relative proportion of CD3-positive T cells in the leukocyte infiltrates is higher in *DA* rats at 3-day postinjury and increases over time in both strains. **c** OX42-IR: A significantly stronger OX42 labeling of microglia is present in the *DA* strain at 7-day postinjury. **d** ED1-IR: ED1 labeling displays a similar temporal profile as OX42, but without discernible differences between strains. **e** RT–PCR: A scatter plot of C3 mRNA assessed by semi-quantitative RT–PCR reveals higher levels in *DA* rats compared to *PVG* rats at 7-day postinjury**. f** C3-mRNA: A semi-quantitative assessment of the in situ hybridization signal for C3 mRNA corroborates the findings by PCR, with significantly stronger labeling in DA rats compared to PVG rats at 7-day postinjury. **g** Fluoro-Jade in pericontusional zone: An increase in Fluoro-Jade labeling is evident in the pericontusional zones of both strains at earlier time points. However, at 30-day postinjury, the labeling is significantly higher in *DA* rats compared to *PVG* rats. (DPI = Days postinjury; ctrl = Sham-operated animal; field = 0.25 mm^2; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; Bar: 1 standard deviation; Mann–Whitney U-test)

the microglial activation results from increased T-cell infiltration (Sunnemark et al. 2003). However, in peripheral nerve injury model, increased microglia activation in the *DA* strain has previously been described even though T-cell infiltration is minimal (Piehl et al. 1999; Lidman et al. 2003). Depletion of T cells did not affect local response, suggesting that these two immune phenotypes depend on different genetic mechanisms (Piehl et al. 1999).

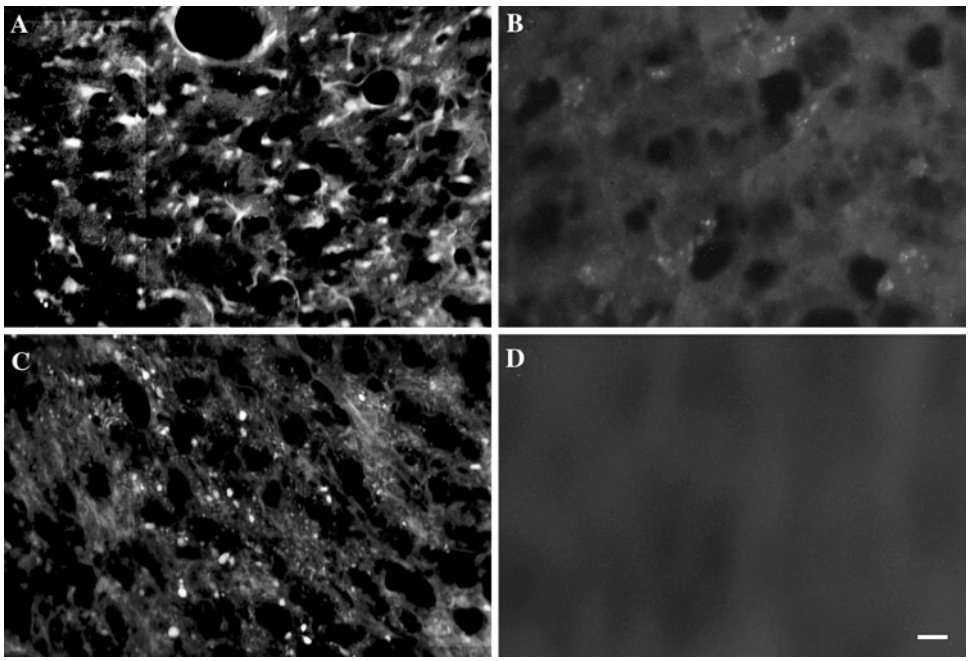

Fig. 6 Semi-quantitative analysis of neuronal necrosis using Fluoro-Jade. **a** Fluoro-Jade in *DA* pericontusional zone (7-day postinjury): An increase in Fluoro-Jade was found in the pericontusional zone of the *DA* rat contusions, though not significant higher compared to *PVG* until 30-day postinjury. This finding indicates that the inflammatory-prone *DA* rat presents an extensive neuronal loss following TBI, possibly due to more intense complement activation that occurs in these animals. **b** Fluoro-Jade in *DA* control: No specific Fluoro-Jade staining was detected in controls. **c** Fluoro-Jade in *PVG* pericontusional zone (7-day postinjury): A modest increase in Fluoro-Jade was found in the pericontusional zone of the contusion in the less inflammatory-prone *PVG* rat, indicating that phenotypes presenting discrete complement activation suffer from less neuronal loss following TBI. **d** Fluoro-Jade in *PVG* control: No increase in Fluoro-Jade was found in controls. (Bar = 50 μm)

Furthermore, activation of microglial cells has been shown to occur in the absence of circulating blood cells (Koshinaga et al. 2000; Bellander et al. 2004).

In contrast to T-cell infiltration and microglial activation, ED1 immunoreactivity did not differ between strains. ED1-positive macrophages may originate from the circulation, but it has recently been shown that ED1-positive cells surrounding neuronal injuries also originate from locally activated microglia (Bellander et al. 2004). The absence of strain variability in this phenotype is somewhat surprising given the robust differences in other assessed inflammatory phenotypes. However, these findings are in line with our previous results in a peripheral nerve injury model, where ED1 labeling was similar between *DA* and *PVG* rats in spite of considerable differences in other phenotypes including microglia activation. This fact supports the notion that nerve injury-induced inflammation is a complex phenomenon that is subject to regulation by multiple genes.

The complement system consists of a set of proteins, C1 to C9, and is activated by one of several pathways, of which the "classical pathway" and the "alternative pathway" are the best known. Activation results in a cascade of events leading to increased vascular permeability, induction of cytokine production with resulting inflammation (Okusawa et al. 1987, 1988; Schindler et al. 1990; Wetsel 1995), chemotactical recruitment of macrophages and opsonization (McGeer et al. 1989; Rosen 1990; Yao et al. 1990; Brown 1991; Bruck and Friede 1991; Frank and Fries 1991; McGeer et al. 1993). Prior studies have shown that the complement cascade is activated after TBI (Bellander et al. 1996, 2001). Complement activation is not only a trigger for further neuroinflammatory events, like stimulating phagocytosis and inducing brain edema but has also been suggested to mediate secondary damages via the terminal pathway. In this complement effector pathway, the membrane attack complex (MAC), which is a ring polymer generated by the assembly of C5b, C6, C7, C8 and multiple C9 molecules, has the ability to damage membranes in any target cells (Vanguri and Shin 1988; Esser 1991; Kinoshita 1991; Stoll et al. 1991; Morgan 1999). In previous studies, we have described complement activation at the surface of injured neurons near contusion injuries in both rodents and humans (Bellander et al. 1996, 2001). In the present study, we found a much greater C3 mRNA expression in the pericontusional zone of the contusion in the *DA* rats compared to *PVG* rats, suggesting that also the complement cascade is influenced by genetic factors.

The resulting damage to the nerve tissue was evaluated using Fluoro-Jade, a marker of neuronal degeneration

(Schmued et al. 1997). The Fluoro-Jade labeling was increased in *DA* rats at both 7- and 30-day postinjuries, albeit significantly increased compared to *PVG* rats only at the later time point, suggesting that increased inflammation in the *DA* strain is accompanied by augmented damage to neuronal networks. In recent years, there have been several reports describing neuroprotective effects of inflammation, at least with regard to T-cell infiltration (Schwartz 2003). The results presented herein may seem contradictory to this view as the increased number of T cells detected in the *DA* strain was associated with more tissue injury. However, it is important to consider that neuroinflammation includes a variety of different biochemical events, some of which may be deleterious and others instead exert protective effects.

Transgenic mice over expressing the complement regulator Crry, a functional homologue of human decay-accelerating factor (CD55), and membrane-cofactor protein (CD46), inhibiting both the classical and the alternative pathways at C3–C5 convertase level, display better blood–brain barrier function, as well as less neurological impairment following closed head injury (Rancan et al. 2003). Furthermore, Leinhase and coworkers have also recently shown that post-trauma treatment in mice using a recombinant Crry molecule significantly improved neurological function up to 7-day postinjury, inhibited hippocampal neuronal destruction in CA3/CA4 layers and significantly up-regulated candidate neuroprotective genes in the injured brain (Leinhase et al. 2006).

These findings further strengthen the hypothesis that complement activation is an important factor for the development of secondary brain damage. With further experimentation, it may be possible to identify those components of the inflammatory response that are instrumental in the development of secondary brain damage following TBI. However, as shown here, it is important to consider the dimension of genetic heterogeneity in the regulation of these events. This may also prove to be a useful approach to provide a better understanding of these complex interactions, since it is possible to genetically map heterogeneous phenotypes in inbred strains of rats or mice in order to find out whether they are connected or not. The results presented here lay the base for such an endeavor in TBI.

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