

# Essential protective roles of reactive astrocytes in traumatic brain injury

D. J. Myer, G. G. Gurkoff, S. M. Lee, D. A. Hovda and M. V. Sofroniew

Departments of Neurobiology and Neurosurgery, University of California, Los Angeles, CA, USA

Correspondence to: Michael V. Sofroniew, Department of Neurobiology, UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-1763, USA

E-mail: [sofroniew@mednet.ucla.edu](mailto:sofroniew@mednet.ucla.edu)

**Astrocytes respond to traumatic brain injury (TBI) by altered gene expression, hypertrophy and proliferation that occur in a graded fashion in relation to the severity of the injury. Both beneficial and detrimental effects have been attributed to reactive astrocytes, but their roles after brain injury are not well understood. To investigate these roles, we determined the effects on cortical tissue of ablating reactive astrocytes after contusion injury generated by controlled cortical impact (CCI) of different severities in transgenic mice that express a glial fibrillary acidic protein–herpes simplex virus–thymidine kinase transgene. Treatment of these mice with the antiviral agent, ganciclovir, conditionally ablates proliferating reactive astrocytes. Moderate or severe CCI were generated with a precisely regulated pneumatic piston, and forebrain tissue was evaluated using immunohistochemistry and quantitative morphometry. Moderate CCI in control mice triggered extensive and persisting reactive astrogliosis, with most neurons being preserved, little inflammation and an 18% loss of cortical tissue beneath the impact site. Ablation of reactive astrocytes after moderate CCI in transgenic mice caused substantial neuronal degeneration and inflammation, with a significantly greater 60% loss of cortical tissue. Severe CCI in control mice caused pronounced neuronal degeneration and loss of about 88% of cortical tissue that was not significantly altered by ablating reactive astrocytes in transgenic mice. Thus, ablation of dividing reactive astrocytes exacerbated cortical degeneration after moderate CCI, but did not alter cortical degeneration after severe CCI. These findings indicate that the reactive astrocytes play essential roles in preserving neural tissue and restricting inflammation after moderate focal brain injury.**

**Keywords:** reactive astrocytes; traumatic brain injury; inflammation; transgenic mice; neural degeneration; glial fibrillary acidic protein; contusion; controlled cortical impact

**Abbreviations:** CCI = controlled cortical impact; TBI = traumatic brain injury

Received February 23, 2006. Revised April 24, 2006. Accepted May 25, 2006. Advance Access publication July 5, 2006.

## Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability with limited treatment options (Nortje and Menon, 2004). Tissue damage varies greatly after different types of TBI (Cunningham *et al.*, 2005). Understanding the roles of different cells types in the response of brain tissue to TBI may help to define mechanisms that lead to preservation or deterioration of tissue after TBI and thus help to identify potential therapeutic targets. Astrocytes are ubiquitous cells throughout brain tissue, and make essential contributions to many homeostatic functions that could directly influence neuronal survival, tissue integrity and functional outcome after TBI. These include maintenance of extracellular ion and fluid balance, clearance of extracellular glutamate, water transport, production of pro- or anti-inflammatory

cytokines and chemokines, production of growth factors, production of glucose and other energy metabolites, and free radical release or free radical scavenging (Katayama *et al.*, 1990; Faden, 2002; Chen and Swanson, 2003; Kettenmann and Ransom, 2004). Astrocytes respond to TBI by pronounced changes in gene expression, cellular hypertrophy and cell proliferation, all of which occur in a graded fashion in relation to the severity of the injury. Although the process of reactive astrogliosis has been characterized extensively at the descriptive level, and many astrocytic activities have been defined in cell cultures, the overarching roles played by reactive astrocytes in the response to TBI *in vivo* are not well understood. For example, it is not clear to what degree reactive astrocytes maintain,

upregulate or downregulate the normal functions of non-reactive astrocytes, or to what degree they adopt new functions. It is also not known how functions exerted by reactive astrocytes influence the progression of the response to injury, the preservation of tissue integrity or the overall outcome after TBI. As might be expected from their wide range of activities, both beneficial and detrimental effects have been attributed to reactive astrocytes. For example, potential tissue protective effects could be provided by glutamate uptake, free radical scavenging or neurotrophin release (Sofroniew *et al.*, 2001; Chen and Swanson, 2003), while potentially harmful effects might be caused by the release of pro-inflammatory cytokines or cytotoxic radicals (Kolker *et al.*, 2001; Chen and Swanson, 2003). Current ideas about the roles of reactive astrocytes in the response to brain insults, and whether these roles are beneficial or detrimental, are dominated by extrapolations from activities that can be elicited *in vitro*. The overall effects of reactive astrocytes on outcome after specific types of TBI *in vivo* have not been experimentally defined.

To investigate potential roles of reactive astrocytes after TBI *in vivo*, we have in this study examined whether the targeted ablation of proliferating reactive astrocytes either exacerbated or reduced tissue loss after TBI. We have previously developed a transgenic mouse model to conditionally ablate reactive astrocytes that are proliferating after CNS injury, and have characterized this model using forebrain stab injury and different types of spinal cord injury (Bush *et al.*, 1999; Faulkner *et al.*, 2004). In this model, expression of the enzyme herpes simplex virus-thymidine kinase (HSV-TK) is transgenically targeted to astrocytes via the mouse glial fibrillary acidic protein (GFAP) promoter. Expression of the GFAP-TK transgene on its own has no detectable detrimental effect on astrocytes, and these cells develop and mature normally in mice of line 7.1. Cell ablation is achieved by delivery of the antiviral agent ganciclovir, a thymidine analogue, which once phosphorylated inhibits DNA synthesis and kills dividing cells. In GFAP-TK mice, delivery of ganciclovir after CNS injury selectively kills dividing reactive astrocytes in the immediate vicinity of the injury, while sparing non-dividing astrocytes distant to the injury (Bush *et al.*, 1999; Sofroniew, 2005).

In the study presented here, we used these GFAP-TK transgenic mice to determine the effects of conditionally ablating reactive astrocytes after controlled cortical impact (CCI), a well-characterized model of focal TBI used extensively to study cellular and molecular changes in injured tissue during and after trauma in mice (Longhi *et al.*, 2001; Hall *et al.*, 2005). We examined the effects of ablating dividing reactive astrocytes on the response to TBI of two different severities, a moderate CCI that on its own did not result in substantial degeneration of neural tissue and a more severe CCI that caused pronounced loss of neural tissue. We reasoned that if reactive astrocytes on balance exert protective effects after CCI, then ablation of these cells would exacerbate moderate CCI, whereas if reactive astrocytes on

balance exert substantive detrimental effects (e.g. by releasing cytotoxins) then ablation of these cells might reduce tissue loss after severe CCI. Our findings show that moderate CCI was exacerbated by ablation of reactive astrocytes, whereas severe CCI was not detectably altered. These findings lend support to the concept that reactive astrocytes exert a net neuroprotective effect after a moderate focal brain injury.

## Material and methods

### Transgenic model for astrocyte ablation

To selectively ablate dividing reactive astrocytes, we used GFAP-TK transgenic mice of line 7.1 previously generated in our laboratory, in which HSV-TK is targeted to astrocytes using the mouse GFAP promoter (Bush *et al.*, 1998). To maximize targeting specificity and to avoid deletion of potential regulatory elements, we used a large mouse GFAP promoter cassette (clone 445) containing the full sequence (>15 kb) of the mouse GFAP gene, including all introns and exons, as well as 2 kb of 5' and 2.5 kb of 3' flanking regions (Johnson *et al.*, 1995). Two base pairs in exon 1 were mutated to prevent GFAP expression from the transgene. GFAP-TK mice of the founder breeding line 7.1 have been characterized extensively and shown to exhibit high expression of transgene-derived TK specifically by GFAP-expressing cells as demonstrated at the single-cell level in the normal and injured forebrain *in vivo* and *in vitro* (Bush *et al.*, 1998; Bush *et al.*, 1999; Imura *et al.*, 2003). GFAP-TK mice of line 7.1 develop normally and exhibit no detectable detrimental effects of transgene expression such that brain structure and histology, including the appearance of astrocytes, are indistinguishable from non-transgenic littermates. Treatment with ganciclovir kills dividing GFAP-TK expressing astrocytes from line 7.1 both *in vivo* and *in vitro* (Bush *et al.*, 1998; Bush *et al.*, 1999). Astrocytes rarely divide in uninjured adult CNS. Thus, after brain or spinal cord injuries that induce astrocyte proliferation, treatment of GFAP-TK mice with ganciclovir causes pronounced loss of dividing reactive astrocytes in the immediate vicinity of the injury, while sparing non-dividing astrocytes distant to the injury (Bush *et al.*, 1999; Faulkner *et al.*, 2004).

### Animals

All GFAP-TK transgenic and non-transgenic mice used in this study were obtained by mating heterozygous females of GFAP-TK line 7.1 (Bush *et al.*, 1998) with wild-type C57/BL6 males. All experimental and control mice were derived from the same breeding colony backcrossed onto a C57/BL6 background for >10 generations. Mice were housed in a 12-h light/dark cycle in a specific pathogen-free facility with controlled temperature and humidity and were allowed free access to food and water. All experiments were conducted according to protocols approved by the Animal Research Committee of the Office for Protection of Research Subjects at University of California Los Angeles. Mice were genotyped by PCR and immunohistochemistry for HSV-TK.

### Control groups

Because our transgenic model for astrocyte ablation requires treating GFAP-TK transgenic mice with ganciclovir, several control groups were examined: (i) non-transgenic mice; (ii) non-transgenic mice receiving ganciclovir; and (iii) GFAP-TK transgenic mice that did not receive ganciclovir. Mice from all three control groups were

evaluated uninjured and after sham craniotomy, moderate CCI and severe CCI. No significant difference was found among the three different types of control mice in any of these conditions, so the values for any given condition were pooled to form single experimental control groups.

### Surgical procedures

Surgical procedures were performed under general anesthesia with isoflurane (1–2%). Rectal temperature was monitored and maintained at 36.5°C. Unilateral CCI was performed as previously described (Kelly *et al.*, 1997) after craniotomy over the left parietal cortex using a stereotaxically positioned 3 mm diameter stainless steel tipped piston centred at 2 mm posterior and 2.5 mm lateral to bregma. A pressure gauge (Wilkerson, Model R16-02-GL0A, Englewood, CO) and stimulus generator (Grass Instruments, Model S88, Warwick, RI) controlled piston velocity. Velocity and depth of penetration of the piston tip were modified to generate CCI of different severities. Analgesic was given every 12 h for 48 h post-injury.

### Parameters for moderate or severe CCI

To identify experimental parameters that would reproducibly generate moderate or severe injuries after CCI we compared various CCI parameters. We defined moderate CCI as causing substantial cortical compression without pronounced neuronal death and little loss of cortical tissue volume beneath the impact site and little damage to hippocampal CA3. We defined severe CCI as causing pronounced cortical compression with extensive neuronal death, rapid loss of cortical tissue and consistent degeneration of hippocampal CA3. To generate different CCI severities using our pneumatic piston, we varied both the depth of penetration and the speed of impact as regulated by the force (psi) driving the piston. Piston depression was sustained for 150 ms in all cases. Using our equipment we found that moderate CCI injuries were reliably generated using the parameters of 0.5 mm depth of piston penetration with 12 psi, corresponding to ~1.2 m/s. Severe CCI injuries were reliably generated using the parameters of 1.0 mm depth of piston penetration with 20 psi, and these injuries appeared equivalent in severity to injuries reported by others using impact speeds of ~4 m/s (Igarashi *et al.*, 2001; Kernie *et al.*, 2001; Nakamura *et al.*, 1999). Mice tolerated these injury severities well. One mouse died prior to its planned survival time, several days after CCI from undeterminable causes that were not detectably related to the CCI (i.e. no obvious brain haemorrhage, oedema and so on).

### Ganciclovir and BrdU treatments

Ganciclovir (Roche, Nutley, NJ) was administered via subcutaneous osmotic minipump (Alzet) at a rate of 100 mg/kg/day in sterile physiological saline for 7 days immediately after CCI (Bush *et al.*, 1999; Faulkner *et al.*, 2004). Bromodeoxyuridine (BrdU) (Sigma, St Louis) was administered by injection at 100 mg/kg/day on Days 2–5.

### Histological procedures

Histological procedures were conducted as described (Bush *et al.*, 1999; Faulkner *et al.*, 2004). Briefly, after terminal anaesthesia with pentobarbital (100 mg/kg), mice were perfused transcardially with buffered 4% paraformaldehyde. Brains were cryoprotected and serial frozen 40 µm coronal sections were prepared (Leica,

Nussloch, Germany). Brightfield and fluorescence immunohistochemistry were performed using biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA), biotin–avidin–peroxidase complex (Vector Laboratories) and diaminobenzidine (Sigma) as the developing agent, or with tagged secondary antibodies, Alexa 488 (green), Alexa 568 (red) from Molecular Probes. Primary antibodies for immunohistochemistry were as follows: rabbit anti-GFAP (1:20 000; Dako); sheep anti-BrdU (1:1000, Maine Biotechnology Services); mouse anti-neuronal nuclei NeuN (1:2500; Chemicon); rat anti-mouse CD45 (1:1000; Pharmingen); and rabbit anti-S100β (1:85 000; Sigma). Sections stained for BrdU were pretreated with 7 M HCl for 30 min and then neutralized in buffer. Staining with cresyl violet was conducted according to the standard procedures. Stained sections were examined and photographed using brightfield and fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

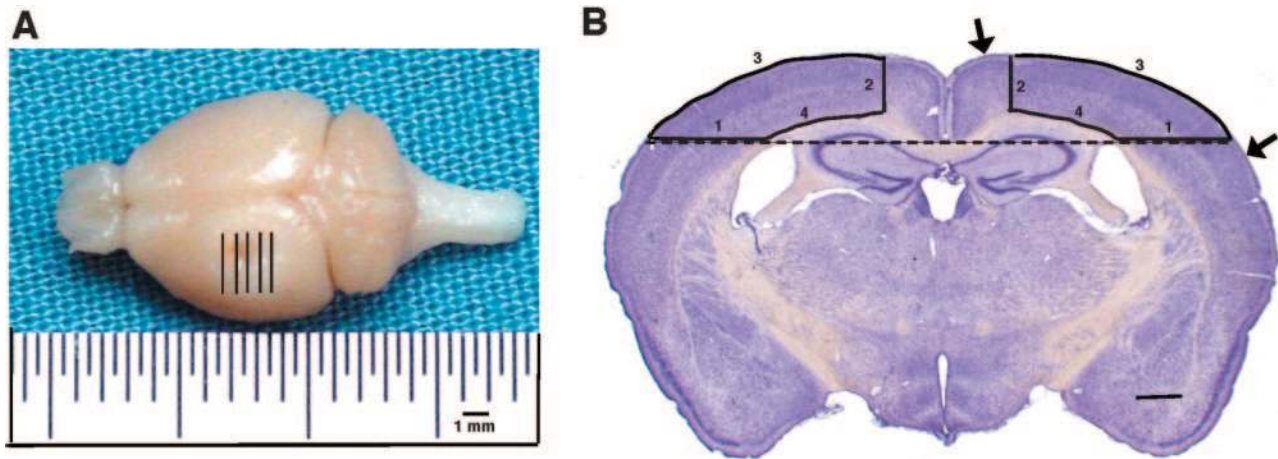
### Morphometric and statistical evaluation

To quantify lesion size after CCI under different experimental conditions, we designed a protocol for measuring the amount of healthy cortical tissue within a precisely defined anatomical region on both sides of the brain based on easily identified and constant anatomical landmarks (Fig. 1). Using this procedure, the amount of healthy cortical tissue ipsilateral to and directly beneath the piston impact site was expressed as a percentage of the amount of cortical tissue in the same, precisely demarcated anatomical region on the contralateral side (Figs 1–3). Healthy cortical tissue was defined as containing an essentially normal density and cytoarchitecture of neurons in cresyl violet and NeuN stained sections. Borders between healthy and degenerated cortex were generally sharp and easily defined because degenerated tissue generally contained few viable neurons either in cresyl violet or NeuN stained sections (Figs 2D, 5B and 6C) or tissue was missing entirely and had been resorbed at longer survival times (Fig. 3D). Statistical analyses were performed by ANOVA with Newman–Keuls' *post hoc* pair-wise analysis (Prism; GraphPad, San Diego, CA).

## Results

### Astrocyte ablation exacerbates tissue loss after moderate but not severe CCI

We determined the effects of ablation of proliferating reactive astrocytes on cortical tissue volume beneath the impact site after moderate or severe CCI using quantitative morphometry (Fig. 1). Control mice that were uninjured (Figs 2A and 3A) or that were examined at 7 days (Fig. 2B and G) or 28 days (Fig. 3B and G) after sham craniotomy exhibited no significant difference in cortical volume on the left versus the right side. After moderate CCI, control mice exhibited no significant loss of cortical volume after 7 days (Fig. 2C and G), and after 28 days exhibited a small but significant loss of 18% of cortical volume beneath the impact site compared with uninjured or sham-operated controls (Fig. 3C and G). Ablation of reactive astrocytes in ganciclovir-treated GFAP-TK caused a significantly greater loss of cortical volume after moderate CCI than was observed in controls at both survival times. Compared



**Fig. 1** Morphometric comparison of viable cortical tissue in defined anatomical regions on both sides of the brain. **(A)** Survey image of a whole mouse brain 28 days after a moderate CCI on the left side. A small depression of the cerebral cortex is evident. Five evenly spaced lines demarcate the levels of five evenly spaced sections taken for measurement from the centre of the lesion and at two 500  $\mu\text{m}$  intervals on either side. Equivalently located sections were evaluated in uninjured or sham-operated mice. **(B)** Survey image of a cresyl violet-stained coronal section through the forebrain of an uninjured mouse illustrating the morphometric procedure. All sections were evaluated using the Stereo Investigator program. A line perpendicular to the midline was drawn across the top of the corpus callosum (dashed line). This line provided a consistent ventral boundary of cortical area measured (1). A second line, perpendicular to the first, was drawn from the apex of the white matter of the corpus callosum to the cortical surface (2), which served as a consistent medial boundary. These ventral and medial boundaries were selected because they were consistently within the boundaries of the craniotomy (demarcated by arrows) and included the entire piston impact site. The area measured was completed by tracing along the top surface of viable cortical tissue (3) and along the surface of white matter (4). Viable tissue was defined as containing cresyl violet-stained neurons and having NeuN-stained neurons in neighbouring sections. The amount of viable cortical tissue within the area ipsilateral to the piston impact (or craniotomy alone in sham mice) was expressed as a percentage of the amount of cortical tissue in the same anatomical area on the contralateral side. Scale bars: A = 1 mm, B = 650  $\mu\text{m}$ .

to controls, ganciclovir-treated GFAP-TK mice had lost 46% of cortical tissue by 7 days (Figs 2D and G) and 60% of cortical tissue by 28 days (Figs 3D and G) after moderate CCI.

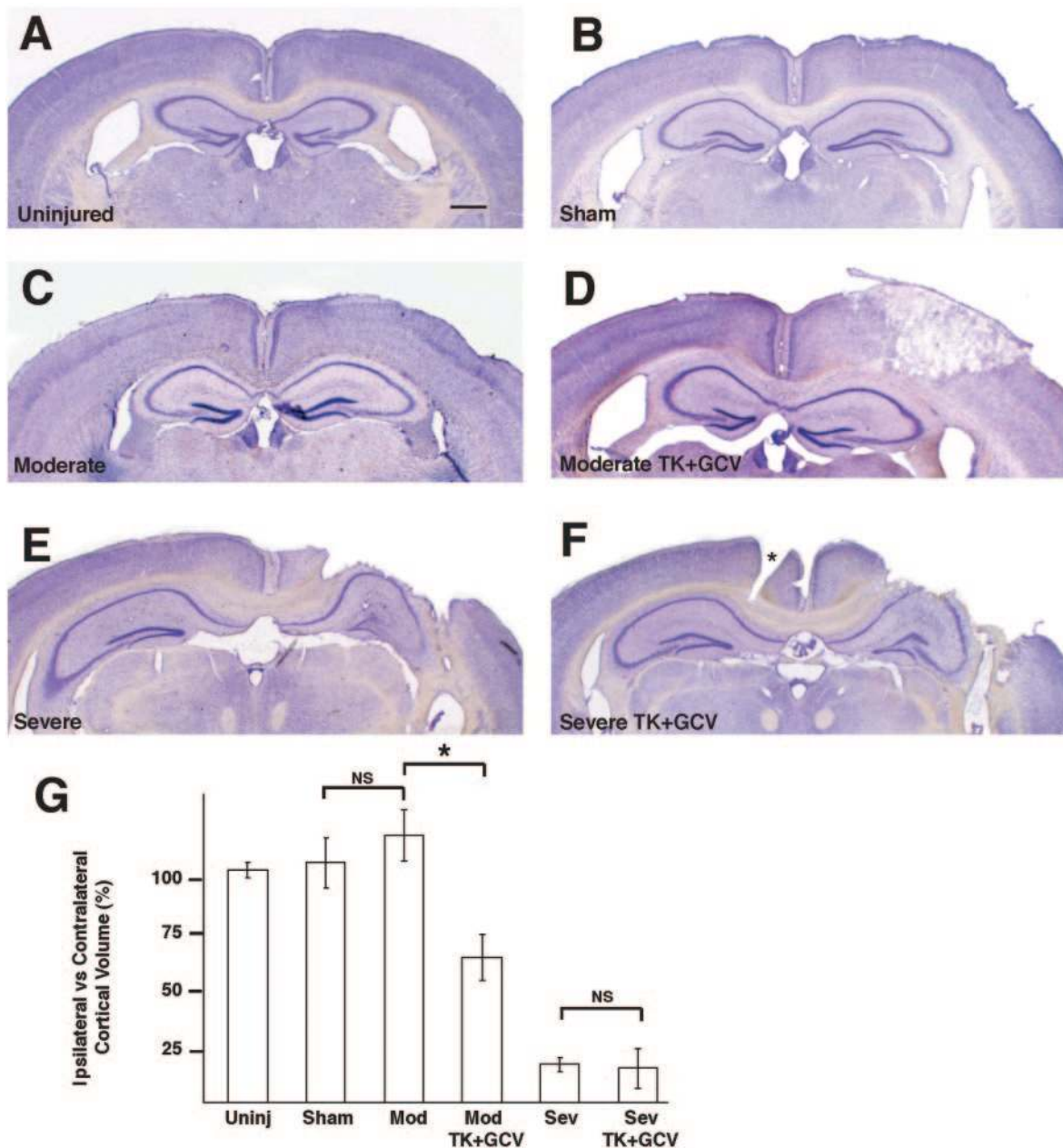
After severe CCI, control mice exhibited a pronounced loss of 86% of cortical tissue beneath the piston impact site by 7 days (Figs 2E and G), which remained of a similar magnitude of 88% loss after 28 days (Figs 3E and G). Ablation of reactive astrocytes after severe CCI in ganciclovir-treated GFAP-TK did not significantly alter the amount of cortical tissue loss as compared with control mice at either time point and was 88% loss after 7 days (Fig. 2F and G) and 87% loss at 28 days (Fig. 3F and G).

### Astrocyte ablation, neuronal degeneration and inflammation after moderate CCI in ganciclovir-treated GFAP-TK mice

Because ganciclovir-treated GFAP-TK mice exhibited a pronounced and significantly greater loss of cortical tissue as compared with control mice after moderate CCI (Figs 2 and 3) we compared the appearance of astrocytes, neurons and inflammatory cells in different treatment groups at 7 days after moderate CCI. Because our transgenic ablation strategy for reactive astrocytes specifically targets proliferating GFAP-expressing cells, we first looked for evidence of dividing reactive astrocytes in cerebral cortex after moderate CCI of control mice. In uninjured cortex, only a minority of

scattered astrocytes exhibited detectable levels of GFAP (Figs 4A and E, 5C and 6D), and dividing BrdU labelled cells were rare, associated with blood vessels and had the appearance of endothelial cells (Fig. 4C and G). At 7 days after moderate CCI in control mice, cerebral cortex exhibited robust reactive astrogliosis with markedly increased expression of GFAP by most if not all astrocytes beneath the piston impact site and pronounced cellular hypertrophy (Figs 4B and F, 5D, 6E and 7C). The cerebral cortex also exhibited many proliferating cells labelled with BrdU (Fig. 4D and H), and a substantial proportion of these proliferating cells were astrocytes double-labelled with GFAP (Fig. 4I<sub>1</sub>–I<sub>3</sub>). Consequently, these findings show that our moderate CCI induced pronounced upregulation of GFAP expression, hypertrophy and cell division of reactive astrocytes in cortical tissue beneath the piston impact site. These GFAP-expressing and proliferating astrocytes would be vulnerable to ablation in GFAP-TK mice treated with ganciclovir (Bush *et al.*, 1999). We therefore looked for evidence of astrocyte ablation in these mice. At 7 days after moderate CCI in GFAP-TK mice treated with ganciclovir, the cerebral cortex beneath the piston impact site exhibited large regions essentially devoid of reactive astrocytes stained with GFAP (Figs 5D, 6F and 7D) or S100 $\beta$  (not shown). Together these findings showed that our experimental model for ablation of reactive astrocytes was effective after moderate CCI.

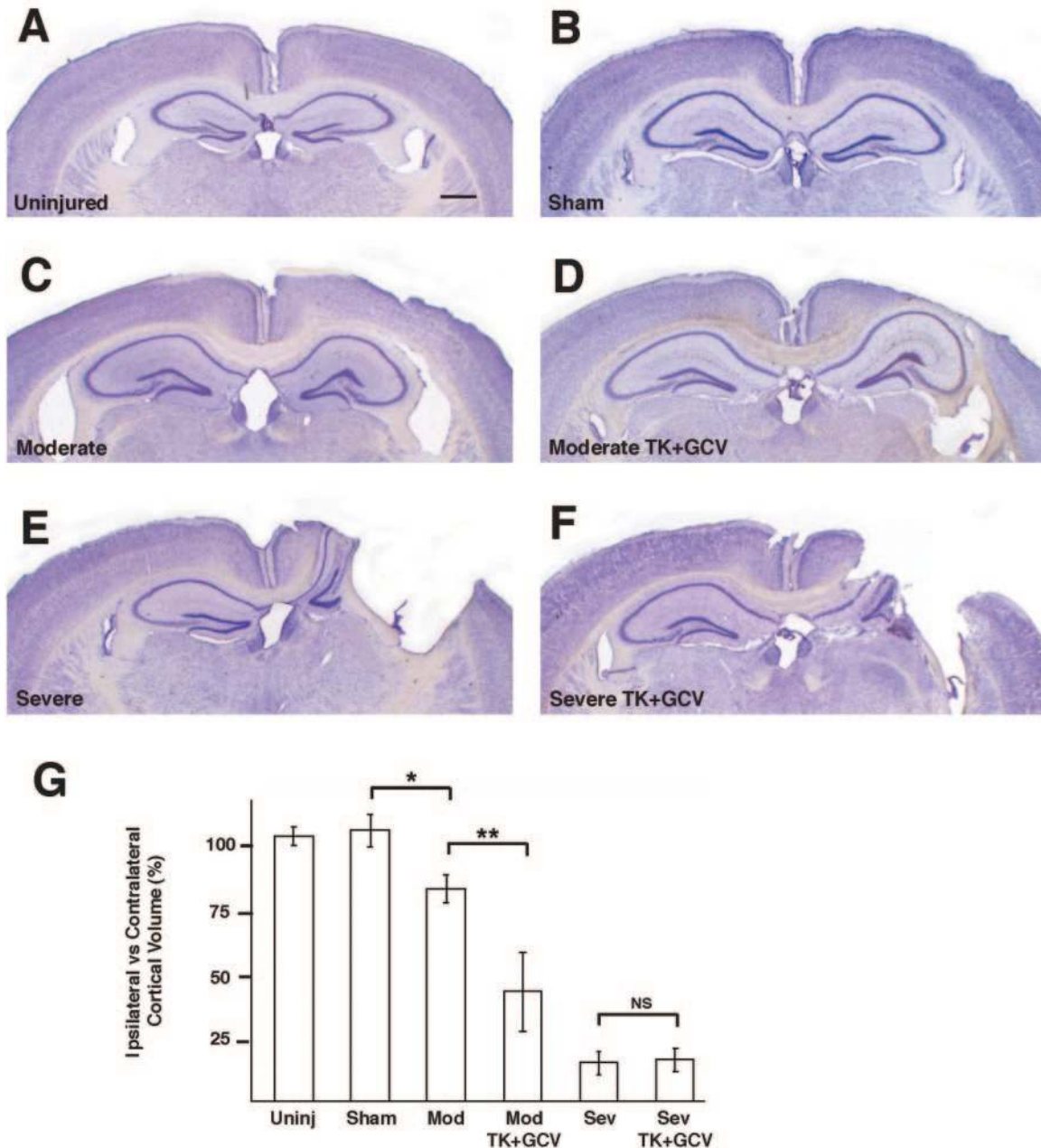
We also examined the effects of astrocyte ablation on cortical neurons and inflammatory cells. Neighbouring



**Fig. 2** Cortical tissue at 7 days after moderate or severe CCI, with or without transgenically targeted ablation of reactive astrocytes. (A–F) Survey images of cresyl violet-stained coronal sections taken from equivalent forebrain regions in mice after various treatments: (A) uninjured control; (B) control, 7 days after sham operation, i.e. craniotomy only; (C) control, 7 days after moderate CCI; (D) ganciclovir-treated GFAP-TK transgenic 7 days after moderate CCI; (E) control, 7 days after severe CCI; (F) ganciclovir-treated GFAP-TK transgenic 7 days after severe CCI. (G) Graph showing mean area  $\pm$  SEM of viable cortical tissue beneath the piston impact site or the equivalent region in uninjured or sham mice, expressed as a percentage of the amount of cortical tissue in the same anatomical area on the contralateral side (see Fig. 1) in each treatment group after 7 days. Control mice exhibited no loss of cortical tissue volume at 7 days after sham or moderate CCI, but exhibited pronounced tissue loss after severe CCI. Ganciclovir-treated GFAP-TK mice exhibited significant loss of tissue volume as compared with control mice at 7 days after moderate CCI, and exhibited a similar degree of tissue loss as controls after severe CCI.  $n = 6$  per group for uninjured, sham, moderate controls and moderate TK + GCV (ganciclovir);  $n = 5$  per group for severe controls and severe TK + GCV; ns = non-significant, \*significantly different  $P < 0.001$  (ANOVA with *post hoc* pair-wise analysis). Scale bar = 600  $\mu$ m.

sections in GFAP-TK mice treated with ganciclovir after moderate CCI showed that cortical regions devoid of reactive astrocytes (Figs 6F, 5D and 7D) corresponded precisely to regions exhibiting pronounced loss of neurons

stained either with NeuN (Figs 5B, 6C and 7B) or cresyl violet (Fig. 2D). In contrast, neighbouring sections in control mice after moderate CCI showed that cortical regions exhibiting well preserved cortical neurons and

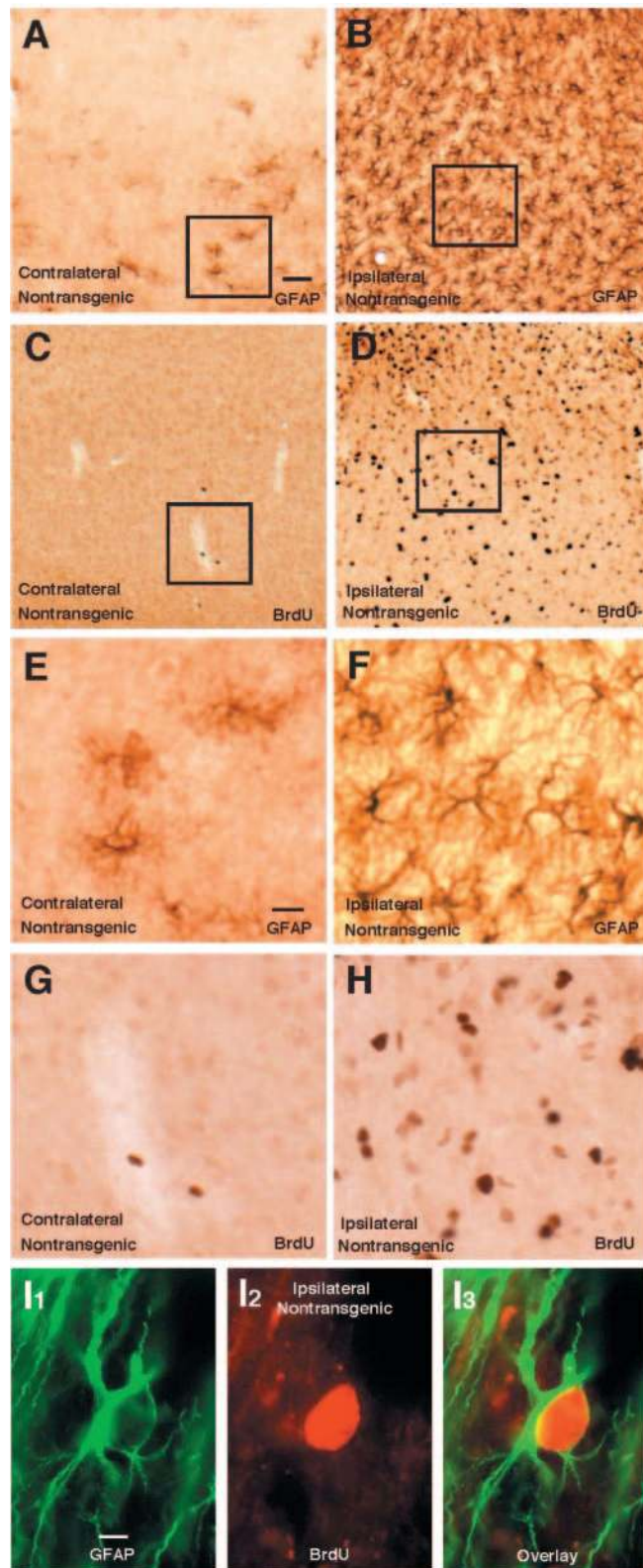


**Fig. 3** Cortical tissue at 28 days after moderate or severe CCI, with or without transgenically targeted ablation of reactive astrocytes. (A–F) Survey images of cresyl violet-stained coronal sections taken from equivalent forebrain regions in mice after various treatments: (A) uninjured control; (B) control, 28 days after sham operation, i.e. craniotomy only; (C) control, 28 days after moderate CCI; (D) ganciclovir-treated GFAP-TK transgenic 28 days after moderate CCI; (E) control, 28 days after severe CCI; (F) ganciclovir-treated GFAP-TK transgenic 28 days after severe CCI. (G) Graph showing mean area  $\pm$  SEM of viable cortical tissue beneath the piston impact site or the equivalent region in uninjured or sham mice, expressed as a percentage of the amount of cortical tissue in the same anatomical area on the contralateral side (see Fig. 1) in each treatment group after 28 days. Control mice exhibited no loss of cortical tissue volume at 28 days after sham injury and a small loss after moderate CCI, but exhibited pronounced tissue loss after severe CCI. Ganciclovir-treated GFAP-TK mice exhibited significantly greater loss of tissue volume as compared with control mice at 28 days after moderate CCI, and exhibited a similar degree of tissue as controls after severe CCI.  $n = 6$  per group for uninjured, sham and moderate controls;  $n = 5$  per group for moderate TK + GCV (ganciclovir), severe controls and severe TK + GCV; ns = non-significant,  $*P < 0.05$  significantly different,  $**P < 0.001$  significantly different (ANOVA with *post hoc* pair-wise analysis). Scale bar = 600  $\mu$ m.

cytoarchitecture (Figs 2C, 5A, 6B and 7A) contained a high density of highly reactive astrocytes (Figs 5C, 6E and 7C). It is also noteworthy that in GFAP-TK mice treated with ganciclovir, non-dividing reactive astrocytes in areas surrounding

the piston impact site were spared (Figs 5D and 6F) and that these areas exhibited substantive preservation of neurons and tissue at both 7 days (Figs 2D, 5B and 6C) and 28 days (Fig. 3D).

To identify inflammatory cells, we used immunohistochemistry for CD45, an antigen associated with microglia and all leucocytes, including macrophages, monocytes, neutrophils and T cells. In uninjured cerebral cortex, CD45



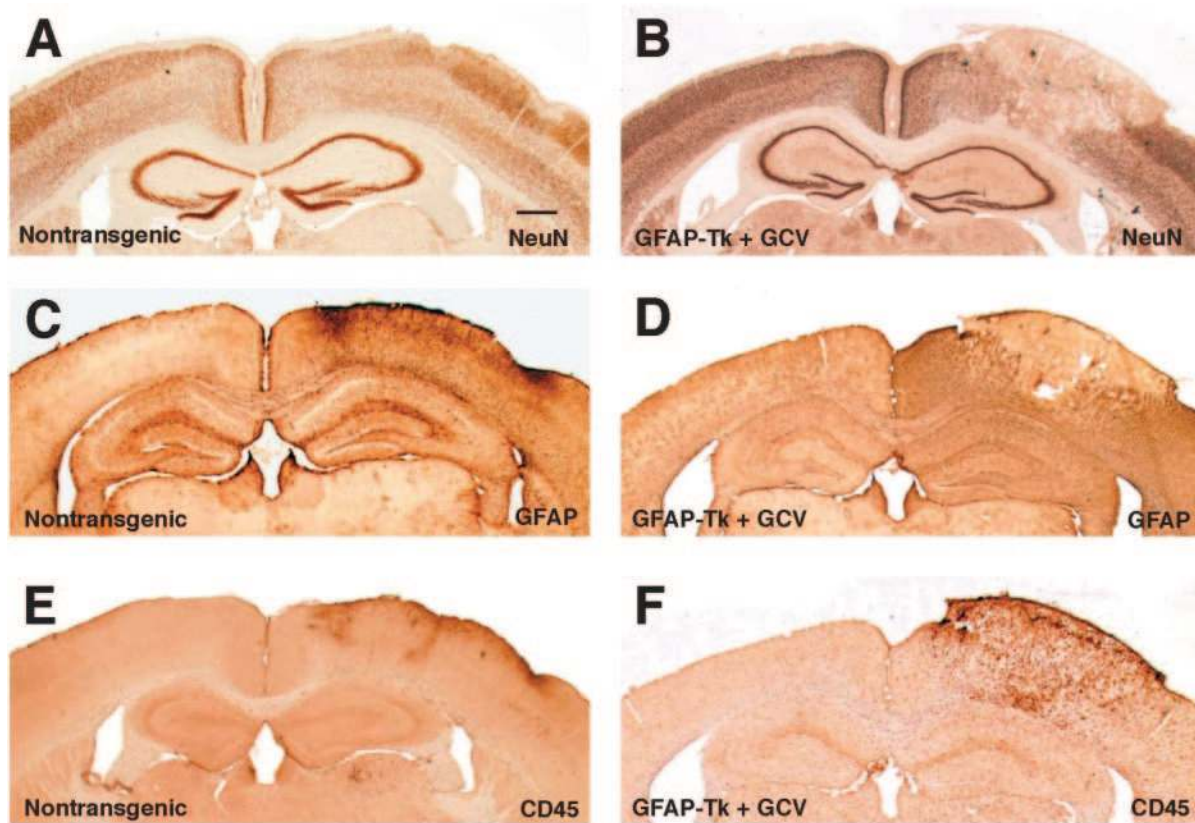
staining was faint and restricted to small quiescent multipolar microglia that were not easily visible at low magnification. At 7 days after moderate CCI in control mice, there was a mild to moderate inflammatory response characterized primarily by microglial hypertrophy and increased CD45 labelling that was most intense at the cortical surface immediately beneath the piston impact site but with few phagocytic macrophages present in the mid or deep cortical tissue (Figs 5E, 6H and 7E) that contained high densities of reactive astrocytes (Figs 5C, 6E and 7C). In contrast, 7 days after moderate CCI in GFAP-TK mice treated with ganciclovir, regions devoid of reactive astrocytes (Figs 5D, 6F and 7D) corresponded precisely to regions that exhibited pronounced infiltration with many round inflammatory cells (Figs 5F, 6I and 7F). Thus, after moderate contusion injury in control mice the presence of reactive astrocytes correlated with the absence of substantial inflammation, whereas the ablation of astrocytes resulted in a pronounced increase in inflammatory cell infiltration.

### Structurally preserved neural tissue exhibits prolonged astrogliosis after moderate CCI in control mice

We looked in more detail at the cytoarchitecture and cellular composition of cortical tissue at a long survival time after moderate CCI in control mice, comparing in particular the distribution of neurons and reactive astrocytes. Our morphometric findings indicated that by 28 days after moderate CCI in control mice the cerebral cortex directly beneath the piston impact site exhibited a small significant loss, but appeared by and large to be well preserved using cytological stains (Fig. 3C and G). These observations were confirmed by immunohistochemistry for NeuN, which showed good preservation of neurons and cortical cytoarchitecture in the area

**Fig. 4** Hypertrophy and proliferation of reactive astrocytes in cerebral cortex 7 days after moderate CCI in control mice.

(A–H) Brightfield images of neighbouring sections of cerebral cortex immunohistochemically stained for GFAP (A, B, E and F) or BrdU (C, D, G and H). E–H show details of boxed areas in A–D, respectively. A, C, E, G are taken from uninjured cortex contralateral to the CCI. B, C, F and H are taken from injured cortical tissue immediately beneath the piston impact site. (I<sub>1</sub>–I<sub>3</sub>) Double-labelling immunofluorescence of GFAP (green) and BrdU (red) shown in single channels (I<sub>1</sub>, I<sub>2</sub>) and as a merged image (I<sub>3</sub>) taken from a neighbouring section of injured cortical tissue immediately beneath the piston impact. Note that few astrocytes express detectable levels of GFAP in uninjured cerebral cortex (A and E), and that by 7 days after moderate CCI astrocytes throughout the cortex have markedly upregulated GFAP and hypertrophied (B and F). Note that uninjured cortex contains few proliferating cells labelled with BrdU and that these are mostly associated with blood vessels and have the elongated appearance of endothelial cells (C and G). By 7 days after moderate CCI there are many BrdU labelled dividing cells throughout the cortex (D, H), many of which double label with GFAP (I<sub>1</sub>–I<sub>3</sub>). Scale bars A–D = 50 μm; E–H = 20 μm, I<sub>1</sub>–I<sub>3</sub> = 5 μm.



**Fig. 5** Effects of ablation of dividing reactive astrocytes on neurons and microglia at 7 days after moderate CCI. (A–F) Survey images of neighbouring coronal sections of cerebral cortex taken either from a control mouse (A, C and E) or ganciclovir-treated GFAP-TK transgenic mouse (B, D and F) 7 days after moderate CCI and immunohistochemically stained for NeuN (A and B), GFAP (C and D) or CD45 (E and F). Note that under the piston impact site in the control mouse, there is pronounced upregulation of GFAP expression by astrocytes (C), with little upregulation of CD45 positive inflammatory cells (E) and good preservation of NeuN positive neurons and their cytoarchitecture (A). In contrast, ablation of reactive astrocytes under the piston impact site (B) is accompanied by pronounced inflammation (F) and loss of NeuN positive neurons (B). Scale bar = 350  $\mu\text{m}$ .

immediately beneath the piston impact site as compared with the uninjured side (Fig. 8C and D). It is noteworthy that astrogliosis, in the form of upregulation of GFAP expression and astrocyte hypertrophy, remained prominent in cerebral cortex, and that large, reactive astrocytes were clearly intermingled with well preserved neurons throughout the cortex beneath the piston impact site at 28 days after moderate CCI (Fig. 8B, D, E<sub>1–3</sub>).

## Discussion

In this study, we investigated the roles of reactive astrocytes after TBI using a transgenically targeted cell ablation strategy combined with experimental CCI of two severities. Our findings show that ablation of reactive astrocytes markedly exacerbated cortical degeneration and inflammation after moderate CCI, but did not alter cortical degeneration after severe CCI. These findings indicate that reactive astrocytes perform roles essential for the preservation of neural tissue and restriction of inflammation after mild or moderate focal brain injury, and thus play a major part in determining outcome after TBI.

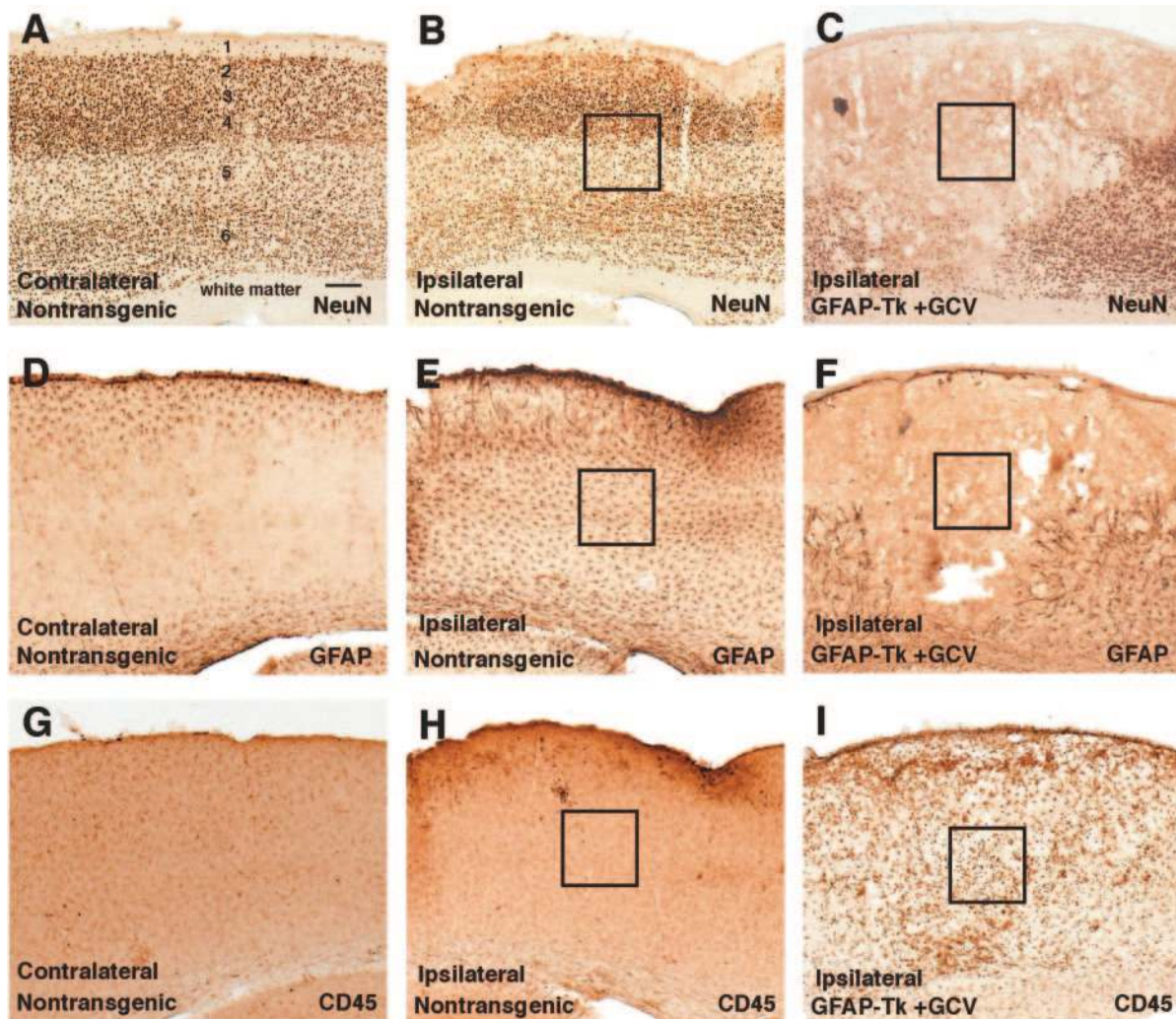
## Technical considerations

The experimental model in this study used a transgenically targeted cell ablation strategy to ablate dividing reactive astrocytes. Numerous findings from our and other laboratories demonstrate the specificity and validity of this model both *in vivo* and *in vitro*.

Because transgenes insert randomly into the genome, transgene expression cannot be assumed automatically to mimic endogenous promoter activity; nevertheless, transgene expression is stable within transgenic breeding lines, and once characterized at the single cell level can be a useful tool (Feng *et al.*, 2000). Our previous studies showed that in the 7.1 line of GFAP-TK mice used here, transgene-derived TK was detectable only in GFAP-expressing cells in forebrain and spinal cord *in vivo* and *in vitro* (Bush *et al.*, 1999; Imura *et al.*, 2003; Faulkner *et al.*, 2004).

Regarding the selectivity of cell ablation using this model, we have previously shown *in vitro* using tissue cultures of intermingled cells that ganciclovir killed only cells that express the GFAP-TK transgene, whereas neighbouring GFAP-TK-negative cells survived well, multiplied and exhibited no evidence of toxicity (Bush *et al.*, 1998; Imura *et al.*,



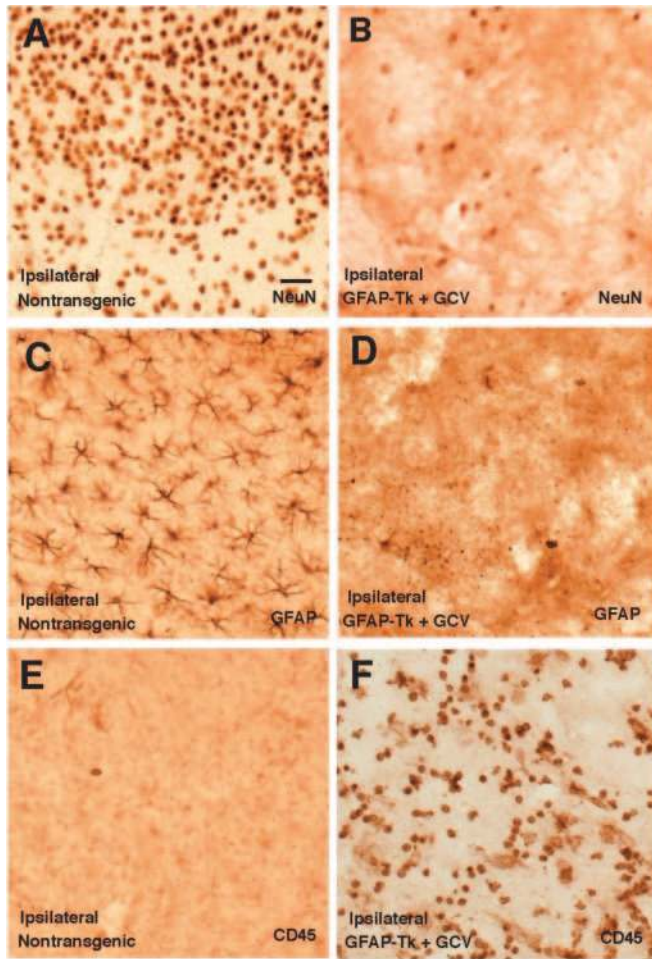


**Fig. 6** Effects of ablation of dividing reactive astrocytes on neurons and microglia at 7 days after moderate CCI. (**A–I**) Detailed images of neighbouring coronal sections of cerebral cortex taken from Fig. 5, showing at 7 days after moderate CCI the cerebral cortex contralateral (**A, D** and **G**) and ipsilateral (**B, C, E, F, H** and **I**) to the piston impact site in a control mouse (**A, B, D, E, G** and **H**) or ganciclovir-treated GFAP-TK transgenic mouse (**C, F** and **I**). The sections are immunohistochemically stained for NeuN (**A–C**), GFAP (**D–F**) or CD45 (**G–I**). Note that under the piston impact site in the control mouse, there is pronounced upregulation of GFAP expression by astrocytes (**E**), with little upregulation of CD45 positive inflammatory cells (**H**) and good preservation of NeuN positive neurons and their cytoarchitecture (**B**) compared with equivalent uninjured contralateral cortex (**A, D** and **G**). In contrast, ablation of reactive astrocytes under the piston impact site (**F**) is accompanied by pronounced inflammation (**I**) and loss of NeuN positive neurons (**C**). Boxes indicate areas that are shown in detail in Fig. 7. Scale bar = 100  $\mu$ m.

2003). Studies by others using different models of HSV-TK transgenically targeted to a variety of cell types in different types of tissues, including oligodendrocytes in the CNS, have also shown that after ablation of TK-expressing cells *in vivo*, immediately adjacent cells that do not express the transgene survive well and do not exhibit signs of non-specific toxicity (Borrelli *et al.*, 1989; Canfield *et al.*, 1996; Mathis *et al.*, 2000; Heppner *et al.*, 2005).

It is noteworthy that the HSV-TK plus ganciclovir cell ablation strategy is regarded as non-inflammatory *in vivo*. Transgene-derived HSV-TK phosphorylates ganciclovir, and the phosphorylated ganciclovir disrupts DNA synthesis during S phase of cell division. At the next cell cycle checkpoint,

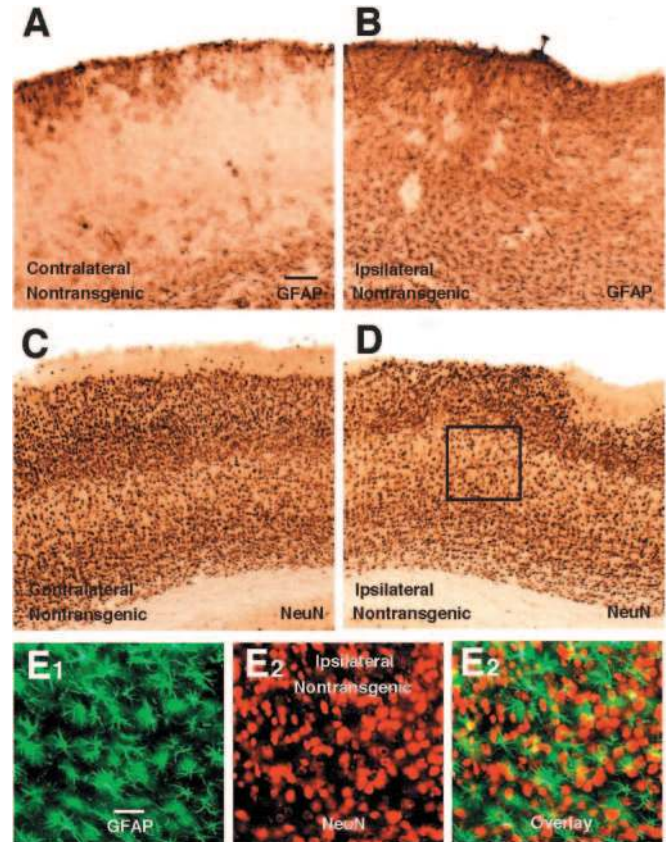
the disrupted DNA triggers apoptotic cell death by modulation of Bcl-2 proteins (Fischer *et al.*, 2005), which does not elicit a major inflammatory response. Previous studies by others support this notion by showing that ablation of different cell types in various peripheral tissues (Borrelli *et al.*, 1989; Canfield *et al.*, 1996), and two separate studies show that ablation of oligodendrocytes in the CNS (Mathis *et al.*, 2000; Jalabi *et al.*, 2005) did not induce pronounced inflammation or tissue loss as observed in our study. In addition, we have previously shown that after CNS stab injury or toxin-induced death of neurons the presence of cellular debris on its own did not induce a pronounced or prolonged invasion of CNS tissue by phagocytic macrophages when



**Fig. 7** Effects of ablation of dividing reactive astrocytes on neurons and microglia at 7 days after moderate CCI. (A–F) Detailed images of neighbouring coronal sections of cerebral cortex taken from Fig. 6, showing at 7 days after moderate CCI the cerebral cortex immediately beneath the piston impact site in a control mouse (A, C and E) or ganciclovir-treated GFAP-TK transgenic mouse (B, D and F). The sections are immunohistochemically stained for NeuN (A and B), GFAP (C and D) or CD45 (E and F). Note that under the piston impact site in the control mouse there is pronounced upregulation of GFAP expression by astrocytes (C), with little upregulation of CD45 positive inflammatory cells (E) and good preservation of NeuN positive neurons and their cytoarchitecture (A). In contrast, ablation of reactive astrocytes under the piston impact site (D) is accompanied by pronounced inflammation (F) and loss of NeuN positive neurons (B). Scale bar = 30  $\mu\text{m}$ .

reactive astrocytes were present, whereas phagocytic macrophages persisted for a long time in CNS tissue in massive numbers (7- to 25-fold greater) when astrocytes had been ablated (Bush *et al.*, 1999; Faulkner *et al.*, 2004). These observations argue that the inflammation we observe is unlikely to be due merely to the presence of cellular debris resulting from astrocyte ablation, and instead results from a loss of astrocyte functions.

Ablation of reactive astrocytes exacerbated cortical degeneration after moderate CCI, but did not detectably alter the response to severe CCI. The effects on moderate CCI provide



**Fig. 8** Appearance of neurons and astrocytes in cortical tissue at 28 days after moderate CCI in control mice. (A–D) Survey images of cerebral cortex contralateral to (A and C) and directly beneath the piston impact site (B and D) 28 days after moderate CCI. (A–D) Brightfield images of neighbouring sections immunohistochemically stained for GFAP (A and B) or NeuN (C and D). (E<sub>1</sub>–E<sub>3</sub>) Double-labelling immunofluorescence of GFAP (green) and BrdU (red) is shown in single channels (E<sub>1</sub> and E<sub>2</sub>) and as a merged image (E<sub>3</sub>). Taken from approximate location of boxed area in D. Note that while most astrocytes in the uninjured cortex do not express detectable levels of GFAP (A), under the piston impact site there is pronounced astrogliosis in the form of upregulated GFAP expression and astrocyte hypertrophy (B), and that this persisting astrogliosis is associated with substantial preservation of NeuN positive neurons and their cytoarchitecture (D), which compare well with the equivalent uninjured contralateral cortex (C). Note also that persisting reactive astrocytes are closely intermingled with surviving neurons (E<sub>1</sub>–E<sub>3</sub>). Scale bars A–D = 100  $\mu\text{m}$ ; E<sub>1</sub>–E<sub>3</sub> = 40  $\mu\text{m}$ .

evidence that reactive astrocytes have an overall protective effect on the response to brain injury. The observation that reactive astrocytes did not improve outcome after severe CCI provides further evidence that these cells do not exert an overall harmful effect; otherwise an improved response might have been expected in a manner similar to the improved response to experimental autoimmune encephalomyelitis that was observed when microglia were ablated using transgenically targeted HSV-TK and ganciclovir (Heppner *et al.*, 2005). Given the evidence for a protective role of reactive astrocytes after CCI, it is interesting that

ablation of these cells did not exacerbate the response to severe CCI. Several observations may provide an explanation. Other investigators have provided evidence that astrocytes die early after severe brain injury (Zhao *et al.*, 2003), in which case our severe CCI may have caused lethal damage to sufficiently large numbers of astrocytes that our transgenically targeted astrocyte ablation had no further effect on depleting the tissue of astrocytes, and thus the lesion volumes would have remained the same. This possibility is supported by the observation that in our model of CCI, the injury is restricted to tissue immediately beneath the piston, and it is only in this area that astrocytes proliferate and would be vulnerable to our ablation technique.

Taken together, these observations allow us to conclude that the neuronal degeneration, pronounced inflammation and tissue loss that occurred when reactive astrocytes were ablated after moderate CCI in ganciclovir-treated GFAP-TK mice were caused by the loss of essential functions provided by reactive astrocytes.

### Roles of astrocytes after TBI

The roles played by reactive astrocytes after TBI are not well understood. It is generally assumed that reactive astrocytes participate in a vaguely defined process of CNS wound repair, but specific contributions to this process are not well defined. In contrast, because reactive astrocytes are ubiquitous in damaged CNS tissue, they are often regarded as uniformly harmful, causing toxic oedema, provoking inflammation, releasing cytotoxins and forming scars that serve no purpose but to inhibit axonal regeneration. The wide range of activities that astrocytes can exhibit *in vitro* contributes to uncertainty over whether these cells exert beneficial or detrimental effects after CNS injuries. For example, potential protective effects could be provided by glutamate uptake and neurotrophin release, while potential detrimental effects might be caused by the release of inflammatory cytokines and cytotoxic radicals. Little information has been available on the roles played by reactive astrocytes in the response to experimental models of specific types of CNS injury *in vivo*. Transgenic models such as the one used here provide a means of obtaining such information.

The findings presented here showed that in the context of moderate contusive brain injury reactive astrocytes were not harmful, but rather played roles essential for preserving cortical neurons and tissue integrity, and for restricting post-injury inflammation. Our findings argue that in this injury context, reactive astrocytes at the very least (i) continued to provide the essential activities of non-reactive astrocytes without which neural tissue would not survive, (ii) upregulated homeostatic activities sufficiently to prevent toxicity by the increased extracellular glutamate load and ion imbalance present in injured tissue and (iii) did not adopt any new activities that might be overtly cytotoxic. The findings are also in agreement with previous evidence that

reactive astrocytes play important roles in restricting the infiltration of inflammatory cells into viable neural tissue after CNS injuries *in vivo* (Bush *et al.*, 1999; Faulkner *et al.*, 2004) as well as with a recent report that astrocytes produce a heat labile small molecule that suppresses microglial activation *in vitro* (Min *et al.*, 2006). The degree to which reactive astrocytes might adopt other mechanisms that might be protective after moderate CNS injuries awaits further investigations, which may be facilitated by other transgenic models that allow more subtle manipulations of astrocyte functions (Sofroniew, 2005). In this regard it is of interest that astrocytes may be lost early after TBI (Zhao *et al.*, 2003). Our findings suggest that such a loss may contribute to neuronal and tissue degeneration.

While the findings of this study support a tissue protective role for reactive astrocytes after mild or moderate TBI, it deserves emphasis that our findings do not rule out the possibility, or even the likelihood, that in other injury contexts reactive astrocytes might exert toxic activities that lead to neuronal dysfunction or death or to tissue degeneration. As discussed earlier, astrocytes are capable of a wide range of activities, including activities that might be detrimental to surrounding cells and tissue. Thus, the potential activities of reactive astrocytes are complex. Moreover, different activities are likely to be context-dependent and regulated by a wide range of signalling cues (Sofroniew, 2005). The activities of reactive astrocytes may differ markedly after different types of injury, or if other interacting factors are present at the time of injury, such as infections, previous injuries, degenerative disease or genetic predispositions. In this context it is of interest that inhibition of the proliferation of both microglia and astroglia was associated with neuroprotection after fluid percussive brain injury in rats (Giovanni *et al.*, 2005). An important goal for future studies will be to determine the different contexts under which astrocytes exert different types of activities that might be tissue protective or cytotoxic, and to identify the specific signalling mechanisms that trigger these different activities.

The findings of this study have important implications for ideas about mechanisms of tissue degeneration and preservation after TBI and for potential therapeutic strategies. Reactive astrogliosis is sometimes regarded as uniformly detrimental to outcome after CNS injury and as a process that should be prevented. Our findings argue that the global and indiscriminant inhibition of reactive astrogliosis would exacerbate mild and moderate injuries, and is likely to cause more damage than good. Our findings also demonstrate that activities played by reactive astrocytes have the potential to play major roles in determining outcome after TBI. Understanding the specific contexts in which astrocytes exert beneficial or detrimental effects after TBI, and identifying the signalling mechanisms that regulate different astrocyte responses may identify important and novel avenues for developing rational therapeutic strategies.

## Acknowledgements

This work was supported by grants from the National Institutes of Health (NIH/NINDS) NS42039, NS016333, NS27544 and the UCLA Brain Injury Research Center. We thank D. Norton, R. Korsak and M.E. Sislak for technical assistance, and P. Borgese and Hoffman La Roche for ganciclovir.

## References

- Borrelli E, Heyman RA, Arias C, Sawchenko PE, Evans RM. Transgenic mice with inducible dwarfism. *Nature* 1989; 339: 538–41.
- Bush TG, NP, Horner CH, Polito A, Ostenfeld T, Svendsen CN, et al. Leukocyte infiltration, neuronal degeneration and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 1999; 23: 297–308.
- Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L, et al. Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell* 1998; 93: 189–201.
- Canfield V, West AB, Goldenring JR, Levenson R. Genetic ablation of parietal cells in transgenic mice: a new model for analyzing cell lineage relationships in the gastric mucosa. *Proc Natl Acad Sci USA* 1996; 93: 2431–5.
- Chen Y, Swanson RA. Astrocytes and brain injury. *J Cereb Blood Flow Metab* 2003; 23: 137–49.
- Cunningham AS, Salvador R, Coles JP, Chatfield DA, Bradley PG, Johnston AJ, et al. Physiological thresholds for irreversible tissue damage in contusional regions following traumatic brain injury. *Brain* 2005; 128: 1931–42.
- Faden AI. Neuroprotection and traumatic brain injury: theoretical option or realistic proposition. *Curr Opin Neurol* 2002; 15: 707–12.
- Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV. Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci* 2004; 24: 2143–55.
- Feng G, Mellor R, Bernstein M, Keller-Peck C, Nguyen Q, Wallace M, et al. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 2000; 28: 41–51.
- Fischer U, Steffens S, Frank S, Rainov NG, Schulze-Osthoff K, Kramm CM. Mechanisms of thymidine kinase/ganciclovir and cytosine deaminase/5-fluorocytosine suicide gene therapy-induced cell death in glioma cells. *Oncogene* 2005; 24: 1231–43.
- Giovanni SD, Movsesyan V, Ahmed F, Cernak I, Schenelli S, Stoica B, et al. Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. *Proc Natl Acad Sci USA* 2005; 102: 8333–8.
- Hall ED, Sullivan PG, Gibson TR, Pavel KM, Thompson BM, Scheff SW. Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury. *J Neurotrauma* 2005; 22: 252–65.
- Heppner FL, Greter M, Marino D, Falsig J, Raivich G, Hovelmeyer N, et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat Med* 2005; 11: 146–52.
- Igarashi T, Huang TT, Noble LJ. Regional vulnerability after traumatic brain injury: gender differences in mice that overexpress human copper, zinc superoxide dismutase. *Exp Neurol* 2001; 172: 332–41.
- Imura T, Kornblum HI, Sofroniew MV. The predominant neural stem cell isolated from postnatal and adult forebrain but not from early embryonic forebrain expresses GFAP. *J Neurosci* 2003; 23: 2824–32.
- Jalabi W, Boehm N, Grucker D, Ghandour MS. Recovery of myelin after induction of oligodendrocyte cell death in postnatal brain. *J Neurosci* 2005; 25: 2885–94.
- Johnson WB, Ruppe MD, Rockenstein EM, Price J, Sarthy VP, Vederber LC, et al. Indicator expression directed by regulatory sequences of the glial fibrillary acidic protein (GFAP) gene: in vitro comparison of distinct GFAP-lacZ transgenes. *Glia* 1995; 13: 174–84.
- Katayama Y, Becker DP, Tamura T, Hovda DA. Massive increases in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury. *J Neurosurg* 1990; 73: 889–900.
- Kelly DF, Lee SM, Pinanong PA, Hovda DA. Paradoxical effects of acute ethanolism in experimental brain injury. *J Neurosurg* 1997; 86: 876–82.
- Kernie SG, Erwin TM, Parada LF. Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice. *J Neurosci Res* 2001; 66: 317–26.
- Kettenmann H, Ransom BR, editors. *Neuroglia*. New York: Oxford University Press; 2004.
- Kolker S, Ahlemeyer B, Huhne R, Mayatepek E, Kreieglstein J, Hoffmann GF. Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures. *Eur J Neurosci* 2001; 13: 2115–22.
- Longhi L, Saatman KE, Raghupathi R, Laurer HL, Lenzlinger PM, Riess P, et al. A review and rationale for the use of genetically engineered animals in the study of traumatic brain injury. *J Cereb Blood Flow Metab* 2001; 21: 1241–58.
- Mathis C, Hindelang C, LeMeur M, Borrelli E. A transgenic mouse model for inducible and reversible dysmyelination. *J Neurosci* 2000; 20: 7698–705.
- Min KJ, Yang MS, Kim SU, Jou I, Joe EH. Astrocytes induce hemeoxygenase-1 expression in microglia: a feasible mechanism for preventing excessive brain inflammation. *J Neurosci* 2006; 26: 1880–7.
- Nakamura M, Saatman KE, Galvin JE, Scherbel U, Raghupathi R, Trojanowski JQ, et al. Increased vulnerability of NFH-LacZ transgenic mouse to traumatic brain injury-induced behavioral deficits and cortical damage. *J Cereb Blood Flow Metab* 1999; 19: 762–70.
- Nortje J, Menon DK. Traumatic brain injury: physiology, mechanisms and outcome. *Curr Opin Neurol* 2004; 17: 711–8.
- Sofroniew MV. Reactive astrocytes in neural repair and protection. *Neuroscientist* 2005; 5: 400–7.
- Sofroniew MV, Howe CL, Mobley WC. Nerve growth factor signaling, neuroprotection and neural repair. *Annu Rev Neurosci* 2001; 24: 1217–81.
- Zhao X, Ahran A, Berman RF, Muizellar JP, Lyeth BC. Early loss of astrocytes after experimental traumatic brain injury. *Glia* 2003; 44: 140–52.