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Tissue sparing and functional recovery following experimental traumatic brain injury is provided by treatment with an anti-myelin-associated glycoprotein antibody

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Abstract

Axonal injury is a hallmark of traumatic brain injury (TBI) and is associated with a poor clinical outcome. Following central nervous system injury, axons regenerate poorly, in part due to the presence of molecules associated with myelin that inhibit axonal outgrowth, including myelin-associated glycoprotein (MAG). The involvement of MAG in neurobehavioral deficits and tissue loss following experimental TBI remains unexplored and was evaluated in the current study using an MAG-specific monoclonal antibody (mAb). Anesthetized rats (n = 102) were subjected to either lateral fluid percussion brain injury (n = 59) or sham injury (n = 43). In surviving animals, beginning at 1 h post-injury, 8.64 μg anti-MAG mAb (n = 33 injured, n = 21 sham) or control IgG (n = 26 injured, n = 22 sham) was infused intracerebroventricularly for 72 h. One group of these rats (n = 14 sham, n = 11 injured) was killed at 72 h post-injury for verification of drug diffusion and MAG immunohistochemistry. All other animals were evaluated up to 8 weeks post-injury using tests for neurologic motor, sensory and cognitive function. Hemispheric tissue loss was also evaluated at 8 weeks post-injury. At 72 h post-injury, increased immunoreactivity for MAG was seen in the ipsilateral cortex, thalamus and hippocampus of brain-injured animals, and anti-MAG mAb was detectable in the hippocampus, fimbria and ventricles. Brain-injured animals receiving anti-MAG mAb showed significantly improved recovery of sensorimotor function at 6 and 8 weeks ($P < 0.01$) post-injury when compared with brain-injured IgG-treated animals. Additionally, at 8 weeks post-injury, the anti-MAG mAb-treated brain-injured animals demonstrated significantly improved cognitive function and reduced hemispheric tissue loss ($P < 0.05$) when compared with their brain-injured controls. These results indicate that MAG may contribute to the pathophysiology of experimental TBI and treatment strategies that target MAG may be suitable for further evaluation.

Keywords

adhesive/sticky paper test; head injury; hemispheric tissue loss; myelin-associated glycoprotein; osmotic minipumps; rat

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Introduction

Each year, traumatic brain injury (TBI) affects 2 000 000−3 000 000 people in the USA (NIH Consensus Development Panel, 1999) and more than 1 000 000 people in the European Union require hospitalization as a result of TBI (International Brain Injury Association, 2005). Currently no standardized pharmacologic therapy with proven clinical benefit is available for the treatment of TBI, despite the promising evaluation of several therapeutic approaches to experimental TBI using clinically relevant experimental models (Bullock *et al*., 1999; Laurer & McIntosh, 2001; Maas, 2002). Most experimental studies, to date, have focused on the evaluation of acute neuroprotection in the immediate post-injury period (for review see Royo *et al*., 2003), rather than an assessment of novel strategies to enhance the inherent regenerative and repair capacity of the central nervous system.

Axonal injury is a common finding following experimental and clinical TBI (Gentleman *et al*., 1995; Povlishock *et al*., 1999; Graham *et al*., 2000; Adams *et al*., 2001). The regeneration of axons post-injury may be limited due to numerous factors including insufficient trophic support, the formation of a physical barrier such as the glial scar, insufficient induction and activation of appropriate growth-promoting proteins and/or the presence of a hostile axonal microenvironment including molecules inhibitory to axonal outgrowth (for review see Fawcett & Asher, 1999; Silver & Stryker, 2001; Filbin, 2003; Lee *et al*., 2003; Sandvig *et al*., 2004; Schwab, 2004). Both *in vitro* and *in vivo* evidence suggests that inhibitors of axonal growth present in myelin, such as Nogo-A, oligodendrocyte-myelin glyco-protein and myelin-associated glycoprotein (MAG), may prevent axonal outgrowth in models of nervous system injury such as cerebral ischemia, traumatic spinal cord injury and peripheral nerve injury (Caroni *et al*., 1988; Caroni & Schwab, 1988; McKerracher *et al*., 1994; Mukhopadhyay *et al*., 1994; Prinjha *et al*., 2000; Kottis *et al*., 2002; Wang *et al*., 2002b). These results suggest that myelin-associated inhibitors of axonal outgrowth may also possibly contribute to the poor functional recovery seen following TBI. Recently, we have evaluated the effects of Nogo-A inhibition using the Nogo-A-neutralizing monoclonal antibodies (mAbs) 11C7 and 7B12 following experimental TBI using the lateral fluid percussion (FP) injury model and observed improved cognitive recovery at 4 weeks post-injury compared with brain-injured rats treated with vehicle (Lenzlinger *et al*., 2005; Marklund *et al*., 2004). These findings support those in other models of central nervous system injury (Papadopoulos *et al*., 2002; Emerick *et al*., 2003; Li & Strittmatter, 2003) where antibody treatment against Nogo and its receptor has resulted in improved functional recovery compared with knockouts (Marklund *et al*., 2005).

Myelin-associated glycoprotein, a 100-kDa transmembrane protein (Quarles *et al*., 1973), plays a role in both signal transduction and cell adhesion (Paivalainen *et al*., 2003). MAG has been localized to cell surfaces of oligodendrocytes and Schwann cells via immunohistochemistry (Bartsch *et al*., 1989; Trapp *et al*., 1989) and interacts with neuronal receptors. The inhibition of axonal outgrowth by MAG, Nogo-66 and oligodendrocyte-myelin glycoprotein is mediated by binding to either the NOGO-66 (NgR1) or the NgR2 receptors and activation of downstream mediators (Wang *et al*., 2002a; Venkatesh *et al*., 2005). However, Nogo-A, oligodendrocyte-myelin glycoprotein and MAG may use additional neuronal receptors, indicating the potential for differing functional activities (Irving *et al*., 2005; Vinson *et al*., 2003). The inhibitory effects of MAG *in vitro* have been restricted to adult neurons (McKerracher *et al*., 1994; Mukhopadhyay *et al*., 1994) and during development, MAG is believed to play a beneficial role in both the process of myelination and the maintenance of myelin (Fruttiger *et al*., 1995; Schachner & Bartsch, 2000; Marcus *et al*., 2002). *In vitro* neutralization of a soluble form of MAG (dMAG) resulted in an increase in neurite outgrowth (Tang *et al*., 2001) and inactivation of MAG *in vitro* immediately post-optic nerve crush injury has been shown to improve regeneration of the optic nerve tract (Wong *et al*., 2003).

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Myelin-associated glycoprotein also plays a role in the maintenance and stabilization of myelin. The cytoplasmic tail interacts with signaling molecules and cytoskeletal components and the absence of MAG results in a progressive 'dying-back oligodendropathy' (Weiss *et al*., 2000). Therefore, under non-disease conditions, the interaction between MAG and neuronal receptors triggers a signaling cascade within oligodendrocytes resulting in maintenance of structure and integrity, possibly via Fyn kinase activation. A pro-survival response in oligodendrocytes is also triggered using an anti-MAG mAb to mimic neuronal receptors (Irving *et al*., 2005).

Recently, the properties of an anti-MAG mAb have been investigated. This antibody binds specifically to MAG, blocks interaction with neuronal MAG receptors and prevents MAG-mediated inhibition of neurite outgrowth (Irving *et al*., 2005). Furthermore, the antibody prevents glutamate-mediated toxicity of oligodendrocytes in culture. *In vivo*, the antibody significantly improves motor function and reduces lesion volume up to 1 week following middle cerebral artery occlusion-induced focal ischemia in rat (Irving *et al*., 2005).

Although these studies suggest that inhibition of MAG results in neuroprotection, the inhibition of MAG as a treatment strategy has not been examined, to date, in TBI. In the present study, we evaluated the effects of MAG inhibition on neurobehavioral deficits and histological damage using neurological motor, sensory and cognitive function, and hemispheric tissue loss up to 8 weeks post-injury following administration of monoclonal mouse anti-MAG antibody into the ipsilateral ventricle of rats from 1 to 72 h following TBI.

Materials and methods

All procedures were approved by the Institutional Animal Care and Use Committee at the The University of Pennsylvania and were performed in accordance with standards published by the National Research Council (1996).

Surgery and fluid percussion brain injury

Adult male Sprague-Dawley rats (350−400 g, n = 102) were anesthetized (sodium pentobarbital, 65 mg/kg, i.p.) and placed into a stereotaxic frame. A subcutaneous injection of bupivicaine (0.25%; 0.2−0.3 ml) was administered at the incision site. At approximately 5 min following injection, a midline scalp incision was made and the underlying periosteum dissected. The scalp and left temporal muscle were reflected, exposing the skull and a left parietal 5-mm craniectomy was performed midway between lambda and bregma, and 4 mm lateral to the midline over the left parietal cortex. A modified Luer-Lok cap was cemented over the craniectomy and filled with saline. At 90 min after administration of anesthesia, lateral FP brain injury of moderate (2.9−3.1 atm) severity (n = 59) was induced as originally described by McIntosh *et al*. (1989). Sham (control) animals (n = 43) received anesthesia and all surgical procedures without FP brain injury. The Luer-Lok fitting was then removed and the incision sutured. Animals were placed on heating pads from the initiation of anesthesia until 60 min post-pump implantation in order to maintain normothermia.

Pump implantation and intracerebroventricular drug administration

At 1 h post-injury, surviving animals were randomized to receive an intracerebroventricular injection of either 0.12 mg/mL inhibitory anti-MAG mAb (72 μL; a kind gift from Glaxo Smith Kline, antibody originally from Chemicon, Hampshire, UK, with additional preparation as per Irving *et al*., 2005) or control antibody (equal volume and concentration of mouse IgG) (Irving *et al*., 2005), injected over 72 h at a rate of 1 μL/h. Separate groups of brain-injured or sham-injured animals received an identical volume of control Ab (IgG). The total dose of anti-MAG mAb administered per animal was 8.64 μg.

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For antibody delivery, osmotic mini-pumps (1003D, ALZET, Cupertino, CA, USA) were attached to a cannula for intracerebroventricular infusion (Brain Infusion Kit I, ALZET) and implanted according to the manufacturer's instructions. The mini-pumps were filled under sterile conditions, attached to a 35-mm-long polyethylene catheter and infusion cannula, and primed overnight at 37 °C in 0.9% sodium chloride. The animals were reanesthetized (isoflurane, 1.5− 3%) and placed into a stereotaxic frame. To accommodate the mini-pumps, the existing scalp incision was reopened and a subcutaneous pocket was created between the scapulae by blunt dissection. The infusion cannula was stereotactically inserted at 0.8 mm posterior to bregma (AP 0.8), 1.3 mm lateral (left) to the midline (ML 1.3) and a depth of 3.8 mm from the surface of the cortex (DV −3.80) into the left lateral ventricle. Using sterile technique, the pump assembly was inserted into the subcutaneous pocket and the cannula was inserted at the above coordinates and secured to the bone with tissue adhesive (Vetbond, 3M, St Paul, MN, USA) over which the scalp was sutured. On day 3 post-injury, all animals were reanesthetized (isoflurane, 1.5− 3%), the polyethylene infusion catheter was tied off and the pump was removed.

Study A. Evaluation of drug penetration and myelin-associated glycoprotein expression post-injury

To examine the penetration of anti-MAG mAb into brain tissue and immunohistochemistry for MAG in the early post-injury phase, a subgroup of brain-injured animals was randomly assigned to receive either the inhibitory anti-MAG mAb (n = 6) or control IgG mAb (n = 5). Sham-injured controls similarly received either anti-MAG mAb (n = 8) or control IgG (n = 6). At 72 h post-injury, animals were overanesthetized with sodium pentobarbital (75 mg/kg) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. The brains were removed and post-fixed overnight at 4 °C in paraformaldehyde, and were then transferred into 30% sucrose solution for 3−4 days, snap frozen in 2-methylbutane at −20 °C, and stored at −80 °C. Brains were cut on a freezing microtome into 40-μm free-floating sections.

Detection of anti-myelin-associated glycoprotein monoclonal antibody or control antibody—Following blocking for 1 h with 3% normal horse serum, donkey anti-mouse IgG biotin (1 : 1000; Jackson ImmunoResearch, West Grove, PA, USA) was applied to every 12th section from Bregma −1.3 to −7.3. The initial order was determined in a random fashion. Following an overnight incubation at 4 °C, the avidin-biotin peroxidase method (Vector Laboratories, Burlingame, CA, USA) was used for visualization of the drug or control antibody. Internal controls included use of non-antibody-treated tissue sections and omission of secondary antibody from the protocol.

Expression of myelin-associated glycoprotein post-injury—Following blocking for 1 h with 3% normal horse serum, goat anti-MAG (1 : 2000; R and D Systems, Abingdon, UK) was applied to every 12th section from Bregma −1.3 to −7.3. The initial section chosen was adjacent to that chosen for drug diffusion. Following an overnight incubation at 4 °C, sections were washed and incubated in biotinylated donkey anti-goat IgG (Jackson ImmunoResearch) at a concentration of 1 : 1000. Following the 1-h secondary antibody incubation period, the avidin-biotin-peroxidase method was used for visualization of MAG within the brain sections. Internal controls included deletion of the primary antibody from the protocol.

Study B. Evaluation of neurobehavioral function and tissue loss

To examine the long-term neurobehavioral effects of anti-MAG mAb following TBI, brain-injured animals were randomized to receive either the inhibitory anti-MAG mAb (n = 25) or control antibody (n = 20, *vide supra*) intracerebroventricularly and sham-injured animals were also randomized to receive control IgG (n = 14) or anti-MAG mAb (n = 15). The total dose administered (8.64 μg) was identical to Study A. Following surgery or injury, neurological

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motor function was evaluated for 2 months in surviving animals in sham-injured (control-treated $n = 13$ and anti-MAG mAb-treated $n = 13$) and brain-injured (control-treated $n = 17$ and anti-MAG mAb-treated $n = 18$) rats using a battery of functional tests which have been previously shown to be sensitive for discriminating injury severity (Dixon *et al*., 1987; McIntosh *et al*., 1989; Mattiasson *et al*., 2000). Experienced observers, blinded to the injury and treatment status of each animal, performed behavioral assessments.

Neuromotor function—Gross neurologic motor function of animals was assessed at 48 h and 1, 2, 4, 6 and 8 weeks after injury using the composite neuroscore, a previously described battery of tests (McIntosh *et al*., 1989). The seven tests used included left and right forelimb contraflexion response during suspension by the tail, left and right hindlimb flexion while hindlimbs are lifted up and back by the tail, the ability to resist right and left lateral pulsion, and the ability to stand on an inclined plane. Scores for each test were given on an integer scale from 4 (uninjured) to 0 (severely impaired) and were combined, yielding a maximum composite neuroscore of 28.

The rotating pole test has been shown to be effective in the evaluation of neurological motor dysfunction following lateral FP brain injury (Mattiasson *et al*., 2000). On each of 2 days prior to injury, each animal was trained to traverse the full length of the pole. On the first training day, animals were trained on a non-rotating pole and on the second training day animals were trained on a rotating pole. At 1, 2, 4, 6 and 8 weeks post-injury, three trials per animal were performed with the pole rotating at 5 r.p.m. in the left rotational (counter-clockwise) direction. Animals were scored using the following scale: 4, rat traverses the pole with less than one footslip; 3, rat traverses the pole with two to three footslips or jumps; 2, rat traverses the pole with four or more footslips or jumps; 1, rat falls off while walking and 0, rat falls off when placed on the pole (Mattiasson *et al*., 2000).

Sensorimotor assessment—The adhesive paper test (Schallert *et al*., 1982; Hernandez & Schallert, 1988) has been previously used successfully to assess neurologic deficits following experimental TBI and pharmacologic treatment (Riess *et al*., 2001). Initial tests were performed prior to brain injury or sham operation to record the baseline latencies for each animal to ensure no significant differences at baseline among the groups. The mean latency (maximum, 120 s) over three trials to remove adhesive tapes (14 × 23 mm, Avery, Pasadena, CA, USA) from the forepaws was recorded at 1, 2, 4, 6 and 8 weeks post-injury for the ipsilateral (left) and contralateral (right) side. Latencies are expressed as mean time to remove the adhesive paper in seconds over the three trials.

Evaluation of visuospatial learning at 6 and 8 weeks post-injury—Animals were introduced to the Morris water maze for the first time beginning on day 42 post-injury in order to evaluate post-traumatic anterograde amnesia using a visuospatial learning paradigm. The maze consisted of a 1.8-m diameter pool where a fixed, hidden, clear, 10-cm-diameter plexiglas platform was submerged 1 cm below the surface of water at 24 °C. Animals underwent 3 days of testing, on post-injury days 42−44, with eight trials per day (two testing blocks of four trials each) during which they learned to find the submerged platform using external visual cues, placed around the periphery of the pool. Learning trials began by placing animals into the maze and releasing them at one of four sites separated by 90° around the periphery of the maze. Each animal was given at most 60 s per trial to find the hidden platform. The animal's latency to reach the platform was recorded for each of the 24 trials and the average for each of the six testing blocks was calculated as a measure of post-injury learning. This paradigm has previously proven effective in detecting deficits in learning ability of brain-injured rats (Saatman *et al*., 1997; Cheney *et al*., 2000). On day 56 post-injury, the platform was moved from its previous location to a new location, and animals were reintroduced to the same 1.8-m maze. Learning trials were conducted in the same manner over days 56−58, as animals were

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released at one of four sites separated by 90° around the periphery of the maze and latency was recorded for up to 60 s for each of the 24 trials. This shift paradigm has been used successfully in detecting alterations in learning ability in rats due to many factors and conditions including FP brain injury (Blokland *et al*., 1999; Cirulli *et al*., 2004; Thompson *et al*., 2006).

Tissue preparation—Following cognitive evaluation at the 8-week time point, animals were killed, perfused and post-fixed with 4% paraformaldehyde as previously described (*vide supra*). Following overnight post-fixation, the brains from Study B were then processed for paraffin embedding. Serial coronal serial brain sections (40 μm thick) were cut on a rotary microtome (Shandon Finesse ME, Thermo Electron, Pittsburgh, PA, USA).

Hemispheric tissue loss—To quantify hemispheric tissue loss following lateral FP brain injury at 8 weeks, coronal sections (40 μm thick) were taken every 1 mm from −0.3 mm bregma to −7.3 mm bregma (Paxinos & Watson, 1994) from a random subset of brain-injured and sham-injured animals ($n = 7$ per group). These regions were selected based on previous studies which demonstrated that cortical and thalamic tissue damage, observed up to 1 month after lateral FP injury, occurred within this region (Soares *et al*., 1995; Hicks *et al*., 1996; Zhang *et al*., 1998; Bramlett *et al*., 1999; Bentzer *et al*., 2001; Bramlett & Dietrich, 2001; Marklund *et al*., 2001). Sections were stained with hematoxylin and eosin and imaged using a digital camera integrated with a light microscope. As previously described (Hoover *et al*., 2004), the peripheries of the contralateral and ipsilateral hemisphere [not including the ventricle(s)] were traced on each image by an evaluator blinded to the injury and treatment status of the animals and the area of each hemisphere was calculated using a calibrated image analysis system (IMAGE 1.62c, Scion Corp., Frederick, MD, USA). The contralateral hemisphere for each section was used to control for interanimal variation in brain size (Zhang *et al*., 1998). Hemispheric tissue loss was calculated as a percentage of the contralateral (uninjured) hemisphere volume (Vc) using the following formula: (Vc – Vi)/(Vc) × 100, where Vi represents the volume of the ipsilateral (injured) hemisphere. To calculate total hemispheric tissue lost, areas were integrated over the 7-mm rostro-caudal distance.

Statistical analyses

Ordinal, non-parametric neurological motor scores and rotating pole scores are presented as median values. Comparisons among groups were made using a Kruskal–Wallis ANOVA followed by individual Mann–Whitney *U*-tests at each time point for these behavior tests. Continuous variables including latency to remove sticky paper and hemispheric tissue loss are presented as means + SD and were evaluated using a two-way ANOVA followed by the Student Newman Keuls post-hoc test. Averages for each treatment group of the six testing blocks in the Morris water maze were evaluated over time using two-way repeated measures ANOVA followed by the Student Neuman Keuls post-hoc test. A *P*-value of less than 0.05 was considered to be statistically significant, with corrections for multiple comparisons made where appropriate. All statistical analyses were performed using spss 12.0 for Windows (SPSS, Chicago, IL, USA).

Results

Mortality

The overall initial mortality for Studies A and B was 23.7% (14/59) similar to that reported previously in our laboratory (Hoover *et al*., 2004). Most animals died due to prolonged apnea immediately following TBI. An additional 12 animals, equally distributed among the treatment and injury groups, were killed during the 2-month study period due to weight loss ($n = 4$), infection ($n = 5$) or aberrant behavior (e.g. extreme lethargy, spinning; $n = 3$) in accordance with animal welfare guidelines. These animals were not included in the behavioral analysis.

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Study A

At 72 h post-injury, anti-MAG mAb or control IgG was detectable in the majority of animals (100% of sham-injured control-treated, 60% of sham-injured anti-MAG mAb-treated, 50% of brain-injured control-treated and 100% of brain-injured anti-MAG mAb-treated animals). Penetration of the anti-MAG mAb was detected primarily in brain areas near the catheter insertion site (Fig. 1A) but diffusion was noted to more distal regions of the hippocampus and fornix (Fig. 1B) as well as the third (Fig. 1C) and fourth ventricles of some animals. The catheter was positioned correctly in the left lateral ventricle in all animals at post-mortem evaluation and pump volumes were assessed pre-insertion and post-removal to confirm antibody delivery.

Using immunohistochemistry, an increased staining profile for MAG was observed in the ipsilateral cortex, external capsule and hippocampus at 72 h post-injury in comparison to sham-injured animals (Fig. 2A–F). In the ipsilateral cortex, hippocampus and external capsule, the immunoreactivity for MAG of brain-injured anti-MAG mAb-treated animals (Fig. 2D–F) was similar to brain-injured animals that received IgG (data not shown).

Study B

Fluid percussion brain injury induced a significant and prolonged deficit in neurologic motor function as assessed using the composite neuroscore from 48 h to 8 weeks post-injury, regardless of treatment status (Kruskal–Wallis test, d.f. = 3, $P < 0.001$, Fig. 3). Treatment with anti-MAG mAb resulted in a statistically significant improvement of gross neuromotor function at 6 weeks post-injury when compared with IgG treatment ($Z = -2.18$, $P < 0.05$, Fig. 3). Additionally, regardless of treatment, using the Friedman's test, brain-injured animals significantly recovered function over time ($P < 0.001$). On post-hoc testing (Wilcoxon sign rank) this functional recovery in injured animals was only significant from 48 h to 1 week ($P < 0.05$) and from 4 to 6 weeks post-injury ($P < 0.01$).

Marked and prolonged post-injury deficits were also present in both IgG-treated and anti-MAG mAb-treated brain-injured animals when compared with their respective sham-injured controls in the rotating pole beginning at 1 week and continuing up to 8 weeks post-injury via Mann–Whitney U post-hoc testing (data not shown). Treatment with anti-MAG mAb did not significantly attenuate the deficits observed on the rotating pole following brain injury in comparison to IgG treatment (P = n.s.; data not shown).

Prior to injury or surgery, no differences were observed in the latency to remove sticky paper among the treatment groups (P = n.s., data not shown). Lateral FP brain injury significantly impaired sensorimotor function assessed by the adhesive paper test from 1 to 8 weeks post-injury on both the right (data not shown) and left ($P < 0.01$, Fig. 4) forepaws when compared with sham-injured animals. Treatment with anti-MAG mAb following brain injury resulted in a significant enhancement (decreased latency) in ipsilateral sensory function at 6 and 8 weeks post-injury in comparison to IgG treatment. Brain-injured animals receiving anti-MAG mAb were significantly faster at removing the adhesive paper from the left forepaw ($P < 0.01$) than those brain-injured animals that received control IgG (Fig. 4). Additionally, administration of anti-MAG mAb significantly improved the latency of the sham-injured animals to remove the adhesive paper from the left forepaw at 1 and 8 weeks post-surgery in comparison to IgG-treated, sham-injured animals ($P < 0.05$; Fig. 4).

All animals, regardless of injury and treatment status, exhibited a pattern of decreasing latencies to find the hidden platform in the Morris water maze test over the six testing blocks at 6 and 8 weeks post-injury ($P < 0.001$, Figs 5 and 6). In comparison to sham-injured animals, brain-injured animals showed a significant deficit in spatial learning ability on days 42−44 post-injury ($F = 61.9$, d.f. = 53, $P < 0.001$; Fig. 5). When the platform was shifted to a new location

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and animals were required to learn a new paradigm, brain-injured animals again showed a significant deficit in spatial learning capacity at 8 weeks post-injury (days 56−58) in comparison to sham-injured animals ($F = 34.75$, $P < 0.001$; Fig. 6). Brain-injured animals receiving anti-MAG mAb exhibited a trend towards shorter latencies to find the hidden platform at 6 weeks post-injury in comparison to IgG-treated animals but this difference was not significant (P 0.09; Fig. 5). However, by 8 weeks post-injury, brain-injured animals that received anti-MAG mAb demonstrated significantly shorter latencies to find the hidden platform than brain-injured animals receiving control IgG ($F = 3.62$, d.f. = 265, $P < 0.05$; Fig. 6).

At 8 weeks post-injury, all brain-injured animals regardless of treatment status lost a significant volume of tissue in the ipsilateral hemisphere in comparison to sham-injured animals ($F = 99.22$, d.f. = 24, $P < 0.001$). Additionally, there was a significant interaction effect between injury status and drug treatment ($F < 6.17$, d.f = 1, $P < 0.05$). Moreover, treatment with anti-MAG mAb following lateral FP brain injury significantly reduced the volume of tissue lost due to injury (12.5%, Fig. 7A, C and E) in comparison to control IgG treatment (18.0%, $P < 0.05$, Fig. 7A, B and D).

Discussion

Acute treatment with a novel anti-MAG antibody for 72 h following TBI when administered intracerebroventricularly, at a dose of 0.12 μg/h, significantly improved motor, sensory and cognitive deficits as well as significantly reduced lesion volume in brain-injured animals. This is the first *in vivo* study supporting the beneficial effects of pharmacologic modulation of MAG on functional and histological outcomes in a model of TBI. These results are marked as it is rare that an experimental compound improves both sensorimotor and cognitive function while concomitantly reducing the amount of post-traumatic tissue injury. The neurobehavioral improvements observed were not seen until 6−8 weeks post-treatment, which is congruent with the antibody's proposed regenerative mechanism of action, and were observed in multiple areas making this a treatment strategy worthy of further investigation for clinical potential.

Our initial observations (Study A) suggest that anti-MAG mAb, when administered centrally, is distributed with a gradient from the infusion site and is capable of penetrating the injured brain. The anti-MAG mAb was found to diffuse into vulnerable brain regions, such as the ipsilateral hippocampus, which may be crucial for the plasticity observed following neutralization of MAG (Tang *et al.*, 2001) and for the beneficial effects observed on cognitive function. We conclude that the osmotic mini-pump with intracerebroventricular infusion was an effective drug-delivery method in this model of brain injury. As diffusion of the antibody occurred mainly around the catheter insertion site and surrounding ventricle, the anti-MAG mAb should also be evaluated in models of diffuse axonal injury (e.g. impact acceleration) to confirm the efficacy of the osmotic mini-pump method vs. the need to explore an intravascular administration paradigm (Irving *et al*., 2005) to optimize predictive validity.

The increase in MAG at 72 h post-injury, as indicated by immunohistochemistry, was similar among the injured treatment groups and was increased in the ipsilateral (injured) cortex, hippocampus and thalamus. There were no differences in the injured groups in injury severity measures including mortality, length of apnea or seizure, indicating that differences seen between the groups are related to the actions of the anti-MAG mAb. As the anti-MAG antibody used for staining at 72 h post-injury in this study was polyclonal, it had multiple epitopes over the entire extracellular region of MAG, whereas the treatment antibody has only one epitope. Thus, it is unlikely that the treatment antibody significantly blocked the signal from the staining antibody, so the similarity in MAG staining patterns seen at 72 h post-injury served as an internal control.

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We observed that treatment with anti-MAG mAb improved neurologic reflex testing using the composite neuroscore test at 6 weeks post-injury. Although this modest improvement in functional motor recovery did not persist in the treated group at 8 weeks post-injury, it is possible that the natural recovery experienced by injured animals may have diluted the treatment effect at 8 weeks.

Improvement of reflexive motor testing following experimental brain injury was not previously observed following inhibition of Nogo-A using the identical experimental lateral FP brain injury model (Marklund *et al*., 2004; Lenzlinger *et al*., 2005), although vestibulomotor improvement was reported at 1 and 4 weeks post-injury (Marklund *et al*., 2004). We were not able to demonstrate improvement in vestibulomotor function with administration of the anti-MAG mAb following FP brain injury. Given the proposed mechanism of action of the anti-MAG mAb in blocking the biological effects of MAG and promoting axonal outgrowth, the timing of initial improvement seen (6 weeks post-injury) is likely to be associated with a more long-term effect of this compound on plasticity. These observations validate the need to plan and conduct studies with long-term time points when evaluating compounds with similar mechanisms of action. The assessment of histopathology at intermediate time points may also be of critical importance in future study design in order to characterize the time course of therapeutic antibody presence, axonal, myelin and neuronal loss, and potential sparing of neurons and/or oligodendrocytes using anti-MAG mAb strategies. Although the optimal target to evaluate compensatory sprouting in experimental models of TBI has yet to be established, future studies of the anti-MAG mAb should also incorporate this analysis into study designs as it may be an additional mechanism of action for the compound's efficacy.

Following lateral FP brain injury, long-term deficits (up to 4 weeks) in sensorimotor function have been previously reported using the adhesive paper test (Riess *et al*., 2001). This study confirms and extends this work as moderately brain-injured animals showed significant sensorimotor deficits in both forelimbs up to 8 weeks post-injury. Treatment with anti-MAG mAb significantly attenuated ipsilateral sensorimotor deficits at 6 and 8 weeks post-injury in comparison to treatment with control IgG. The significantly lower latencies in sham-injured animals treated with the anti-MAG mAb are interesting given that there were no differences at baseline among the groups. Similar to human TBI, over time there is a progressive development of the injury cavity and hydrocephalus seen with the lateral FP model (Bramlett *et al*., 1997; Pierce *et al*., 1998). As treatment with the anti-MAG mAb following brain injury significantly preserved brain tissue in the ipsilateral (injured) hemisphere, including the cortex and thalamus, the anti-MAG mAb may be one mechanism for the significant differences seen in the adhesive paper test. Following glutamate toxicity, significant preservation of oligodendrocytes *in vitro* by the anti-MAG mAb has been previously reported (Irving *et al*., 2005) and this mechanism may be a component of the tissue sparing seen in the present study. It is therefore possible that the administration of the anti-MAG mAb, via oligodendrocyte-sparing mechanisms, may attenuate the progressive white matter loss observed following TBI, resulting in improved functional outcomes. However, given the current limitations of the lateral FP model for assessment of oligodendrocyte loss (Grady *et al*., 2003), this was not evaluated in the present study. Future studies should be planned to include additional sensorimotor evaluation as well as evaluation of the effects of the compound on axonal sprouting within the rubrospinal tract and within both control and injured animals.

In the present study we also demonstrate significant long-term improvement in cognitive function following treatment with the anti-MAG mAb. The antibody was able to diffuse into the injured (ipsilateral) hippocampus of brain-injured animals and was present up to 72 h post-injury, and the observed cognitive effects seen could be related to antagonism of MAG and/or stabilization of myelin in this vulnerable region. Improvement in memory function has recently been reported following TBI with the inhibition of Nogo-A (Marklund *et al*., 2004; Lenzlinger

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et al., 2005). Although Nogo-A and MAG both inhibit axonal regeneration, this effect does not seem to be mediated by the same receptor. Although Nogo-66 and MAG share a common receptor (NgR) (McKerracher & Winton, 2002), the receptor mediating inhibition of regeneration in response to Nogo-A is not known. Furthermore, MAG may utilize other receptors in addition to NgR (Vinson *et al*., 2001, 2003; Vyas *et al*., 2002). Thus, it is certainly possible that, although the effect of MAG and Nogo on axonal outgrowth is similar, the effect of modulating the pathways in experimental TBI may not be. Taken together, our data suggest that MAG may be involved in the development of motor and cognitive deficits post-injury and that the inhibition of MAG following experimental TBI is neuroprotective.

In the present study, the administration of the anti-MAG mAb began at 1 h post-injury and continued for 72 h; we show that there is an increased immunostaining of MAG in the ipsilateral cortex, external capsule and hippocampus at 72 h post-injury. Given the ability of dMAG to diffuse from damaged white matter (Tang *et al*., 1997, 2001) and the diffuse staining pattern seen after lateral FP brain injury, the diffusion of anti-MAG mAb into areas known to be vulnerable to injury as well as areas distal to the site of infusion were also important findings.

The behavioral improvements seen beginning at 6 weeks and tissue sparing following administration of the anti-MAG mAb to FP brain-injured animals support its function as a neuroprotective agent. This study confirms the findings of tissue sparing and improvement of motor function following anti-MAG mAb administration by Irving *et al*. (2005) in a cerebral ischemia model, and extends the previous work with the findings of sensorimotor and cognitive improvement. The discovery of the ability of MAG antagonism to exert beneficial effects across multiple behavioral endpoints is rare in the setting of FP injury (for review see Thompson *et al*., 2005). Thus, these novel observations suggest that MAG may be an important and differential contributor to the pathophysiology of TBI and treatment strategies targeting MAG may be suitable for further clinical evaluation.

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Abbreviations

FP, fluid percussion; mAb, monoclonal antibody; MAG, myelin-associated glycoprotein; TBI, traumatic brain injury..

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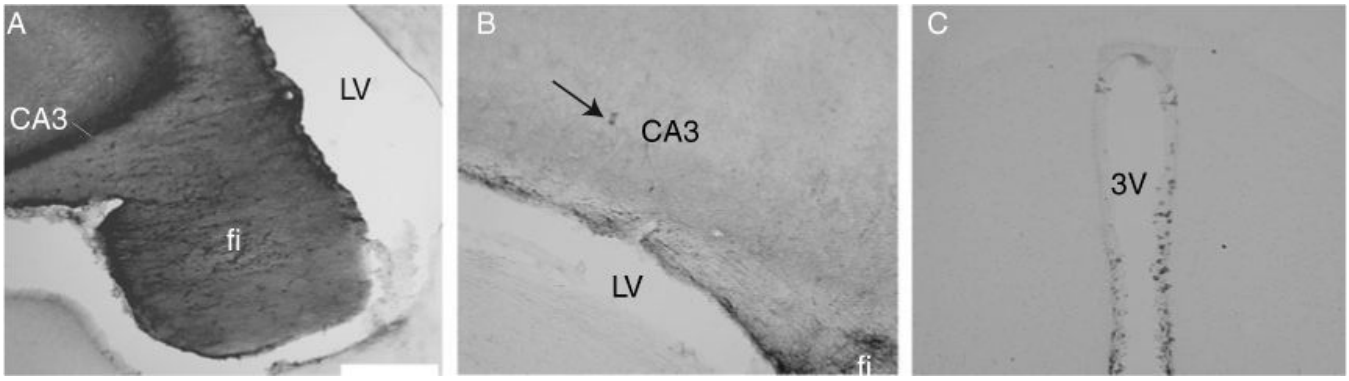

Fig. 1.

Penetration and diffusion of anti-myelin-associated glycoprotein monoclonal antibody into rat brain at 72 h post-injury in a brain-injured animal. (A) At Bregma −1.8 (Paxinos & Watson, 1994) into CA3 near catheter insertion site. (B) At Bregma −2.8 showing penetration into the ipsilateral hippocampus (CA3 arrow) and fimbria (fi). (C) At Bregma −3.6 demonstrating diffusion of the drug to the third ventricle (3V). Scale bar, 0.5 mm in A, 200 mm in B and C. LV, lateral ventricle.

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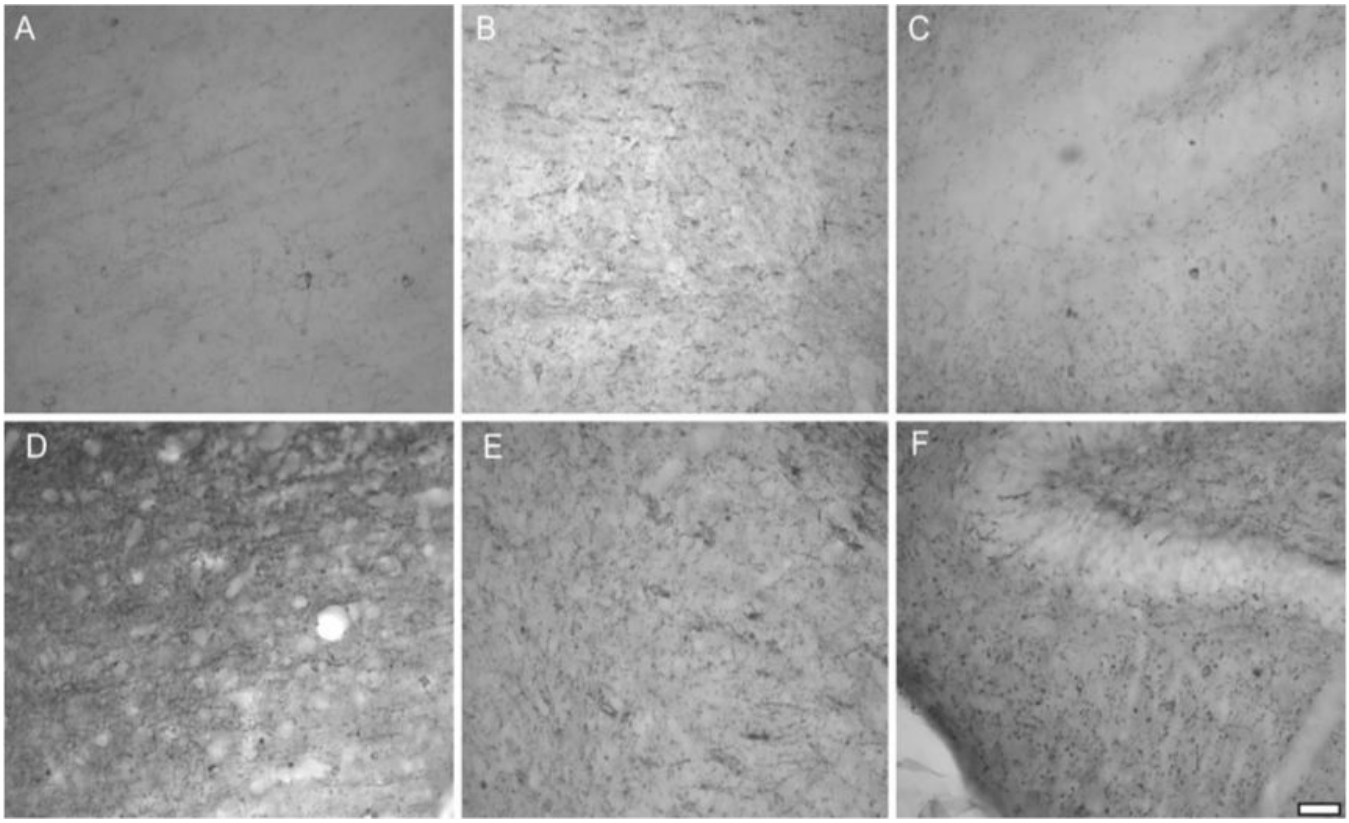

Fig. 2.

Immunohistochemistry characterizing patterns of myelin-associated glycoprotein (MAG) at 72 h post-injury. Representative sections at 72 h post-surgery from ipsilateral (A) cortex, (B) thalamus and (C) hippocampus of a sham-injured animal reveal a small amount of native MAG. Representative sections from a brain-injured anti-MAG antibody-treated animal show an increased manifestation of MAG post-injury in ipsilateral (D) cortex, (E) thalamus and (F) hippocampus in comparison to sham injury. All panels 200× magnification.

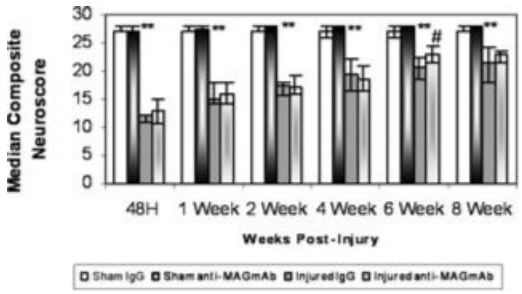

Fig. 3.

Assessment of composite neuroscore (median +25th and 75th percentile) among the treatment groups. Following injury, brain-injured IgG-treated animals had significantly lower neuroscores than sham-injured IgG-treated animals (***P* < 0.01). At 6 weeks post-injury, brain-injured anti-myelin-associated glycoprotein (MAG) monoclonal antibody (mAb)-treated animals had a significantly higher neuroscore than their brain-injured IgG-treated counterparts (#*P* < 0.05).

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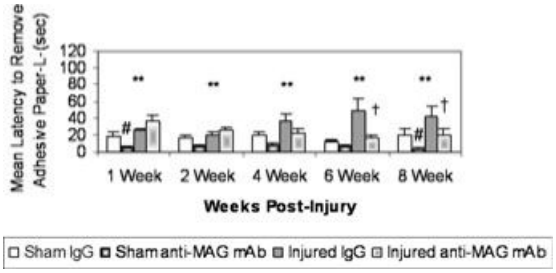

Fig. 4.

Latency to remove adhesive paper from left forepaw (mean + SEM). Following injury, brain-injured IgG-treated animals had significantly longer latencies to remove the sticky paper from the left forepaw than sham-injured IgG-treated animals (***P* < 0.01). At 1 and 8 weeks post-surgery sham animals given anti-myelin-associated glycoprotein (MAG) monoclonal antibody (mAb) had significantly shorter latencies to remove the paper than their IgG-treated counterparts (#*P* < 0.05). At 6 and 8 weeks post-injury, brain-injured animals treated with anti-MAG mAb were able to remove the paper significantly faster than their IgG-treated counterparts (†*P* < 0.05).

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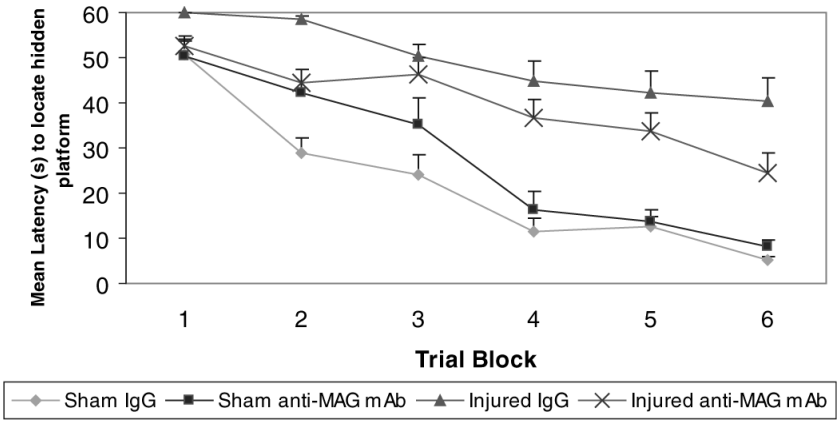

Fig. 5.

Learning at 6 weeks post-injury. Latencies to reach the hidden platform using the Morris water maze paradigm to assess visuospatial learning at 6 weeks post-injury (means + SEM). Brain-injured vehicle-treated animals had significantly longer latencies to reach the platform when compared with sham-injured, IgG-treated controls ($P < 0.001$). Treatment with the anti-myelin-associated glycoprotein (MAG) monoclonal antibody (mAb) following lateral fluid percussion brain injury resulted in consistently shorter latencies compared with IgG-treated brain-injured controls but this did not reach statistical significance ($P = 0.094$).

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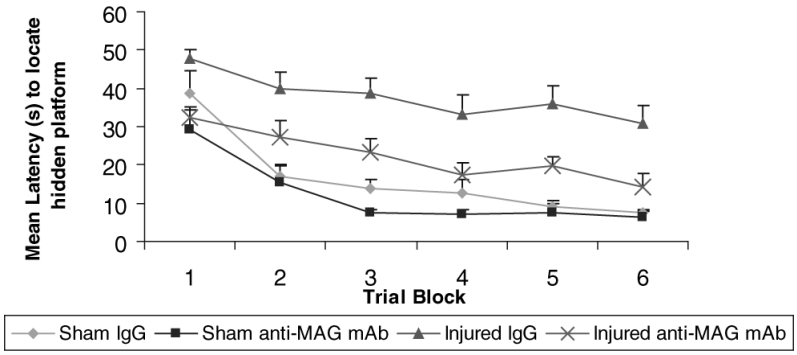

Fig. 6.

Learning at 8 weeks post-injury. At 2 weeks following the original learning paradigm, the animals were reintroduced to the Morris water maze in a paradigm shift and challenged to learn a new visuospatial task. Brain-injured vehicle-treated animals had significantly longer latencies to reach the platform when compared with sham-injured, IgG-treated controls. Treatment with the anti-myelin-associated glycoprotein (MAG) monoclonal antibody (mAb) following brain injury resulted in significantly shorter latencies to reach the platform when compared with brain-injured IgG-treated controls ($P < 0.05$).

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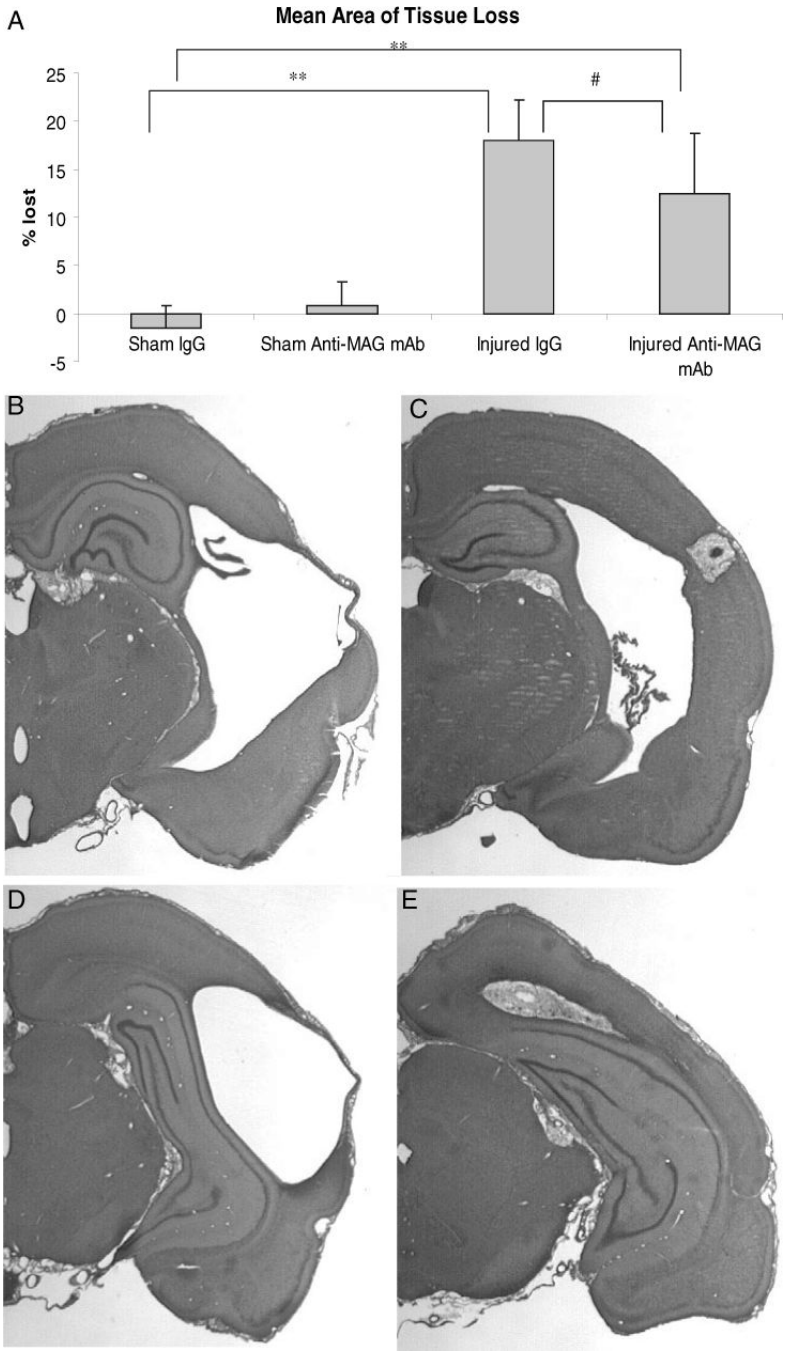

Fig. 7.

Mean percent hemispheric tissue loss + SD at 8 weeks post-injury. (A) Lateral fluid percussion (FP) brain injury resulted in significant ipsilateral hemispheric tissue loss at 8 weeks post-injury (**$P < 0.001$) and treatment with the anti-myelin-associated glycoprotein (MAG) monoclonal antibody (mAb) significantly reduced this tissue loss (#$P < 0.05$) in comparison to brain-injured animals given control IgG. (B and C) Lateral FP injury resulted in a significant loss of ipsilateral tissue at 8 weeks post-injury with visual lesions. Sections from Bregma −2.8 of (B) control IgG-treated and (C) anti-MAG mAb-treated animals and from Bregma −3.8 of (D) control IgG-treated and (E) anti-MAG mAb-treated animals demonstrating significant amelioration of tissue loss following brain injury.