Neuroprotection in a rabbit model of intraventricular haemorrhage by cyclooxygenase-2, prostanoid receptor-1 or tumour necrosis factor-alpha inhibition

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Intraventricular haemorrhage is a major complication of prematurity that results in neurological dysfunctions, including cerebral palsy and cognitive deficits. No therapeutic options are currently available to limit the catastrophic brain damage initiated by the development of intraventricular haemorrhage. As intraventricular haemorrhage leads to an inflammatory response, we asked whether cyclooxygenase-2, its derivative prostaglandin E2, prostanoid receptors and pro-inflammatory cytokines were elevated in intraventricular haemorrhage; whether their suppression would confer neuroprotection; and determined how cyclooxygenase-2 and cytokines were mechanistically-linked. To this end, we used our rabbit model of intraventricular haemorrhage where premature pups, delivered by Caesarian section, were treated with intraperitoneal glycerol at 2 h of age to induce haemorrhage. Intraventricular haemorrhage was diagnosed by head ultrasound at 6 h of age. The pups with intraventricular haemorrhage were treated with inhibitors of cyclooxygenase-2, prostanoid receptor-1 or tumour necrosis factor-α; and cell-infiltration, cell-death and gliosis were compared between treated-pups and vehicle-treated controls during the first 3 days of life. Neurobehavioural performance, myelination and gliosis were assessed in pups treated with cyclooxygenase-2 inhibitor compared to controls at Day 14. We found that both protein and messenger RNA expression of cyclooxygenase-2, prostaglandin E2, prostanoid receptor-1, tumour necrosis factor-α and interleukin-1β were consistently higher in the forebrain of pups with intraventricular haemorrhage relative to pups without intraventricular haemorrhage. However, cyclooxygenase-1 and prostanoid receptor 2–4 levels were comparable in pups with and without intraventricular haemorrhage. Cyclooxygenase-2, prostanoid receptor 1 or tumour necrosis factor-α inhibition reduced inflammatory cell infiltration, apoptosis, neuronal degeneration and gliosis around the ventricles of pups with intraventricular haemorrhage. Importantly, cyclooxygenase-2 inhibition alleviated neurological impairment, improved myelination and reduced gliosis at 2 weeks of age. Cyclooxygenase-2 or prostanoid receptor-1 inhibition reduced tumour necrosis factor-α level, but not interleukin-1β. Conversely, tumour necrosis factor-α
Keywords: cyclooxygenase-2; prostanoid receptor; germinal matrix haemorrhage; intraventricular haemorrhage; premature rabbit pups; tumour necrosis factor-alpha; celecoxib

Abbreviations: COX-2 = cyclooxygenase-2; EP = prostanoid receptor; GFAP = glial fibrillary acidic protein; IL-1β = interleukin-1β; IVH = intraventricular haemorrhage; PBS = phosphate buffered saline; PCR = polymerase chain reaction; PGE2 = prostaglandin E2; TNF-α = tumour necrosis factor-alpha; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labelling

Introduction

Germinal matrix haemorrhage-intraventricular haemorrhage (IVH) occurs in about 12,000 premature infants every year in the USA, and predisposes the survivors to cerebral palsy and cognitive deficits (Heuchan et al., 2002). IVH is not substantially preventable and the treatment of the resultant brain injury is non-existent. The development of IVH results in an inflammatory response, oxidative stress and neural cell death, predominantly around the lateral ventricles of the forebrain (Georgiadis et al., 2008; Zia et al., 2009). Cyclooxygenase 2 (COX-2)—an inducible enzyme catalyzing synthesis of prostanoid—plays a key role in cerebral pathologies associated with inflammation, oxidative injury and glutamate excitotoxicity (FitzGerald, 2003; Minghetti, 2007). Therefore, we asked whether COX-2 derived prostanoids contribute to IVH-induced brain injury in premature newborns and if so, whether inhibition of COX-2 or its downstream mediators would offer neuroprotection.

COX-2 expression is markedly upregulated in a wide range of brain diseases including traumatic, ischaemic and degenerative diseases, and inhibition of this enzyme confers neuroprotection in animal models of brain injury (Chu et al., 2004; Gopez et al., 2005). Accordingly, celecoxib, a COX-2 inhibitor, reduces cerebral inflammation and brain oedema and facilitates functional recovery in a rat model of hypoxia-ischaemia (Chu et al., 2004). Emerging evidence indicates that prostaglandin E2 (PGE2) mediates neurotoxicity of COX-2 (Cimino et al., 2008). PGE2 activates G-protein coupled receptors, including prostanoid receptor (EP1, EP2, EP3 and EP4). Activation of EP1 elicits neurotoxicity while EP2, EP3 and EP4 are neuro-protective (Bilak et al., 2004; Ahmad et al., 2005). Indeed, gene inactivation or pharmacological blockade of EP1 provides neuroprotection in models of excitotoxicity, cerebral-ischaemia and oxygen glucose deprivation (Kawano et al., 2006).

IVH results in an inflammatory response consisting of cell death, microglia infiltration and gliosis (Georgiadis et al., 2008). Activated microglia and reactive astrocytes produce pro-inflammatory cytokines—to tumour necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β), which are associated with neonatal cerebrovascular injuries (Kaur and Ling, 2009). Importantly, several studies demonstrate a regulatory cross-talk between COX-2 and TNF-α pathways (Ikawa et al., 2001; Martinet et al., 2009). COX-2 inhibition suppresses TNF-α production in neuronal-glial culture experiments and alleviates the cellular inflammatory response in a rat stroke model (Brambilla et al., 1999; Araki et al., 2001; Ahmad et al., 2009). Conversely, pharmacological inhibition of TNF-α suppresses COX-2 levels in an in vitro model of peritoneal macrophages (Crisafulli et al., 2009; Martinet et al., 2009). Thus, it is important to determine how COX-2, prostanoid receptors, pro-inflammatory cytokines, cell death and gliosis are mechanistically linked in the signalling cascade in order to develop an optimal therapeutic strategy for IVH. On this basis, we hypothesized that COX-2, its derivative PGE2, EP1 receptor and pro-inflammatory cytokines (TNF-α and IL-1β) might be elevated in preterm newborns with IVH, and that inhibitors of COX-2, EP1 receptor or TNF-α would attenuate inflammation, neuronal cell death and gliosis, thereby offering neuroprotection. We also postulated that COX-2 or EP1 receptor inhibition might reduce pro-inflammatory cytokines, and that TNF-α inhibition would suppress COX-2 levels.

To test our hypotheses, we adopted our rabbit pup (E29, term = 32 days) model of IVH, as this model mimics preterm infants with IVH (Ballabh et al., 2007; Georgiadis et al., 2008). The present study revealed that IVH resulted in upregulation of COX-2, TNF-α, IL-1β and EP1, but not EP2-4. Suppression of COX-2, EP1 or TNF-α offered neuroprotection. Furthermore, EP1 and TNF-α are downstream to COX-2 in the signalling cascade induced by IVH. Hence, the present study identifies a novel strategy for neuroprotection in premature newborns with IVH and substantially unravels the mechanistic links not only between the three interwoven molecules—COX-2, EP1 and TNF-α—but their relation to cell death and gliosis in brain haemorrhage of premature infants.

Materials and methods

Animal experiment

The Institutional Animal Care and Use Committee of New York Medical College approved the animal protocol. The details of brain injuries in our model of glycercol-induced IVH have been previously established and published (Georgiadis et al., 2008; Chua et al., 2009). We obtained timed pregnant New Zealand rabbits from Charles River Laboratories, Inc. (Wilmington, MA, USA). We delivered the pups prematurely by Caesarean section at E29 (full-term = 32 days). Pups were dried and kept in an infant incubator pre-warmed to a temperature of 35°C. Pups were fed 1 ml rabbit milk at 4 h of age.
and then ~2 ml every 12 h (100 ml/kg/day) for the first 2 days using 3.5 French feeding tube. After Day 2, we used kitten milk formula (KMR, PETAG Inc. IL, USA) and advanced feeds to 125, 150, 200, 250 and 280 ml/kg on postnatal Days 3, 5, 7, 10 and 14, respectively.

At 2 h of age, we treated rabbit pups with 50% glycerol (6.5 g/kg) intraperitoneally to induce IVH. Head ultrasound was performed at 6 h of age to assess the presence and severity of IVH using an Acuson Sequoia C256 (Siemens) ultrasound machine. IVH was classified as (i) mild, no gross signs but microscopic haemorrhage detected in haematoxylin and eosin stained brain sections; (ii) moderate, gross haemorrhage into lateral ventricles (two separate lateral ventricles discerned); or (iii) severe, gross IVH leading to fusion of lateral ventricles into a common chamber (Chua et al., 2009). About 80% of rabbit pups develop moderate-severe IVH and 15% mild IVH after glycerol treatment (Georgiadis et al., 2008). As microscopic IVH cannot be diagnosed by head ultrasound, a diagnosis of absence of IVH in glycerol-treated pups indicates that the kit had either microscopic haemorrhage or no IVH. To evaluate the effect of haemorrhage on parameters (COX-2, EP1, TNF-α, IL-1β) or Caspase activity, we included three groups of pups: (i) glycerol-induced IVH; (ii) glycerol-induced non-IVH (control); and (iii) saline treated non-IVH (control). Inclusion of two control groups determined whether intraperitoneal glycerol treatment would confound the IVH-induced morphological (cell infiltration and death) and molecular changes (COX, cytokines) in the forebrain. Accordingly, our previous study had shown that there was no significant cell death or cellular infiltration in the forebrain of glycerol-treated non-IVH pups, just as in saline-treated non-IVH pups (Georgiadis et al., 2008). To assess the effect of treatment (celecoxib/SC51089/etanercept), we alternately assigned pups with moderate-to-severe haemorrhage (glycerol-induced) into two groups—treatment group and vehicle controls. We confirmed that the two groups were balanced with respect to the severity of IVH.

Celecoxib, SC51089 or etanercept treatment

To suppress COX-2, we treated pups with subcutaneous celecoxib (Pfizer Inc., 20 mg/kg/day once daily for 3 days). For evaluation of long-term outcome, we treated the pups with subcutaneous celecoxib for 7 days (20 mg/kg/day once daily) starting at Day 1 and performed neurological and histochemical evaluation at Day 14. To block EP1, we treated another subset of pups with SC-51089 (8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl] hydrazide hydrochloride; Sigma, St Louis, MO; 10 mg/kg twice daily for 3 days). TNF-α was inhibited by using intracerebroventricular etanercept (Amgen and Wyeth pharmaceuticals, CA, USA; 2 mg/kg diluted to 20 µl once in both lateral ventricles). To assess the effect of COX-2, EP1 or TNF-α inhibitors, pups with IVH were alternatively assigned at 6 h age to receive either the compound or the vehicle (control). The severity of IVH was comparable between the comparison groups. Etanercept was injected into the cerebral ventricle using the following coordinates from Bregma: 1 mm posterior, 4 mm lateral and 3 mm deep. We used a 30 gauge needle mounted on Hamilton syringe for drug administration. The doses of these medications were based on previous studies (Chu et al., 2004; Kawano et al., 2006; Tobinick and Gross, 2008).

Rabbit tissue collection and processing

We processed the tissues as described previously (Ballabh et al., 2007). The brain slices were immersion-fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; 0.01 M, pH 7.4) for ~18 h and then were cryoprotected by immersing into 20% sucrose in 0.01 M PBS for 24 h followed by 30% sucrose for the next 24 h. Tissues were frozen into optimum cutting temperature compound (Sakura, Japan). Frozen coronal blocks were cut into 12 µm sections using cryostat.

Immunohistochemistry

Immunostaining was performed as described before (Ballabh et al., 2007). The primary antibodies used in experiments included goat polyclonal COX-2 (Catalogue #160112, Caymen Chemicals, MI, USA), goat polyclonal COX-1 (Catalogue sc 1752; Santa Cruz, CA, USA), goat polyclonal TNF-α (sc-1350, Santa Cruz, CA, USA), mouse monoclonal gliab fibillary acidic protein (GFAP) (Catalogue #G3893, St Louis, MO, USA), monoclonal mouse anti-rabbit neutrophil (Catalogue #801, Serotec, NC, USA), monoclonal mouse anti-rabbit CD11b (Catalogue #MCA802, Serotec, NC, USA) and rat monoclonal myelin basic protein (Catalogue #7349, Abcam, MA). The secondary antibodies used were Cy-3 conjugate goat anti-mouse and fluorescein isothiocyanate conjugate goat anti-rat (Jackson Immunoresearch, West Grove, PA, USA). Briefly, fixed sections were hydrated in 0.01 M PBS and incubated with the primary antibodies diluted in PBS overnight at 4°C. After washing in PBS, the sections were incubated with secondary antibody diluted in 1% normal goat serum in PBS at room temperature for 60 min. Finally, after washes in PBS, sections were mounted with Slow Fade Light Antifade reagent (Molecular Probes, Invitrogen, CA, USA) and were visualized under fluorescent microscope (Axioskop 2 Plus, Carl Zeiss, Inc. Germany).

Neuronal degeneration and fluorescent in situ detection of DNA fragmentation

We performed Fluro-Jade (Chemicon) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining on fixed brain sections as described previously (Georgiadis et al., 2008). For TUNEL staining, 15 µm tissue sections were air dried on slides, hydrated in 0.01 M PBS and permeabilized for 5 min in 1:1 ethanol:acetic acid. An ApopTag-fluorescein in situ DNA fragmentation detection kit (Chemicon, CA, USA) was used to visualize TUNEL-labelled nuclei.

Quantification of cell infiltration, death, gliosis and myelination

We counted neutrophil, microglia, TUNEL positive nuclei, degenerated neurons and astrocytes in coronal brain sections of treatment (celecoxib, SC51089 or etanercept) pups with IVH compared with vehicle-treated IVH controls. From each brain, six coronal sections (30 µm) taken as every third section at the level of midseptal nucleus were used for the study. Counting was performed in an unbiased fashion and random basis in the periventricular zone (germinal matrix, caudate nucleus, deep corona radiata and corpus callosum around the ventricle) and the cerebral cortex by two blinded investigators using a fluorescent microscope with 40x objective (Zeiss Axioscope 2 plus, Carl Zeiss Inc, Germany). We counted objects in ~120 images (7–10 images x two brain regions x six coronal sections) per brain (n = 5 pups per each group) for each parameter.

To evaluate myelination, we analysed images acquired from corona radiata and corpus callosum of brain sections double labelled with myelin basic protein and panaxonal filament antibodies, as previously described (Chua et al., 2009). We used Metamorph version 6.1 from
Universal Imaging Corporation 1993–2003 (Downington, PA). The two sources of image, myelin basic protein (red) and panaxonal filament (green), were displayed on the Metamorph screen. Both images were thresholded. The software calculated the percentage overlap of red (myelin basic protein) over green signal (panaxonal filament). We compared images from non-IVH, vehicle-treated IVH and celecoxib-treated IVH pups (n = 5 pups each).

### Western blot analyses

The frozen brain tissue was homogenized in sample buffer (3% sodium dodecyl sulphate, 10% glycerol, 62.5 mM Tris-HCl and 100 mM Dithiothreitol) using a mechanical homogenizer and the samples were boiled immediately for 5 min. The protein concentration in the sample was determined using RC–DC Protein Assay Kit (Biorad, CA, USA) and dilutions of bovine serum albumin were used as the standard. Total protein samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis according to the previously described method (Laemmli et al., 1970). Equal amounts of protein (30 μg) were loaded into 4–15% gradient precast gel (Biorad, CA, USA). The separated proteins were transferred to polyvinylidene difluoride membrane by electrophoresis. The membranes were then incubated with primary antibodies. Target proteins were detected with enhanced chemiluminescence system (ECL system; Amersham) by using secondary antibodies conjugated with horseradish peroxidase (Jackson immunoresearch, PA, USA). The blots were then stripped with stripping buffer (Pierce) and incubated with β-actin primary antibody followed by secondary antibody and detection with ECL system.

As described previously (Ballabh et al., 2007), the blots from each experiment were densitometrisedly analysed using J-image. We used pre-calibrated optical density step tablet to calibrate an image J (rsweb.nih.gov/ji/) and then measured the optical density of the bands. The optical density values were normalized by taking the ratio of the target protein and β-actin. The antibodies used for immunohistochemistry and western blot analyses were the same for each parameter; except in the GFAP western blot when we used mouse monoclonal GFAP from BD biosciences (Catalog # 556327).

### Prostaglandin E2 assay

Rabbit pup brain tissue was weighed, snap frozen and stored at –80°C until use. Frozen tissues were homogenized using a Polytron (Brinkman) in 100% methanol, centrifuged and the supernatants were collected, dried under a stream of nitrogen, resuspended in 500 μl of enzyme immunoassay buffer and subsequently assayed for PGE2 by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI).

### Caspase-Glo assay

We used the Caspase-Glo R 3/7, 8 and 9 Assay Kit (Promega, catalogue #G8091, G8201 and G8211) to measure their activity in the tissue. Briefly, forebrain extracts made in extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM Pefabloc and 1 Ul/ml each pepstatin, leupeptin and aprotonin) were treated with luminogenic caspase substrate and luminometre readings were taken at 1 h.

### Neurobehavioural assessment

We performed neurobehavioural testing at postnatal Day 14 based on the scoring protocol described previously (Derrick et al., 2004; Georgiadis, 2008; Chua et al., 2009). The evaluation was performed by two physicians blinded to group assignment. We examined cranial nerves by testing smell (aversive response to ethanol), sucking and swallowing (formula delivered by a plastic pipette). We graded the responses on a scale of 0–3, 0 being the worst response and 3 the best. Motor evaluation included tone (modified Ashworth’s scale), motor activity, locomotion at 30° angle, righting reflex and gait. Tone was examined by active flexion and extension of forelegs and hindlegs (score 0–3). The righting reflex was assessed by ability and rapidity to turn prone when placed in supine position. Sensory examination included touch on face (touching face with cotton swab) and extremitities as well as pain on limbs (mild pin prick). Grading of tone, gait and locomotion at 30° angle are illustrated in the footnote of Table 1. To assess coordination and muscle strength in fore and hindlegs, we evaluated the ability of the pups to hold their position on a 60° slope. The test was conducted on a rectangular surface (18 x 6 inch) kept at 60° inclination. We placed the pup at the upper end of the inclination and measured the latency to slip down the slope. We performed the visual cliff test for the assessment of vision. All animals could detect the cliff.

### Statistics and analysis

Data are expressed as mean and SEM. The parameters were compared between pups with and without IVH as well as a function of postnatal age 12, 48 and 72 h. We used t-test (parametric variable) or Mann–Whitney U-test (non-parametric variable) to perform pairwise comparison and ANOVA to compare multiple groups. A probability value of 0.05 was considered significant.
**Results**

**Induction of COX-2 and PGE2 after intraventricular haemorrhage, but not COX-1**

Since COX-2 is upregulated in hypoxia-ischaemic, traumatic and degenerative brain injuries, and as COX-2 activation exacerbates tissue damage (FitzGerald, 2003; Minghetti, 2007), we asked whether COX-2 expression was enhanced in the forebrain of premature animals with IVH. To this end, we injected glycerol intraperitoneally to induce IVH in premature rabbit pups (E29) at 2 h of age and performed head ultrasound at 6 h of age to determine the presence and severity of IVH (Supplementary Fig. 1A). We evaluated COX-2 expression by immunohistochemistry, western blot analysis and real-time PCR in the forebrain of pups with and without IVH at 12, 48 and 72 h of postnatal age. Immunohistochemistry revealed that COX-2 was abundantly expressed in immature neurons and moderately to weakly in astrocytes and microglia, located around the lateral ventricle (periventricular zone, including germinial matrix, deep white matter and caudate nucleus) of pups with IVH (Fig. 1A and Supplementary Fig. 18 and C). By contrast, COX-2 was weakly expressed in the neuronal and glial cells of the periventricular zone of pups without IVH. COX-2 immunoreactivity was weak in the cerebral cortex of both pups with and without IVH. Western blot analysis confirmed that COX-2 levels were higher in pups with IVH than in glycerol-treated controls without IVH at all three epochs—12, 48 and 72 h postnatal age (P = 0.02, 0.01 and 0.02, respectively, n = 8 per group at each time point, Fig. 1B). Similarly, real time PCR revealed that COX-2 mRNA expression was greater in pups with IVH compared to saline- and glycerol-treated controls without IVH at 12, 48 and 72 h postnatal age (P < 0.05 each, n = 6 per group at each time point, Fig. 1C). In contrast, both mRNA and protein expression of COX-1 were comparable between pups with IVH and controls without IVH at all the three epochs—12, 48 and 72 h of postnatal age (n = 8 per group at each time point) (Fig. 1D and E).

PGE2 is a COX-2 derivative and mediates neurotoxicity through prostanoid receptors (Kawano et al., 2006). It was, therefore, important to determine whether IVH enhanced PGE2 levels. PGE2 levels, in homogenates made from coronal slice taken at the level of mid-septal nucleus, were significantly higher in pups with IVH compared to saline- and glycerol-treated controls without IVH at 12 h (39.2 ± 11.8 versus 12.2 ± 3.2 ng/g, n = 8 each, P < 0.05) and 48 h (18.4 ± 3.4 versus 6.7 ± 0.9 ng/g, n = 8 each, P < 0.05) of age. In conclusion, the development of IVH was associated with upregulation of COX-2 and PGE2 synthesis, while levels of COX-1 remained the same.

### Table 1 Neurobehavioural evaluation of premature pups (E29) at postnatal Day 14

<table>
<thead>
<tr>
<th>System</th>
<th>Test</th>
<th>Glycerol-treated pups without IVH, n = 17</th>
<th>Glycerol-treated pups with IVH, vehicle-treated, n = 17</th>
<th>Glycerol-treated pups with IVH, celecoxib-treated, n = 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial nerves</td>
<td>Aversive response to alcohol</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td></td>
<td>Sucking and swallowing</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td></td>
<td>Vision</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td>Motor</td>
<td>Motor activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Head</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td></td>
<td>Forelegs</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td></td>
<td>Hind legs</td>
<td>3 (3,3)</td>
<td>3 (2.7,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td></td>
<td>Righting reflex</td>
<td>5 (5,5)</td>
<td>5 (4,5)</td>
<td>5 (4,5)</td>
</tr>
<tr>
<td></td>
<td>Locomotion on 30° inclination</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td></td>
<td>Tone</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
</tr>
<tr>
<td></td>
<td>Forelimb</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
</tr>
<tr>
<td>Gait</td>
<td>Hold their position at 60° inclination (latency to slip down the slope in seconds)</td>
<td>12.0</td>
<td>7.1</td>
<td>14.7***</td>
</tr>
<tr>
<td></td>
<td>Distance walked in 60 s (in inches)</td>
<td>96 (12.0)</td>
<td>66 (11.8)</td>
<td>85 (14.7)**</td>
</tr>
<tr>
<td></td>
<td>Motor impairment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weakness in extremities (%)</td>
<td>0%</td>
<td>23.52%</td>
<td>6.25%</td>
</tr>
<tr>
<td></td>
<td>Facial touch</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td></td>
<td>Pain</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
</tbody>
</table>

Values are median and interquartile range. 0 is the worst response and 3 is the best response.

a Score (range 1–5): number of times turns prone within 2 s when placed in supine out of 5 tries.
b Score (range 0–3): 0 = does not walk; 1 = takes a few steps (less than 9 inches); 2 = walks for 9–18 inches; 3 = walks very well beyond 18 inches.
c Score (range 1–5): number of times turns prone within 2 s when placed in supine out of 5 tries.
d Gait was graded as 0 (no locomotion), 1 (crawls with trunk touching the ground for few steps and then rolls over), 2 (walks taking alternate steps, trunk low and cannot walk on inclined surface), 3 (walks taking alternate steps, cannot propel its body using synchronously the hind legs, but walks on 30° inclined surface), 4 (walks, runs, and jumps without restriction, propels the body using synchronously the backlegs, but limitation in speed, balance, and coordination manifesting as clumsiness in gait), or 5 (normal walking).

*P < 0.05, **P < 0.01 for the comparison between celecoxib-treated and vehicle-treated pups with IVH. Mann–Whitney U-test used.
Celecoxib suppresses PGE2, cellular infiltration, cell death and gliosis in pups with intraventricular haemorrhage

Since COX-2 contributes to an inflammatory response (Minghetti, 2007), we next postulated that COX-2 inhibition could suppress PGE2 and inflammation in pups with IVH. To this end, we alternately assigned pups with glycerol-induced IVH (detected by head ultrasound) to receive either subcutaneous celecoxib (20 mg/kg/day once daily for 3 days) or vehicle (30 µl dimethyl sulphoxide) at 6 h of age. The severity of IVH was comparable between the treatment and control (vehicle) pups. PGE2 levels were significantly reduced in treated pups compared to vehicle controls at both 12 and 48 h of age ($P = 0.01$ and 0.04, respectively, $n = 8$).
We then assessed the density of neutrophils and microglia in the periventricular zone and cerebral cortex of treated pups and vehicle controls. The neutrophil density was diminished in the periventricular zone at 72 h ($P = 0.045$, $n = 5$ each), but not at 24 h of age, in celecoxib-treated pups with IVH compared to vehicle controls (Fig. 2A). Likewise, microglial density was reduced in the periventricular zone of celecoxib-treated pups compared to vehicle controls at 72 h ($P = 0.027$,

**Figure 2** Reduced cell infiltration and cell death in celecoxib-treated pups with IVH compared to controls. (A) Representative immunofluorescence of cryosections immunolabelled with rabbit neutrophil specific antibody and propidium iodide (nuclear stain). The section is from the periventricular zone of pups with IVH that were treated with either celecoxib (lower panel) or vehicle (upper panel). Neutrophils were few in celecoxib-treated pups with IVH and abundant in vehicle-treated pups with IVH. Inset shows neutrophil labelling under high magnification. Data for neutrophil count are depicted as mean ± SEM ($n = 5$ per group at each time point). Neutrophil count in the periventricular zone was significantly lesser in celecoxib-treated pups with IVH compared to controls at 72 h of age, but not at 24 h. (B) Cryosections from forebrain of celecoxib-treated and vehicle-treated pups with IVH were labelled with CD11b specific antibody and propidium iodide. CD11b positive microglia were scarce in the brain region around the ventricles in celecoxib-treated pups with IVH, but numerous in vehicle-treated controls. Inset shows microglia labelling under high magnification. Data are mean ± SEM ($n = 7$ per group at each time point). CD11b positive microglia were significantly lower in density in the periventricular zone of celecoxib-treated pups with IVH compared to vehicle controls at both 24 and 72 h of age. In the cerebral cortex, microglial density was similar in celecoxib-treated pups and vehicle controls. (C) Representative TUNEL labelling of the periventricular zone from pups with IVH that were treated with either celecoxib or vehicle. The section was counterstained with propidium iodide. TUNEL positive nuclei were less abundant in celecoxib-treated pups compared to vehicle controls. Data are mean ± SEM ($n = 5$ per group at each time point). TUNEL positive nuclei were lesser in number in celecoxib-treated pups with IVH compared to vehicle controls at both 24 and 72 h of age. Apoptotic nuclei were similar in density in the cerebral cortex of celecoxib-treated pups and vehicle controls. (D) Rabbit pups with IVH were treated with either celecoxib or vehicle. Cryosections were stained with FluorJadeB. Data are mean ± SEM ($n = 7$ per group at each time point). Degenerated neurons were lesser in density in celecoxib-treated pups with IVH versus vehicle treated controls at 24 h; *$P < 0.05$ for celecoxib-treated pups with IVH versus vehicle controls at 24 h; **$P < 0.05$ for celecoxib-treated pups with IVH versus vehicle controls at 72 h. All scale bars = 20 μm. Coxib = celecoxib treatment; PVZ = periventricular zone.


n = 7 each, Fig. 2B), but not at 24 h of age. In the cerebral cortex, neutrophil and microglial density were similar between treated pups and vehicle controls.

Since celecoxib treatment reduced cellular infiltration around the ventricle, we next assessed cellular death in celecoxib-treated pups with IVH relative to vehicle-treated controls with IVH at 24 and 72 h postnatal age. We performed TUNEL staining for the evaluation of apoptosis and Fluoro Jade B labelling for the assessment of neuronal degeneration. We noted lower density of TUNEL positive nuclei in the periventricular zone of celecoxib-treated pups compared to vehicle controls at both 24 and 72 h of age (P = 0.03 and 0.045, n = 5 each, Fig. 2C). Likewise, neuronal degeneration was reduced in the periventricular zone of celecoxib-treated pups relative to vehicle controls at both 24 and 72 h of age (P < 0.016 and 0.014, n = 7 each, Fig. 2D). In the cerebral cortex, neuronal degeneration and apoptosis were comparable between treatment group and vehicle controls.

IVH results in gliosis (Chua et al., 2009) and PGE2, a COX-2 derivative, is considered a key mediator of gliosis (Hwang et al., 2006). Thus, we compared gliosis in celecoxib-treated pups with IVH and vehicle-treated controls with IVH. We observed only few hypertrophic astrocytes—with relatively large cell bodies and several processes making a dense network—in the periventricular zone of celecoxib-treated pups, but these were abundant in vehicle controls. Accordingly, astrocyte counts confirmed that there were fewer GFAP positive astroglial cells in the periventricular zone of celecoxib-treated pups than in vehicle controls at 72 h of age (P < 0.001, n = 6 each) (Supplementary Fig. 2A). We then quantified GFAP protein in the forebrain of glycerol-treated pups without IVH, untreated pups with IVH and celecoxib-treated pups with IVH by western blot analyses. Consistent with immunohistochemical data, GFAP protein level was significantly elevated in pups with IVH compared to controls without IVH (P < 0.01) and more importantly, celecoxib treatment substantially reduced GFAP level in pups with IVH (P < 0.01, n = 5, Supplementary Fig. 2B). In conclusion, celecoxib treatment reduced PGE2 synthesis, cellular infiltration, cell death and gliosis around the lateral ventricle of pups with IVH, and thus, conferred neuroprotection.

**Celecoxib attenuates elevation of caspase-3/7, -8 and -9 in intraventricular haemorrhage**

IVH may trigger both the intrinsic and extrinsic pathways of apoptosis (Broughton et al., 2009). Therefore, we compared caspase-3/7 (effector caspase), -8 and -9 activities among the forebrains of (i) saline-treated pups without IVH; (ii) glycerol-treated pups without IVH; (iii) vehicle-treated pups with IVH; and (iv) celecoxib-treated pups with IVH, at 12, 48 and 72 h postnatal age.

Expression of all three caspases were significantly higher in pups with IVH compared to saline- and glycerol-treated pups without IVH (P < 0.001 all, n = 6 each). Caspase-3/7 and -9 activity were reduced in celecoxib-treated pups compared to vehicle controls at 12 h (P < 0.004 and 0.008 for caspase-3/7 and -9, respectively) and 48 h (P = 0.02, 0.03, respectively), but not at 72 h (P = 0.3, 0.07, respectively, n = 6 each) (Supplementary Fig. 3). Of note, caspase-8 activity was significantly less in celecoxib-treated pups with IVH compared to vehicle-treated controls with IVH at 12, 48 and 72 h of age (P = 0.004, 0.02, 0.012, respectively, n = 6 each). In conclusion, IVH induced both intrinsic and extrinsic pathways of apoptosis and celecoxib treatment suppressed both the cascades.

**COX-2 inhibition suppresses EP1 in pups with intraventricular haemorrhage and EP1 inhibition offers neuroprotection**

Since PGE2 acts through EP receptors (EP1-4) (Alvarez-Soria et al., 2007), we chose to (i) compare the expression of EP1-4 receptor between pups with and without IVH; and (ii) determine the effect of COX-2 inhibition on EP1-4 receptors in pups with IVH. IVH was induced by intraperitoneal glycerol at 2 h of age, head ultrasound was performed at 6 h of age and then pups were sequentially assigned as (i) glycerol-treated controls without IVH, (ii) celecoxib-treated pups with IVH or (iii) vehicle-treated pups with IVH.

Real-time PCR showed that EP1 mRNA accumulation was higher in pups with IVH compared to glycerol-treated controls without IVH at 48 and 72 h postnatal age (P < 0.05 each, n = 5, Fig. 3A), but not at 12 h of age. However, EP2-4 mRNA expressions were comparable between pups with IVH and glycerol-treated controls without IVH (Fig. 3B-D). Importantly, EP1 expression was significantly suppressed in celecoxib-treated pups with IVH compared to vehicle controls at both 48 and 72 h (P < 0.05 each, n = 5), but not at 12 h of age (Fig. 3A).

We next asked whether EP1 receptor inhibition would suppress cell death and gliosis in pups with IVH. Thus, we alternatively treated pups with IVH with either subcutaneous SC51089 (10 mg/kg twice daily for 3 days) or vehicle (30 μl sterile water), starting at 6 h of age. The pups were euthanized at 72 h of age and severity of IVH was similar in SC51089- and vehicle-treated controls. TUNEL staining showed lower density of TUNEL positive cells in SC51089-treated pups compared to vehicle controls in the periventricular zone (P = 0.016, n = 5), but not in the cortex (Fig. 3E). Accordingly, caspase-3/7 activity and neuronal degeneration were significantly reduced in SC51089-treated pups compared to vehicle controls (P = 0.001 and 0.013, respectively, n = 5) in the periventricular zone, but not in the cerebral cortex (Fig. 3F and G). Furthermore, EP1 receptor inhibition substantially reduced both the astrocyte count in the periventricular region (P < 0.01, n = 5) and GFAP protein levels (western blot analyses) in the forebrain of pups with IVH compared to controls (Supplementary Fig. 2A and B).

In conclusion, EP1 receptors were elevated in IVH, COX-2 inhibition alleviated EP1 expression, and more importantly, EP1 suppression conferred neuroprotection, just as COX-2 inhibition did.
COX-2 inhibition alleviates consequences of intraventricular haemorrhage—neurological impairment, impaired myelination and gliosis

To determine whether COX-2 inhibition attenuates neurological impairment, we compared motor and sensory evaluation between three sets of premature rabbit pups at Day 14: (i) glycerol treated pups without IVH (n = 17); (ii) celecoxib-treated pups with IVH (n = 16); and (iii) vehicle-treated pups with IVH (n = 17) (Table 1). The severity of IVH in celecoxib-treated pups was comparable to those of vehicle-treated controls with IVH. We noted significant weakness in the foreleg of one and the hind legs of three vehicle-treated IVH pups (23.5%), whereas one pup in the celecoxib-treated group (6%) had weakness in right hind-leg manifesting as asymmetry in gait. The scores for gait were significantly better in celecoxib-treated pups than in vehicle-treated IVH controls (P = 0.02). The average distance walked in 60 s was longer in treated pups compared with vehicle controls (P = 0.027). The latency to slip down the 60° inclination was substantially longer in the celecoxib-treated pups relative to vehicle-treated IVH controls (P < 0.001). The tone in the back legs of three pups with motor impairment in the vehicle-treated group with IVH was slightly increased (score = 1). No difference was observed in sensory and cranial nerve assessment of the three sets of rabbit pups. Importantly, we did not observe any apparent adverse effect attributable to celecoxib treatment among pups with IVH receiving this medication.

We next compared myelination among the three groups of pups at Day 14 (n = 5, Fig. 4). Immuno-labelling showed that expression of myelin basic protein was significantly less in vehicle-treated pups with IVH compared to controls without IVH manifesting as asymmetry in gait. The scores for gait were significantly better in celecoxib-treated pups than in vehicle-treated IVH controls (P = 0.02). The average distance walked in 60 s was longer in treated pups compared with vehicle controls (P = 0.027). The latency to slip down the 60° inclination was substantially longer in the celecoxib-treated pups relative to vehicle-treated IVH controls (P < 0.001). The tone in the back legs of three pups with motor impairment in the vehicle-treated group with IVH was slightly increased (score = 1). No difference was observed in sensory and cranial nerve assessment of the three sets of rabbit pups. Importantly, we did not observe any apparent adverse effect attributable to celecoxib treatment among pups with IVH receiving this medication.

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in corona radiata and corpus callosum \( (P < 0.001 \) each), and that celecoxib treatment enhanced the expression of myelin basic protein in these two white matter regions \( (P < 0.01 \) each, Fig. 4A).

Accordingly, western blot analyses revealed that myelin basic protein level was reduced in vehicle-treated pups with IVH when compared to controls without IVH \( (P = 0.045) \), and celecoxib treatment enhanced its expression in pups with IVH \( (P = 0.01, \) Fig. 4B).

We next measured GFAP expression in a) pups without IVH, b) pups with IVH who were treated with celecoxib, and c) vehicle treated pups with IVH at Day 14. Western blot analyses showed that IVH elevated GFAP level \( (P = 0.04, \) \( n = 5) \) and celecoxib treatment reduced GFAP expression in pups with IVH at Day 14 \( (P = 0.006, \) Fig. 4C). In conclusion, celecoxib treatment reduced motor impairment, presumably by enhancing myelination and attenuating gliosis at Day 14.

**TNF-α and IL-1β are elevated in intraventricular haemorrhage and COX-2 or EP1 inhibition reduce TNF-α levels, but not IL-1β**

Pro-inflammatory cytokines TNF-α and IL-1β are increased in adult animal model of brain haemorrhage, contributing to the brain damage (Mayne et al., 2001); and COX-2 inhibition suppresses TNF-α production in neuronal culture experiments (Araki et al., 2001). It was, therefore important to determine whether TNF-α
and IL-1β were elevated in pups with IVH and if COX-2 or EP1 inhibition reduced TNF-α and IL-1β levels. To this end, we evaluated protein expression of TNF-α in pups both with and without IVH using immunohistochemistry and western blot analyses. TNF-α was abundant in the periventricular zone of pups with IVH, but sparse in controls without IVH (Fig. 5A and B). TNF-α was weakly expressed in the cerebral cortex of both pups with and without IVH. Double immunolabelling of brain section with TNF-α combined with Tuj1 or GFAP confirmed that TNF-α was expressed in several immature neurons and also in some of the astrocytes of the periventricular zone in pups with IVH (Fig. 5A and B). Furthermore, TNF-α was also weakly expressed in microglia labelled with tomato lectin (data not shown). Western blot analyses show two sets of bands: 17 and 110kDa. The 17kDa band matches with the molecular weight of TNF-α (Fig. 5C and D). To determine the specificity of these bands we performed peptide competition. Briefly, we incubated in polyvinylidene difluoride membrane after protein transfer into primary antibody or primary antibody pre-absorbed with peptide (specific to TNF-α antibody). The membrane incubated with primary antibody depicted bands at 17 and 110kDa, whereas membrane incubated with antibody pre-absorbed with peptide showed only the 110kDa band.
This suggested that the 17 kDa band was specific to TNF-α. TNF-α levels (17 kDa) were significantly greater in pups with IVH compared to controls at 12, 48 and 72 h of age ($P = 0.03, 0.05$ and $0.02$).

We compared TNF-α levels between celecoxib-treated pups with IVH and vehicle-treated controls without IVH. Immunolabelling showed that TNF-α expression in the periventricular zone was diminished in celecoxib-treated pups compared to vehicle-treated controls (Fig. 6A). Western blot analyses confirmed that TNF-α protein levels were markedly reduced in celecoxib-treated pups with IVH compared to vehicle-treated controls at 48 and 72 h ($P <0.03$ and $0.02$, $n=8$ at each epoch, $P = 0.02$).

Human breast carcinoma tissue lysate was the positive control. Data are mean ± SEM ($n = 8$ each time point). Values were normalized to β-actin. TNF-α mRNA expression was assayed by real time PCR. Data are mean ± SEM ($n = 6$ for each time point). TNF-α gene expression was substantially higher in pups with IVH compared to saline- and glycerol-treated controls without IVH at 12, 48 and 72 h of age, and celecoxib treatment reduced TNF-α mRNA expression in pups with IVH at 12 and 48 h, but not at 72 h of age. *$P <0.05$, **$P <0.01$ saline-treated pups without IVH versus vehicle treated pups with IVH; # $P <0.05$, ## $P <0.01$ glycerol-treated pups without IVH versus vehicle treated pups with IVH; † $P <0.05$ celecoxib- versus vehicle-treated pups with IVH. (D) Representative western blot analysis of TNF-α in forebrain of pups without, vehicle-treated pups with and SC51089-treated pups with IVH at 72 h of age. Data are mean ± SEM ($n = 5$). Values were normalized to β-actin. TNF-α protein levels were significantly greater in pups with IVH than controls without IVH, and SC51089 attenuated TNF-α levels in pups with IVH. *$P <0.05$ for pups without versus those with IVH; ## $P <0.01$ for SC51089- versus vehicle-treated pups with IVH. (E) TNF-α mRNA expression was assayed by real time PCR. Data are mean ± SEM ($n = 6$). TNF-α gene expression was significantly higher in pups with IVH compared to saline-treated and glycerol-treated controls without IVH and SC51089 reduced TNF-α gene expression. *$P <0.05$ for pups without versus those with IVH; † $P <0.05$ for SC51089- versus vehicle-treated pups with IVH.
Fig. 6B), but not at 12 h of age ($P = 0.07, n = 8$ each). Accordingly, real-time PCR showed that TNF-α mRNA accumulation was significantly higher in pups with IVH compared to saline- and glycerol-treated controls without IVH at 12, 48 and 72 h ($P < 0.05$ each, $n = 6$ each, Fig. 6C). In addition, celecoxib treatment significantly reduced TNF-α mRNA expression in pups with IVH compared to vehicle controls at 72 h of age ($P < 0.01$, Fig. 6D). Similarly, real-time PCR revealed that TNF-α expression was significantly reduced in pups with IVH upon SC51089 treatment at 72 h of age ($P < 0.04, n = 6$ each, Fig. 6E).

We next evaluated IL-1β levels in the same paradigm as TNF-α expression. Immunohistochemistry showed that IL-1β was strongly expressed in the periventricular zone of pups with IVH and weakly in controls without IVH (Supplementary Fig. 4). Double immunolabelling confirmed that IL-1β was expressed in the immature neurons and some of the astrocytes in the periventricular zone of pups with IVH. IL-1β immunoreactivity was weak in the cerebral cortex of pups with and without IVH. Consistent with protein expression, IL-1β mRNA expression was higher in pups with IVH compared to controls without IVH at 72 h postnatal age. SC51089 did not substantially affect IL-1β gene expression. *$P < 0.05$ for saline-treated pups without versus those with IVH; **$P < 0.05$ for glycerol-treated pups without versus those with IVH.
expression; and celecoxib or SC51089 alleviates TNF-α, but not IL-1β levels in pups with IVH.

**Etanercept reduces cell death and gliosis, and does not affect COX-2 expression**

As both COX-2 and EP1 inhibition distinctly attenuated TNF-α expression in our experiment, and since TNF-α mediates inflammatory brain damage (Crisafulli et al., 2009), it was crucial to determine whether TNF-α inhibition offers neuroprotection in pups with IVH. We used etanercept as a TNF-α antagonist. It is a dimeric fusion protein that binds specifically to TNF-α and blocks its interaction with cell surface TNF receptors. We treated pups with IVH with either intra-cerebroventricular etanercept (2 mg/kg diluted to 20 μl—one dose in each lateral ventricle) or vehicle at 6 h of age. The pups were euthanized at 72 h of age and severity of IVH was similar in etanercept- and vehicle-treated controls. We found that caspase-3/7 levels were significantly reduced in treated pups compared to vehicle controls (P < 0.05, n = 6 each, Fig. 8A). We next assessed apoptotic cell death and neuronal degeneration in treated and control pups. Both TUNEL positive nuclei and
Fluorochrome positive neurons were substantially less in etanercept-treated pups compared to vehicle controls in the periventricular zone \((P<0.001\) each, \(n=6\) each), but not in the cortex (Fig. 8B and C). Finally, the number of astrocytes was significantly reduced in etanercept-treated pups compared to vehicle controls in the periventricular zone \((P<0.01\) each, \(n=6\) each), but not in the cortex (Supplementary Fig. 2A). GFAP content, measured by western blot analysis, was also substantially reduced in the etanercept-treated pups compared to controls (Supplementary Fig. 2B).

As pharmacological inhibition of TNF-\(\alpha\) suppresses COX-2 levels in a culture model of peritoneal macrophages (Crisafulli et al., 2009), it was important to assess whether TNF-\(\alpha\) inhibition reduced COX-2 levels in our animal model. Western blot analysis revealed that COX-2 protein expression in etanercept-treated pups with IVH and vehicle-treated controls were comparable (Fig. 8D). Consistent with protein measurement, COX-2 gene expression in etanercept-treated pups was substantially reduced in the etanercept-treated pups compared to controls (SUPPLEMENTAL Fig. 2A). Indeed, COX-2 inhibition by celecoxib treatment induces neuroprotective in adult animals following intracerebral haemorrhage (Chu et al., 2004). In addition, selective COX-2 inhibition alleviates motor impairment in the G93A superoxide dismutase mouse model of amyotrophic lateral sclerosis (Pompl et al., 2003), and celecoxib is undergoing clinical trials to mitigate neuroinflammation in amyotrophic lateral sclerosis (Gordon et al., 2008). Hence, COX-2 inhibition could be a viable strategy of neuroprotection for IVH in premature infants.

**Discussion**

As both birth rate and survival of premature neonates have increased over the last few decades, IVH and its attendant complications—cerebral palsy and cognitive deficits—have developed as major public health concerns (Shennan and Bewley, 2006; Stephens and Vohr, 2009). No therapeutic strategy exists to curtail the devastating cascades of pathological events triggered in the haemorrhaging brain of premature infants. The present study highlights a unique set of mechanistically linked molecules—COX-2, EP1 and TNF-\(\alpha\)—whose levels were elevated in pups with IVH compared to controls without IVH; and more importantly, that inhibition of any of the three prevented cell infiltration, neural cell death and gliosis during the first 3 days of life. We found that COX-2 inhibition by celecoxib treatment restored neurological impairment, enhanced myelination and reduced gliosis at Day 14. Furthermore, our study revealed novel molecular links indicating that EP1 and TNF-\(\alpha\) were downstream of COX-2 in the inflammatory cascade triggered by IVH.

The most important and novel observation in this study was the identification of a set of mechanistically interwoven molecules: COX-2, EP1 and TNF-\(\alpha\), which were upregulated in IVH. Importantly, each of the three molecules offered short-term neuroprotection on separate preclinical testing. Moreover, neurobehavioral assessment at Day 14 showed that celecoxib-treated (7-day regimen) pups display superior motor function and greater myelination compared to vehicle-treated controls. Consistent with our findings, COX-2 is markedly upregulated in ischaemic, degenerative and traumatic brain injuries and mediates tissue damage (Gomez et al., 2005; Candelario-Jalil and Fiebich, 2008). COX-2 activation exerts neurotoxicity primarily by (i) inducing oxidative stress; (ii) contributing to glutamate excitotoxicity; and (iii) promoting cell-cycle activity (Candelario-Jalil and Fiebich, 2008). Indeed, COX-2 inhibition by celecoxib treatment induces neurological recovery in adult animals after intracerebral haemorrhage (Chu et al., 2004). In addition, selective COX-2 inhibition alleviates motor impairment in the G93A superoxide dismutase mouse model of amyotrophic lateral sclerosis (Pompl et al., 2003), and celecoxib is undergoing clinical trials to mitigate neuroinflammation in amyotrophic lateral sclerosis (Gordon et al., 2008). Hence, COX-2 inhibition could be another important strategy of neuroprotection for IVH in premature infants.

We demonstrated the neuroprotective ability of TNF-\(\alpha\) inhibition in our model of IVH. TNF-\(\alpha\) signalling is involved in every facet of inflammatory brain injury and results in two major responses: apoptosis and inflammation (Hallenbeck, 2002). It has a large signalling network, which initiates activation of caspases (apoptosis), matrix metalloproteinases, xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase and inducible nitric oxide synthase (generation of free radicals), and potentiates N-methyl-D-aspartate receptor-mediated neurotoxicity (Hallenbeck, 2002; Hosomi et al., 2005; Hua et al., 2006). Both TNF-\(\alpha\) knockout mice and antisense oligodeoxynucleotide-treated adult rats with brain haemorrhage exhibit diminished cell death and inflammation compared to controls (Mayne et al., 2001; Hua et al., 2006). Indeed, anti-TNF-\(\alpha\) therapy is in extensive clinical use for inflammatory disorders including rheumatoid arthritis, psoriasis and other conditions (Palladino et al., 2003). Similar, and in agreement to these observations, we found that etanercept, a TNF-\(\alpha\) inhibitor, attenuated cell death and inflammation. Hence, TNF-\(\alpha\) inhibition could be another important strategy to abrogate brain injury in premature infants with IVH.

Our experiments revealed a novel mechanistic connection between TNF-\(\alpha\), COX-2 and EP1. Specifically, COX-2 and EP1 suppression reduced TNF-\(\alpha\) levels, while TNF-\(\alpha\) inhibition did not influence COX-2 expression. In agreement to our findings, it has been reported that COX-2 inhibition by NS-398 reduces TNF-\(\alpha\) elevation and neuronal cell death in neuronal culture experiments (Araki et al., 2001). However, in contrast to our results, both genetic and pharmacological inhibition of TNF-\(\alpha\) significantly reduces COX-2 expression in peritoneal macrophages in culture experiment; and TNF-\(\alpha\) triggers upregulation of COX-2 expression in HT-29 cells (Ikawa et al., 2001; Crisafulli et al., 2009). We
could not find any in vivo experiments in brain showing that TNF-α directly regulates COX-2. Therefore, it is plausible that COX-2 and EP1 are upstream molecules that regulate TNF-α expression in premature infants with brain injuries. Our previous work has shown that TuJ1 positive immature neurons constitute the predominant population of degenerating and dying cells around the ventricle of the haemorrhaging brain (Georgiadis et al., 2008). Accordingly, the present study revealed an abundance of COX-2 and TNF-α in the TuJ1 positive neurons in the periventricular zone, and suppression of either COX-2 or TNF-α reduced neuronal cell death in this brain region. A study, which is also consistent with our findings, has shown that intracerebral injection TNF-α in neonatal rat (P5) results in astroglisis and neuronal cell death (Cai et al., 2004). In another study performed on APP/PS1 mice, inhibition of nuclear factor-κB—a downstream mediator of TNF-α—has attenuated gliosis (Zhang et al., 2009). Indeed, TNF-α is a mediator of cell death and gliosis both in vitro and in vivo (Hallenbeck, 2002; Hosomi et al., 2005; Hua et al., 2006). Taking these facts together, COX-2 seems to be an upstream molecule in a neonatal model of brain injuries that induces cell death and gliosis via upregulating TNF-α and not the reverse.

IVH results in cerebral palsy and cognitive deficits in premature infants. At this time, there is no treatment of IVH and active withdrawal of life support from premature infants with severe IVH based on quality of life concerns, although infrequent, does occur (Sawyer, 2008). Our study showed clinical recovery in treated pups compared to untreated controls. Furthermore, we did not observe any apparent adverse effects attributable to celecoxib in pups with IVH receiving this medication. Unfortunately, neither celecoxib nor the other two treatments—EP1 and TNF inhibitor—are approved by the Food and Drug Administration for use in newborns. Nevertheless, celecoxib has a broad experience in human application and therefore, testing its utility in a phase I-type clinical trial in human neonates with severe IVH (grade III or IV, Papile Scale) appears to have merit.

Of note, neurological outcome of IVH is principally determined by the severity of haemorrhage and extent of parenchymal involvement (Brouwer et al., 2008). Similarly in our model, we observed three grades of IVH, including microscopic (mild), gross blood in the ventricle (moderate) or large haemorrhage resulting in fusion of the two lateral ventricles (severe). In our previous study, we observed abundant inflammatory cells (neutrophil and microglial), apoptosis and neuronal degeneration in the periventricular region at 24, 48 and 72 h of age in pups with IVH, whereas these inflammatory changes were not significantly present in glycerol-treated or in saline-treated controls without IVH (Georgiadis et al., 2008). Furthermore, cellular infiltration and death are minimal in microscopic IVH in contrast to pups with moderate-to-severe IVH. Therefore, in the present study, we included only pups with moderate-to-severe IVH to evaluate the effect of the three neuroprotectants. We also ascertained that the comparison groups are balanced with respect to the severity of IVH. However, the present study did not assess COX-2, EP-1 and cytokine levels as a function of severity of IVH—moderate versus severe haemorrhage.

In conclusion, we demonstrated that COX-2, EP1 or TNF-α inhibition attenuated inflammation, cell death and gliosis induced by IVH in premature rabbit pups, and that COX-2 inhibition by celecoxib treatment also promotes long term recovery in motor function and myelination. The three molecules—COX-2, EP1 and TNF-α—are mechanistically linked in a signalling cascade with COX-2 and EP1 upstream to TNF-α. The suppression of any of the three might minimize the brain injury in premature infants with IVH. If translated into human investigation, the results of this study might positively impact the survival and neurological outcome of premature infants with IVH. These data provide a fundamental and mechanistic rationale to conduct a phase I-type therapeutic trial.

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Supplementary material

Supplementary material is available at Brain online.

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