Experimental subarachnoid haemorrhage results in multifocal axonal injury

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The great majority of acute brain injury results from trauma or from disorders of the cerebrovasculature, i.e. ischaemic stroke or haemorrhage. These injuries are characterized by an initial insult that triggers a cascade of injurious cellular processes. The nature of these processes in spontaneous intracranial haemorrhage is poorly understood. Subarachnoid haemorrhage, a particularly deadly form of intracranial haemorrhage, shares key pathophysiological features with traumatic brain injury including exposure to a sudden pressure pulse. Here we provide evidence that axonal injury, a signature characteristic of traumatic brain injury, is also a prominent feature of experimental subarachnoid haemorrhage. Using histological markers of membrane disruption and cytoskeletal injury validated in analyses of traumatic brain injury, we show that axonal injury also occurs following subarachnoid haemorrhage in an animal model. Consistent with the higher prevalence of global as opposed to focal deficits after subarachnoid haemorrhage and traumatic brain injury in humans, axonal injury in this model is observed in a multifocal pattern not limited to the immediate vicinity of the ruptured artery. Ultrastructural analysis further reveals characteristic axonal membrane and cytoskeletal changes similar to those associated with traumatic axonal injury. Diffusion tensor imaging, a translational imaging technique previously validated in traumatic axonal injury, from these same specimens demonstrates decrements in anisotropy that correlate with histological axonal injury and functional outcomes. These radiological indicators identify a fibre orientation-dependent gradient of axonal injury consistent with a barotraumatic mechanism. Although traumatic and haemorrhagic acute brain injury are generally considered separately, these data suggest that a signature pathology of traumatic brain injury—axonal injury—is also a functionally significant feature of subarachnoid haemorrhage, raising the prospect of common diagnostic, prognostic, and therapeutic approaches to these conditions.

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Introduction

Acute brain injury is one of the leading causes of death and long-term disability in the USA (Langlois et al., 2006; Go et al., 2014; http://www.cdc.gov/traumaticbraininjury; http://www.cdc.gov/stroke/facts.htm). While brain trauma is the greatest source of injury at younger ages, ischaemic and haemorrhagic brain injury dominate in older individuals. Although traumatic and vascular brain injury are generally considered separately, identification of common injury pathways could have a greater impact on management approaches. Axonal injury and ischaemia are the key instigators of cellular brain injury following trauma and ischaemic stroke, respectively. The mechanisms of brain injury following intracranial haemorrhage are less well established.

Subarachnoid haemorrhage (SAH) from the rupture of a cerebrovascular aneurysm is a particularly devastating form of intracranial haemorrhage (Suarez et al., 2006). It afflicts 30–40,000 Americans (Rincón et al., 2013) and costs the USA $5.6 billion annually in 1990 dollars, a cost equivalent to that of all other forms of intracranial haemorrhage combined (Taylor et al., 1996). Compared to other vascular brain injuries, SAH strikes younger people in their prime working years, has high mortality, and is characterized by highly prevalent cognitive, emotional, and social impairment (Johnston et al., 1998; Al-Khindi et al., 2010; Passier et al., 2011). Consequently, despite representing 3–5% of all stroke, SAH accounts for 27.3% of stroke-related years of productive life lost—a burden on par with that of ischaemic stroke (38.5%) and all other varieties of intracranial haemorrhage (34.2%) (Johnston et al., 1998). Despite its tremendous clinical impact, frustratingly few acute treatment options exist for SAH. Development of new treatments to limit brain injury is hampered by both an incomplete understanding of cellular injury mechanisms and a lack of diagnostic modalities that can identify and track them.

Although ischaemic stroke and SAH share a dysregulation of cerebral blood flow, we questioned whether SAH may be pathophysiologically more similar to trauma than it is to ischaemic brain injury, as both SAH and traumatic brain injury (TBI) involve hyperacute forces applied to the brain and abrupt changes in intracranial pressure (Powers, 2010). Moreover, while ischaemic stroke generally results in discrete lesions causing focal neurological deficits, victims of both TBI and SAH suffer most from global cognitive and emotional dysfunction and associated diffuse cerebral atrophy (Ogden et al., 1993; Suarez et al., 2006; Bendel et al., 2009; Passier et al., 2011). We therefore hypothesized that diffuse or multifocal axonal injury, a primary determinant of outcome following TBI (Smith et al., 2003; Johnson et al., 2013), may also be an important feature of SAH. To approach this question, we looked for evidence of axonal injury following SAH in a mouse model using histological and ultrastructural methods. We then applied a translational radiological technique, diffusion tensor imaging (DTI), to these same specimens. We validated radiological biomarkers generated from diffusion imaging against quantitative histological and functional outcomes, and used this approach to further characterize the distribution of axonal injury following SAH.

Materials and methods

See the online Supplementary material for full details of all experimental procedures.

Injury

Endovascular perforation SAH was performed as described (Parra et al., 2002) on male C57BL/6 mice at 11 weeks of age with slight modification. Briefly, mice were anaesthetized and a 5-0 nylon suture was advanced 3 mm past the bifurcation of the left internal carotid artery to induce arterial rupture, withdrawn 3 mm and re-advanced 3 mm. In sham injured animals the suture was not advanced past the internal carotid artery bifurcation. Twice daily postoperatively mice were administered 50 mg/kg ampicillin in a 10% dextrose solution (Days 0–3) or 0.5 ml of 10% dextrose in saline (Days 4–7). Controlled cortical impact TBI was performed as described (Brody et al., 2007; Jiang and Brody, 2012).

Membrane permeability assay

Assays of traumatic membranoporation were carried out as previously described (Farkas et al., 2006) by infusing Alexa Fluor® 488 conjugated to 10 kD dextran in sterile saline into the right lateral ventricle to a final dose of 10 mg/kg 1 h prior to injury.

Behavioural analysis

Neurobehavioural outcome was examined on Day 0 and on postoperative Days 1, 3 and 7 using NeuroScore and rotarod tests as described (Parra et al., 2002; Gao et al., 2006).

Histology

Immunohistostaining was assessed on Day 1 and silver staining on Day 7 post-injury. Mice were perfused transcardially with 0.3% heparin and their brains fixed ex vivo in paraformaldehyde. MRI was performed ex vivo (see below) prior to immunohistostaining. Evaluation was conducted on sections taken at 250 µm intervals along the anterior–posterior axis of the brain.
The following primary antibodies were used for immunohistochemistry: rabbit anti-amyloid precursor protein (APP) and the mouse monoclonal anti-neurofilament (NF) antibodies RM014.9, SMI-31, and SMI-34. For staining with mouse primary antibodies, a complex of primary antibody and biotinylated anti-mouse IgG Fab fragments was first prepared followed by incubation with tissue sections. APP labelling was followed by incubation with biotinylated goat anti-rabbit antibody. APP and neurofilament labelling was visualized with ABC Elite (Vector Laboratories). For silver staining, a Neurosilver Kit (FD NeuroTechnologies) was used according to the manufacturer’s instructions with slight modification as previously described (Shitaka et al., 2011). Stereology and silver stain quantification was performed as described (Shitaka et al., 2011). Region of interest parameters were the same as used for DTI analysis (see below). Electron microscopy was performed as described (MacDonald et al., 2007).

**Magnetic resonance imaging**

Mouse brains were imaged ex vivo with a 4.7 T Agilent DirectDrive™ Small-Animal MRI system using a 1.8 cm (ID) linear transmit/receive RF coil. Animals in the injury group without evidence of bleeding or with evidence of cerebral infarction were not analysed. The following DTI acquisition parameters were used: repetition time = 1500 ms, echo time = 45 ms, and field of view/matrix 15 mm × 15 mm/128 × 128, voxel size 117 μm × 117 μm × 0.5 mm, six diffusion gradient directions, B-values 0 and 2000. Software written in MATLAB was used to calculate the elements of the diffusion tensor and relative anisotropy. Analysis was performed with ImageJ. Hand-drawn, multislice regions of interest were defined by prespecified anatomical boundaries on relative anisotropy and T2 images. Regions of haemorrhage on the GRE scan were avoided. A voxel-weighted relative anisotropy measure was calculated for each region of interest.

**Results**

**Subarachnoid haemorrhage results in axonal injury**

To test the hypothesis that SAH results in axonal injury, we first looked for classic histological evidence in a widely-adopted mouse model of SAH (Parra et al., 2002). In this model, the middle cerebral artery is perforated with a filament (Fig. 1A). This results in self-limited subarachnoid bleeding and acute neurobehavioural impairment. We subjected mice to filament perforation SAH and analysed their behavioural outcomes at three time points. Neuroscore and rotarod testing in these animals revealed significant functional deficits compared to sham operated littermates at Day 1 and Day 3 post-injury (Fig. 1B).

We first asked whether experimental SAH results in axolemmal injury, which is among the first histological changes following moderate-severe traumatic brain injury (Pettus et al., 1994). We subjected animals to experimental SAH after a 10 kD fluorescent dextran conjugate was preloaded into the contralateral cerebral ventricle. Confocal microscopy of ipsilateral white matter tracts revealed entry of fluorescent dextran into injured axons indicative of disruptions in the axolemma. Many labelled axons exhibited varicosity formation characteristic of traumatic injury (Fig. 1C and Supplementary Fig. 1).

Axonal accumulation of APP and side-arm rearrangement of neurofilament are classic markers of traumatic axonal injury indicative of cytoskeletal damage (Pettus and Povlishock, 1996; Johnson et al., 2013). Immunohistological evidence of both were observed in multiple white matter regions ipsilateral to the site of haemorrhage following SAH (Fig. 2A and Supplementary Figs 2 and 3). Blinded quantitation revealed a significant increase in injury markers in both the cerebral peduncle (APP and neurofilament) and fimbria (APP) compared to control animals (Fig. 2B and Supplementary Fig. 2). Interestingly, two patterns emerged: first, a ‘geographic’ distribution of APP staining that affected both grey and white matter was occasionally observed directly adjacent to the ruptured artery (Supplementary Fig. 2A). Second, at sites further removed from the epicentre of haemorrhage, APP staining was patchy and preferentially affected white matter (Fig. 3A, Supplementary Figs 2A and 3). These alternate patterns may result from spatially distinct mechanisms of axonal injury (see ‘Discussion’ section).

Antibodies against phosphorylated neurofilaments, additional histological markers of traumatic axonal injury (Yaghmai and Povlishock, 1992), also labelled white matter regions after SAH (Supplementary Fig. 4). These markers revealed axonal morphology—multiple varicosities and axonal dystrophy—comparable to that seen after experimental TBI (Johnson et al., 2013).

Silver staining, which we have previously shown highlights injured white matter regions after experimental concussive TBI that lack classic histological features (APP and neurofilament) of axonal injury (Bennett et al., 2012), was also positive in multiple ipsilateral white matter regions after SAH (Fig. 2C and D). Notably, white matter tracts distant from the focus of haemorrhage including the contralateral internal capsule, cerebral peduncle, optic tract, and anterior commissure were also argyrophilic (Fig. 2D and Supplementary Fig. 5) compared to controls. These findings demonstrate a broad, multifocal distribution of white matter injury following SAH.

To confirm these histological findings, we analysed the ipsilateral cerebral peduncle and fimbria from control and injured animals with electron microscopy. We found multiple ultrastructural features indicative of myelinated and unmyelinated axonal injury (Christman et al., 1994) in injured animals including enlarged axons, neurofilament compaction, organelle clumping, and varicosity formation (Fig. 2E and Supplementary Fig. 6). Taken together, these results indicate that histological and ultrastructural axonal injury is a prominent feature of neurological injury after experimental SAH.
DTI reveals a gradient of axonal injury following SAH

Histochemical and ultrastructural techniques are rarely applicable as diagnostic or monitoring tools in humans. We therefore turned to a radiological assay for axonal injury with greater translational potential, DTI. Axonal injury after TBI is well known to reduce the anisotropy of diffusion within white matter regions (Arfanakis et al., 2002; MacDonald et al., 2007). We performed DTI analysis of 10 prespecified white matter regions over three time points following experimental SAH. In control experiments we measured anisotropy before and after a clot was manually applied to the ex vivo brain and found no gross image distortion or artefactual reduction of anisotropy (Supplementary Fig. 7). The lack of a consistent effect of the clot on relative anisotropy indicates that the influence of haemorrhage-induced magnetic field inhomogeneities is unlikely to be significant. We furthermore confirmed that similarly-sized regions were measured between all groups (Supplementary Fig. 8).

DTI analysis of ipsilateral white matter regions revealed decrements in anisotropy in injured versus sham-operated animals consistent with axonal injury in all regions and at every time point (Fig. 3). Subsequent histological analysis of these specimens showed accumulation of APP in many of these same areas (Fig. 3A–C and see Figs 2 and 4), including in some areas distant from the epicentre of haemorrhage (Fig. 3A). Mean relative anisotropy trended toward a nadir on Day 3 (Fig. 3F). Relative anisotropy recovered slightly by Day 7, but this may have resulted from mortality between Days 3 and 7 post-injury. Several contralateral regions also exhibited reduced relative anisotropy versus controls (Supplementary Fig. 9), confirming a widespread injury process.

Although the injury was multifocal, its severity varied by region. As expected, relative anisotropy relative to control regions fell the most near the epicentre of haemorrhage and in general was less affected with greater distance (Fig. 3G,
Interestingly, this gradient was non-uniform: contralateral regions near the base of the brain (anterior commissure and the composite region containing the cerebral peduncle, internal capsule, and optic tract), despite their proximity to the site of injury, were relatively protected compared to the contralateral fimbria, corpus callosum, and external capsule (Fig. 3G and Supplementary Fig. 10). The mechanisms underlying this regional protection and vulnerability are unknown.

Radiological axonal injury correlates with functional deficits and histological injury

To further evaluate the utility of DTI as a marker for axonal injury following SAH, we compared relative anisotropy from two white matter regions proximate to the site of bleeding with measurements of functional disability and histological axonal injury. At 1 and 3 days after injury, when functional deficits are most pronounced, relative
Figure 3. Evolution and distribution of axonal injury following experimental SAH. (A) Relative anisotropy (RA) maps of control (left) and injured (right) animals. Site of haemorrhage identified (red asterisk; SAH n = 31, control n = 17). Decreased relative anisotropy in ipsilateral corpus callosum (arrowhead in region boxed in red) and in cerebral peduncle/optic tract (blue box) highlighted. Immunostaining against APP in corpus callosum (red boxes below) from the same animals shown with APP staining highlighted (black arrowheads). Cerebral peduncle and optic tract from control (B) and injured (C) animals presented at higher magnification. Region of relative anisotropy measurement overlaid on control. Inset shows blood-sensitive sequence (GRE) for same field, demonstrating lack of haemorrhage within the region of analysis. Immunostaining...
anisotropy in both the ipsilateral fimbria and the composite region encompassing the cerebral peduncle, internal capsule, and optic tract was strongly correlated with deficits in neuroscore (Fig. 4A and B). Rotarod score correlated significantly with relative anisotropy in the ipsilateral composite region, but not in the fimbria (Fig. 4C). These correlations were no longer apparent at Day 7, possibly due to improvement in mean neurobehavioural scores with time in this model (Fig. 1B) (Milner et al., 2014).

We next compared anisotropy measurements with histological indicators of axonal injury. APP labelling in the fimbria was significantly and inversely correlated with relative anisotropy (Fig. 4D), as expected. The correlation did not reach statistical significance in the composite region (Fig. 4E), though silver densitometry in this region was significantly and inversely correlated with relative anisotropy (Fig. 4F). Taken together, these findings reveal a substantial though incomplete correlation between DTI metrics consistent with axonal injury and both functional and histological outcomes, similar to results in experimental TBI (Bennett et al., 2012). This suggests that axonal injury is a functionally significant component of neurological damage after SAH, and that DTI can serve as a useful tool for its diagnosis and monitoring.

**Discussion**

**A connection between traumatic and SAH-induced brain injury**

We provide histological, ultrastructural, and radiological evidence of multifocal axonal injury following SAH in a rodent model. Our results suggest that axonal injury is an important component of diffuse brain injury following SAH, a particularly deadly form of intracranial haemorrhage. Thus SAH may share a closer pathological link with TBI than it does with ischaemic vascular brain injury. Indeed aspects of acute SAH-induced brain injury with TBI than it does with ischaemic vascular brain hemorrhage. Thus SAH may share a closer pathological link with TBI than it does with ischaemic vascular brain hemorrhage. We and others have reported that axonal cytosolic proteins (neurofilament subunits and tau) can be detected acutely in the CSF (Nylén et al., 2006; Petzold et al., 2006; Zanier et al., 2013), serum (Hu et al., 2012; Cai et al., 2013), and peri-anneurysmal brain interstitial fluid (Helbok et al., 2015 and our unpublished results) of patients after SAH and intraparenchymal haemorrhage. Levels of these biomarkers correlate with long-term neurological recovery, suggesting that injury to axons may be an important determinant of outcome following SAH and potentially other forms of brain haemorrhage. APP accumulation has also been described in perihematoma axon bundles in the striatum in a rat collagenase model of intraparenchymal haemorrhage (Wasserman and Schlichter, 2008). Egashira and colleagues (2014) reported that after SAH axon and myelin injury markers were reduced in animals deficient in lipocalin 2, an iron transport protein implicated in brain injury. Thus iron may be an important modulator of some forms of white matter pathology after SAH and therefore a promising therapeutic target.
Potential mechanisms of SAH-induced axonal injury

A key feature of haemorrhage and TBI is widespread cerebral dysfunction. It is therefore intriguing that we observed multifocal white matter pathology in our model system. We postulate that barotrauma resulting from sudden arterial rupture plays an important role in this process. Wallerian degeneration, toxic serological factors present in the haematoma, and ischaemia may also contribute. Wallerian degeneration has been observed chronically in the corticospinal tract following rostral injury to the same fibres by intraparenchymal haemorrhage and stroke (Yu et al., 2009; Venkatasubramanian et al., 2013). However, the time course of injury in our system is more rapid than would be expected given the characteristically slow progress of Wallerian degeneration in the CNS (Vargas and Barres, 2007), and injury was not confined to white matter regions directly impacted by haemorrhage. A diffusable serological factor damaging to axons and released from the haematoma would also be expected to have a more restricted distribution than we found, and cannot explain the regional vulnerability and resilience to injury we observed after SAH.

The observation that the pattern of APP staining after experimental SAH can vary with distance from the ruptured artery argues that two or more spatially distinct mechanisms may be involved. A ‘geographical’ pattern of APP accumulation has been reported in TBI autopsy series to demarcate ischaemic areas, while a patchy distribution with dystrophic axons restricted to white matter defines traumatic injury (Geddes et al., 2000; Reichard et al., 2005). In some animals we noted an ischaemic pattern of APP staining directly adjacent to the haemorrhage following experimental SAH. At more distant sites a traumatic pattern that respected grey-white but not vascular boundaries was seen exclusively. While we excluded the small subgroup of animals with cerebral infarcts apparent by MRI after SAH, this does not rule out the possibility of small areas of ischaemia. One consistent explanation is that axons in the immediate vicinity of the haemorrhage are subjected to both mechanical and ischaemic insults, while those at greater distance are injured mechanically (Fig. 5). This situation is frequently encountered following TBI in humans where diffuse axonal injury often coexists with focal cerebral contusions and ischaemic injury near the site of impact (Geddes et al., 2000; Reichard et al., 2005; Frattalone and Ling, 2013).

Regional protection and vulnerability to SAH-induced axonal injury

In humans, the orbitofrontal regions, cerebellum, and brainstem are known to be more susceptible to stretch injury during TBI on the basis of radiographic studies and computer simulations of barotraumatic shear forces (Chafi et al., 2010; MacDonald et al., 2011). Related biomechanical processes may shape global injury patterns after SAH as well. In particular, our studies suggest that fibre
A strength of our approach is the use of multiple histological and radiological techniques with demonstrated validity for detecting axonal injury in TBI. Several limitations, however, must be acknowledged. Although the injury model we employed, the filament perforation model, has greater face validity than techniques involving the intracranial injection of blood, it does not completely replicate the human condition. Mice subjected to filament perforation experience a less-morbid injury compared to humans. The mortality rate after experimental SAH was ~20% with relatively modest supportive measures, compared with ~40% in human victims of SAH despite modern ICU care (Suarez et al., 2006; Van Gijn et al., 2007). This is likely due to a less severe experimental injury, though it is also possible that mice are more resistant to SAH-induced neurological or systemic injury. The filament perforation model is also variable. Although this further replicates the human condition, it limits the ability to detect modest effects of injury. In our experiments we prescreened animals to eliminate those with ischaemic strokes or radiologically undetectable haemorrhage from further analysis. To fully understand the relevance of our results to human health, it will be important to complete carefully controlled studies of SAH patients using the imaging methods validated in this report. We are currently undertaking these studies.

We detected axonal injury using several unrelated approaches, but the results of these techniques did not completely overlap. This situation is similar to previous observations in experimental TBI. Specifically, APP and neurofilament markers do not completely colocalize (Marmarou et al., 2005; DiLeonardi et al., 2009; Bennett et al., 2012) nor do they identify an identical population as that revealed in axolemmal permeability assays (Stone et al., 2004). Silver staining also highlights axons following TBI that are not clearly dystrophic by APP/NF immunohistochemistry, as does DTI (Bennett et al., 2012). A qualitative but not always quantitative agreement between these methods is therefore to some degree expected. A further limitation is that each histological and radiological technique we report may detect non-specific changes in ischaemia, as does DTI (Bennett et al., 2012). A qualitative but not always quantitative agreement between these methods is therefore to some degree expected. A further limitation is that each histological and radiological technique we report may detect non-specific changes in ischaemia, as does DTI (Bennett et al., 2012). A qualitative but not always quantitative agreement between these methods is therefore to some degree expected.

Implications and conclusions

The observation that SAH and TBI share pathophysiological underpinnings suggests that treatment approaches applied in TBI may also be of value following haemorrhage. For instance, it is widely recognized that clinical radiographic pathology (e.g. cerebral contusions and skull fractures) underestimate the degree of parenchymal brain injury in TBI. As a result, TBI patients with a depressed level of consciousness often undergo invasive neuromonitoring of radiographically-normal brain, regardless of imaging findings, allowing treatments to be tailored to

Figure 5 Fibre orientation relative to stress vector predicts severity of injury at sites distant from the epicentre of haemorrhage. Fibre orientation in the relatively vulnerable contralateral corpus callosum (illustrated by curved double-headed arrows) is perpendicular to the direction of the predicted compressive vector (upper thick arrow). Orientation of fibres in the relatively protected contralateral composite region of interest (internal capsule, cerebral peduncle, and optic tract), in contrast, is close to parallel with the direction of the predicted compressive vector (lower thick arrow). At very close proximity (e.g. the ipsilateral IC/CP/OT) a fibre orientation-independent process may predominate (see text). RA = relative anisotropy; Fi = fimbria; IC/CP/OT = internal capsule/cerebral peduncle/optic tract; CC = corpus callosum; EC = external capsule.

Limitations and future directions

A strength of our approach is the use of multiple histological and radiological techniques with demonstrated
intracranial physiology (Brain Trauma Foundation, 2007). Outside of hydrocephalus management this is rarely practiced for SAH or for other forms of intracranial haemorrhage. Our analysis reveals that brain regions distant from the site of haemorrhage are nonetheless impacted in a similar fashion to that seen after TBI; they may also benefit from tailored therapy.

DTI scans can be performed relatively quickly on standard clinical MRI scanners. If our results are borne out in ongoing human studies of SAH, DTI metrics may find a role in the diagnosis, triage, monitoring, and prognosis of SAH patients. DTI-based assessments may furthermore provide a surrogate endpoint for clinical trials targeting white matter injury in SAH and TBI. The current lack of such endpoints is a significant barrier to such studies.

A National Institute of Neurological Disorders and Stroke workshop (2005) identified mechanisms of white matter injury in haemorrhage as a translational research priority. Our results suggest that white matter is particularly vulnerable to injury by SAH, possibly via multiple mechanisms. Axonal injury is, however, likely one part of a multifaceted cellular injury cascade triggered by SAH, just as multiple forms of cellular injury occur following TBI (Frattalone and Ling, 2013). Further work is needed to conclusively identify the mechanism of axonal injury following SAH, and to determine its relevance to patient outcomes.

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Supplementary material

Supplementary material is available at Brain online.

References


