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Ceruloplasmin and β-amyloid precursor protein confer neuroprotection in traumatic brain injury and lower neuronal iron.

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Abstract
Traumatic brain injury (TBI) is in part complicated by pro-oxidant iron elevation independent of brain hemorrhage. Ceruloplasmin (CP) and β-amyloid protein precursor (APP) are known neuroprotective proteins that reduce oxidative damage through iron regulation. We surveyed iron, CP and APP in post mortem control and TBI-affected brain tissue, which was stratified according to time of death following injury. We observed CP and APP induction following TBI accompanying iron accumulation. Elevated APP and CP expression was also observed in a mouse model of focal cortical contusion injury concomitant with iron elevation. To determine if changes in APP or CP were neuroprotective we employed the same TBI model on APP⁻/⁻ and CP⁻/⁻ mice and found that both exhibited exaggerated infarct volume and iron accumulation post injury. Evidence supports a regulatory role of both proteins in an iron defence system to oxidative damage after TBI, which presents as a tractable therapeutic target.

Highlights
• Pro-oxidant iron elevation occurs in TBI in a temporally distinct pattern.
• Expression of iron exporting proteins APP and CP is induced following TBI.
• Loss of APP or CP exaggerates iron deposition and consequent lesion severity in TBI.
**Introduction**

Traumatic brain injury (TBI) is the major cause of death in young individuals (14-24 years) from industrialised countries, with traumatic head injuries accounting for 25-33% of all trauma related deaths[1]. Following the initial insult, injury is exacerbated by secondary factors such as oxidative stress, inflammation and excitotoxicity[2]. The manifestation of these insults after TBI arises from vascular effects, distinct cellular responses, apoptosis and chemotaxis[3]. Our ability to identify therapeutic targets and devise strategies for the treatment of TBI relies on our understanding of the early molecular processes that are initiated following brain injury as well as the delayed molecular events, which together propagate extensive neuronal loss. Iron elevation has been observed in the brain after TBI[4-7] independent of the heme-bound iron associated with the blood leakage within the site of injury. While the cause of non-heme iron elevation after TBI is not understood, this likely contributes to secondary cell loss and is a possible tractable therapeutic target.

Iron is an essential nutrient required as a cofactor in metabolic processes throughout the body and specifically in tissues of high oxygen consumption, such as the central nervous system. The ability of iron to freely receive and donate electrons is critical for myelination, DNA synthesis, energy production and cell cycling as well as a cofactor for enzymes involved in neurotransmitter production and metabolism[8] and a deficiency in iron can lead to metabolic stress upon these processes[9]. However, the increased presence of unbound iron is also detrimental as this may catalyze the production of toxic reactive oxygen species and is a major compounding factor to oxidative stress, inflammation and excitotoxicity.

Since too much or too little iron can compromise cell viability, cellular iron homeostasis is tightly regulated[10] and within the brain is specialised according to cell type[11]. Reducing harmful iron accumulation within the central nervous system can be regulated by reducing iron import, via transferrin receptor, divalent metal transporter or Slc39a14 (Zip14)[12], as well as by intracellular iron storage via ferritin, neuromelanin and hemosiderin. Excess cellular iron is exported via ferroportin (FPN). Ceruloplasmin (CP) and the Alzheimer’s-associated β-amyloid precursor protein (APP) both bind to FPN and are proposed to facilitate iron export through their ability to prolong FPN’s presence on the cell surface [13, 14].

Both CP and APP exist in membrane-bound and soluble forms. CP is located as a
soluble form in plasma and as a glycosylphosphatidylinositol (GPI)-anchored isoform on the membrane of select cell types[15]. Ablation of CP function in aceruloplasminemia[16] or within a knockout mouse model[17, 18] causes age-dependent toxic iron accumulation in the brain. APP is ubiquitously expressed as a full-length type 1 transmembrane protein and as processed fragments including the soluble species found in the extracellular fluid (sAPP). Decreased retention of APP on the neuronal cell surface, as found in some neurodegenerative diseases (e.g. Alzheimer’s disease) or decreased expression of the functional protein in knockout mouse models, also leads to age-dependent toxic iron accumulation in the brain, increased susceptibility to oxidative injury and increased glutamate excitotoxicity[13, 19]. Soluble APP and CP are both neuroprotective to a range of insults and to animal models of TBI[20-24]. The present paper uses TBI post mortem tissue and a focal cortical contusion model of TBI to investigate whether the capacity for CP and APP to protect neurons against TBI may be partially through neuronal iron regulation and if the expression of these proteins could offset iron elevation post lesion to confer neuroprotection.

**Methods**

*Human post-mortem brain tissue*

All procedures were conducted in accordance with the Australian National Health & Medical Research Council’s *National Statement on Ethical Conduct in Human Research* (2007), the Victorian Human Tissue Act 1982, the National Code of Ethical Autopsy Practice and the Victorian Government Policies and Practices in Relation to Post-Mortem.

Trauma brain samples from 37 individuals who died after closed head injury were obtained from the Australian Neurotrauma Tissue and Fluid Bank. Cases were aged between 17 and 78 years (mean 48 years) and the causes of injury include motor vehicle accident, motorbike accident, nursing home accident, household accident, stair accident and falls. The *post-mortem* intervals varied between 33 and 129 hours (mean 81 hours). Patients were divided in 3 groups to compare the acute and delayed times after injury: 10 ‘acute’ cases (8 males and 2 females) had survival time of less than 20 minutes, when death occurred upon arrivals of the paramedics; 8 ‘early’ cases (7 males and 1 female) were selected with a survival time up to 3 hours; and 9 ‘late’ cases (7 males and 2 females) had survival time 12-40 hours. As previously reported[25-27], the human cortical brain region analysed was located within 1 cm of the injured tissue and was identified macroscopically by a neuropathologist (Prof. Catriona McLean). A second brain region was also
analysed for the cases with a survival time of more than 6 hours that was located in the contralateral brain hemisphere at the same locus of injury in the ipsilateral hemisphere where no macroscopic damage was detectable. Control brain samples of 10 individuals, aged between 16 and 78 (mean 56 years), without brain injury or other neuropathologies were obtained from the National Neural Tissue Resource Centre of Australia. Clinical information and epidemiological details of all patients are described in Table S1.

**Mice**

All mouse studies were performed with the approval of the IACUC and in accordance with statutory regulations. For focal cortical contusion head trauma APP-/- mice[28] and CP-/- mice[29] as well as their respective littermate controls (C57/BL6) were all aged ~12 weeks. When applicable trauma was given to mice in an identical stereotaxic position and then sacrificed 3, 6 or 24 hours after injury. Control mice were given a sham injury and sacrificed at the same points as in the experimental group. After pericardial perfusion, brains were either immediately prepared for biochemical and infarct analysis, stored as hemispheres at -80°C until required or fixed in 4% paraformaldehyde for 24 h, dehydrated in ascending ethanol and embedded in paraffin for histological staining of iron.

**Focal Cortical Contusion Head trauma model**

Trauma to mice was implemented similar to previously described[30]. Briefly, APP-/-, CP-/- and littermate control mice were anaesthetised with an intra-peritoneal injection of Ketamine (100mg/kg, Parnell)/Xylazine (10mg/kg, Parnell). A sagittal scalp incision was made to expose the underlying parietal skull. A 2 mm diameter plate of bone (centred 1.5 mm posterior to bregma and 2.5 mm lateral to the midline) was then removed using a Dremel 10.8V drill with a 0.8mm tip (Dremel, Europe) to expose the underlying right parietal cortex. A 1.5mm deep impact into the exposed cortex was made at 5m/s. Following impact by the computer-controlled 2mm tip, the bone plate was replaced and held in place with a small section of parafilm to cover the injury site. The skin incision was then closed with sterile silk 5.0 metric sutures (Syneture Tyco Healthcare). Mice were administered intra-peritoneal Buprenorphine (0.6 mg/kg, Reckitt Benckiser Healthcare) and placed on a heat mat for post-surgical recovery. Sham operated mice, designated ‘Control’ underwent the same anesthesia, scalp incision and bone plate removal, but were not injured.
Western blot analysis.
Human and mouse fresh frozen tissue (kept at -80°C until required) either ipsolateral or contralateral to the site of injury were homogenized in 1 x Tris-Base saline (TBS) with 0.1% Triton-X100 (TBST) with 20 x 1 sec pulses on a sonicator. As determined by BCA assay, 20 μg total protein from each experimental condition, was separated on 4-20% PAGE (Bis-Tris, Invitrogen) and transferred to nitrocellulose membrane using iBlot (Invitrogen). Primary antibodies used were mouse anti-APP (1:100, in house 22C11), rabbit anti-CP (1:5000, Sigma) and rabbit anti- total Tau (1:5000, Sigma). The load control was mouse anti-β-actin (1:10,000). Proteins were visualized with ECL (Amersham) and a LAS-3000 Imaging suite, and analyzed using Multi Gauge (Fuji). Densitometry using Image J (NIH) of APP, CP and Tau was performed in triplicate on 3 separate experiments. All quantitation was standardized against β-actin levels before calculating to % of control.

Total Iron Assay.
Total homogenate in TBST was lyophilised from hemispheric brain samples before dissolving in conc. HNO₃ and H₂O₂ (Aristar, BDH). Metal levels were measured by inductively coupled plasma mass spectrometry with an Ultramass 700 (Varian, Victoria, Australia) as described[31].

Histochemical detection of non-heme iron by modified Perl's staining.
For direct visualization of non-heme iron in paraffin embedded sections containing both hemispheres, a modified Perl’s technique was used, as previously described[32, 33]. Briefly, deparaffinized and rehydrated tissue sections (7 μm) were incubated at 37°C for 1 h in 7% potassium ferrocyanide with aqueous hydrochloric acid (3%) and subsequently incubated in 0.75 mg/ml 3,3’-diaminobenzidine and 0.015% H₂O₂ for 5-10 min. When required, sections were counterstained in Mayer’s haematoxylin for 2 min and washed in Scott’s tap water before mounting.

All sections were imaged on a Northern Light Illuminator (Imaging Research Inc, Ontario, Canada) using a Spot RT-KÉ 2MP digital camera (Diagnostic Instruments, MI, USA) equipped with a Nikkor 55 mm lens and 56 mm extension tube set (Nikon). Each image was analysed using ImagePro Plus 5.1 (Media Cybernetics, MD, USA).

Histological measurement of infarct size
On a separate cohort from animals used for iron analysis, mice were killed 24 hours after TBI or sham with brains immediately removed and sliced in a mouse brain
matrix to 500 μm thickness. These were placed in a 2% 2,3,5-Triphenyltetrazolium chloride (TTC) and PBS solution at 35°C for 15 minutes. Photomicrographs were captured using a Zeiss Axioskop microscope and infarct area was determined using the Image J software (v1.47; NIH) as previously described[34]. Tissue swelling in the injured side was accounted for by dividing the infarct area from each section by the ratio of the areas of the injured relative to uninjured side. Areas of infarct and the entire rostral surface area were delineated and area values given in pixels. All infarct areas on the section surfaces of the same brain were added, as were the total rostral surface areas. A two-dimensional approximation of the infarct volume was calculated as a percentage of these two sums.

Statistical Analysis
Statistical analysis was typically carried out in Prism (GraphPad) or Excel (Microsoft) software. Primarily analysis was carried out with a 2-tailed t-test with the level of significance set at $P=0.05$. For multiple comparisons one-way ANOVA was used to identify significant differences between trauma and control groups. The Pearson product correlation moment was used to confirm whether confounding relationships existed between post-mortem delay, age and protein expression or iron levels (Table S2).

Results
Post mortem tissue iron accumulation after TBI parallels changes to CP and APP expression.
We surveyed iron, CP and APP in post mortem brains of individuals who had died following a closed head trauma at acute (within 20 mins, n=10), early (~3 hrs, n=8) or late (12-40 hrs, n=9) intervals. Cortical brain tissue on the ipsilateral side (ILS) to lesion site was compared to control post-mortem brains from patients of a similar age with no history of trauma (n=10). Cortical tissue from the contralateral side (CLS) of the injury was also available for comparison from TBI patients who died after the late interval following TBI. Iron accumulation as determined by ICP-MS was not evident before 3 hours, but increased by ~250% after 12 hrs post injury (Fig. 1). This change was specific to the ILS (Fig. 1A).

Protein expression studies on the same patient cohort indicated a late CP response at the site of injury that varied with iron changes (~200% elevation at 12-40 hrs) (Fig. 1B). An inverse relationship between APP protein expression and total iron content was evident with APP rapidly increased in patients 20 minutes after TBI (~450%) and
then steadily decreased over time until no significant difference was seen after 12-40hrs post TBI (Fig. 1C).

As to be expected when using post mortem tissue, a wide range of post mortem interval and age within and between the groups was observed (Table S1). Therefore, Pearson product moment correlation was used to identify any confounding relationship between these two variables and protein expression or iron levels. All analysis on CP and APP expression showed that the correlation coefficient r for each pair analyzed were all near 0, with P values greater than 0.05 indicating no significant relationship between the variables PMI or age (Table S2). Similarly, no significant relationship was seen for iron levels with PMI. While iron was significantly elevated with age, as previously reported[35], further investigation of age variance within each group revealed no significant difference between all groups.

Iron related changes in a focal cortical contusion injury mouse model of TBI

To more closely examine the relationship between APP and CP expression with iron elevation in TBI we employed a focal cortical contusion injury mouse model. Compared to a study of post mortem patients, the model has the advantage of eliminating variables such as location of injury, intensity of trauma and PMI. Using a previously established TBI injury mouse model[36] time-dependent total iron content was measured by ICP-MS. Iron levels were raised after injury in the ILS compared to CLS, which peaked at 6 hours following the procedure. After this time point, iron levels on the ILS declined to levels comparable to the CLS (Fig. 2A). Bulk iron measurement by ICP-MS is complicated by hemoglobin contamination from unavoidable hemorrhage upon lesion. To determine if intraneuronal iron is likewise elevated after the insult, we performed histology with modified Perls’ staining within the region of the injury site. As Perls’ staining is unable to detect heme bound iron[37, 38], the neuronal iron accumulation present at the site of injury (Fig. 3Ai & Bi) is not simply blood contamination, however reflects the cellular response to injury.

CP and APP protein expression from the same samples also increased significantly after injury (Figs. 2B & C). Elevations in both proteins compared to control mice were observed to be more pronounced in the hemisphere of the ILS compared to CLS. Recently we have reported that tau’s ability to assist transport of APP to the cell surface facilitates its correct location for iron efflux[19]. Total tau expression was also measured in these animals but shown not to significantly change (Fig. 2D) as
previously reported in an alternative TBI model [39] and in humans <24hrs after a single acute trauma[40].

The iron response to focal cortical contusion in mice deficient in CP or APP. To investigate if expression level changes in CP or APP after TBI protect against iron elevation and/or neuronal damage, the same trauma model was performed on APP<sup>+/−</sup> and CP<sup>+/−</sup> mice. As both mouse strains have previously been shown to have significant neuronal iron accumulation by 6 months of age[13, 18], studies were performed on animals less than 3 months old. 24 hours after injury, iron was visualized using modified Perls’ staining histology in both genotypes. An increase in intracellular iron within the cortex (immediately adjacent to the site of lesion) and hippocampus (ventral to the site of lesion) of mice deficient in either CP (Fig. 3Aii & Bii) or APP (Fig. 3Aiii & Biii) was visually apparent within the region of injury (ILS) compared to wild-type littermates (Fig. 3Ai & Bi). Parallel regions on the CLS were also stained and while iron was marginally elevated in the CLS hemisphere of mice deficient in either CP (Fig. 2Av & Bv) or APP (Fig. 3Avi & Bvi) compared to wild-type (Fig. 3Aiv & Biv), levels were not as high as those observed on the ILS of injury (Fig. 3Ai-iii & Bi-iii). Minor hemorrhage associated iron confined to the site of injury was discriminated morphologically by the characteristic shape of red blood cells compared to brain cells.

Next, we appraised infarct volume of both APP<sup>−/−</sup> and CP<sup>−/−</sup> mice to determine if lack of induction of APP or CP impacted lesion severity. Infarct volume was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining on sections throughout the region of interest. In accordance with the accumulation of iron, infarct size in mice deficient in either protein was significantly increased (APP<sup>−/−</sup> +~20%, CP<sup>−/−</sup> +~25% Fig. 3C) compared to littermate controls given an identical injury.

Discussion
The extant literature demonstrates iron accumulation that does not originate from hemorrhage is a feature of the TBI-effected brain [6, 7] and parallel studies have shown alterations in APP and CP post TBI[20-22]. Here we suggest that post injury changes in APP and CP expression observed in human (Fig. 1) and mouse (Fig. 2) act to reduce iron elevation and, in the case of the mouse model, alter infarct volume since the absence of these proteins exacerbates lesion severity (Fig. 3).

Understanding the cellular events that cause iron accumulation post TBI might
present new therapeutic targets to prevent iron elevation from occurring. Supporting a role for CP and APP in iron homeostatic response after TBI, both APP\(^{-/-}\) and CP\(^{-/-}\) mice exhibited exaggerated iron elevation in response to injury (Fig. 3) and correlative changes between iron and CP were observed in mice (Fig. 2) and humans (Fig. 1). While expression changes between mouse and human APP appeared to differ in their temporal profile (Fig. 1 & 2), this difference may have been highlighted by the collection time points in the mouse model of TBI being marginally different to post mortem human tissue, thereby preventing the identification of mouse APP expression at time points earlier than 3 hrs. In addition, it is worth noting that the mouse model data may not represent TBI in full and additional compounding factors may be present in human trauma.

CP\(^{-/-}\) mice have not previously been studied in TBI, however a spinal cord contusion injury model is exacerbated in CP null mice[41] similar to what we report here using a model of TBI (Fig. 3). Mice deficient in APP have been previously reported to have increased neuronal death and worse cognitive and motor outcomes using several models of TBI[42-44]. Significantly, the intracranial introduction of exogenous sAPP\(_\alpha\) to various murine models of TBI reduces neuronal injury and improves functional motor outcome following TBI[24, 42-45]. While recently sAPP’s ability to rescue TBI has been found to be mediated via the heparin binding site in the D1 region of the protein[42], a more C-terminal region of APP is also known to be neuroprotective in TBI[45]. Here we propose that the presence of APP also protected against iron elevation in a TBI model and hypothesize that this may in part be the neuroprotective mechanism of the protein.

Iron accumulation likely contributes to neuronal free radical injury[46, 47] that is known to be part of a degenerative cascade in TBI [48-50]. In our study iron accumulation emerged later in the temporal sequence, after 3 hours in both mice (Fig. 2) and human (Fig. 1) tissues, suggesting either a secondary degenerative mechanism such as an increase in heme oxygenase-1 expression[51] or that iron elevation is prevented by an early cellular response (potentially involving APP) which fatigues over time. Regardless, attenuating the iron loading is beneficial in TBI models since several studies have shown varying levels of neuroprotection in rodent models of trauma using a range of chelators with differing affinities for iron[52-55].

Iron is an accessible drug target, with multiple selective chelators approved for use in neurological disorders of iron metabolism[9, 56]. The delay of 3 hours before iron
induction also allows for a therapeutic window to treat patients before iron-related damage emerges. However the use of small molecule chelators of iron is limited by their BBB penetration and off-target effects of iron depletion in the brain and periphery, indeed in some TBI models their use has shown limited efficacy [52, 57]. Alternatively, the use of APP [24, 42-45] or CP[18] protein supplementation or a way of boosting their iron regulatory function might be an alternate avenue to restore iron homeostasis following injury.

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Competing Interests statement: Dr. Bush is a shareholder in Prana Biotechnology Pty Ltd., Eucalyptus Pty Ltd., Mesoblast Pty Ltd. And a paid consultant for Collaborative Medicinal Developments LLC.

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Figure Legends

Figure 1. Levels of iron and expression of iron efflux proteins in post mortem tissue from patients that died at different times after close head trauma. Brain tissue was analyzed for iron by ICP-MS and protein expression from patients who died <20 minutes post a TBI (n=10), <3 hours post a TBI (n=8), and between 12-40 hours post injury (n=9). These were compared to tissue from 10 control patients. A. Elevated total iron (~250% control) was observed in the ILS cortex from patients that died 12-40hrs post-injury. B. CP measured in the same tissue was likewise elevated after 12-40 hours post injury (~200% control). C. APP expression was elevated by ~450% in patients who died within 20 minutes of injury, while the expression profile declined stepwise in patients that died in the subsequent pooled intervals. Data are means ± SEM, minimal group N=8, ***=p<0.001 & *=p<0.05 as analyzed by two-tailed t test compared to control and CLS.
Figure 2. Levels of iron in wild-type mice after close head trauma correlate with changes in APP and CP expression. **A.** Cortical total iron (measured by ICP-MS) is elevated in the ipsilateral hemisphere (ILS) of injury in mice killed at 6 hours post injury. Iron is returned to contralateral hemisphere (CLS) levels in the ILS at 24 hours post injury and is significantly unchanged in the CLS at all time points. **B.** CP expression in the ILS hemisphere of injury steadily increased over time compared to expression in control and the CLS hemisphere reaching maximum expression (~350% of control) 24hrs after injury. **C.** Similar to CP expression, APP also steadily increased (~175% control) in expression after injury on the ILS compared to control and CLS. **D.** In contrast, tau; previously shown to facilitate transport of APP to the cell surface for iron export, is not significantly altered over time after injury incurred. Data are means ± standard error of the mean (SEM), N=3, *=p<0.05 as analyzed by two-tailed t test compared to control and CLS.

Figure 3. Non-heme iron accumulation and infarct volume after close head injury are exacerbated in APP and CP knockout mice. **A.** Cortical sections stained with modified Perls’ stain to detect non-heme iron (brown) and counterstained with hematoxylin (blue) for cell localization show increased iron in ILS sections from wildtype mice 24 hours after injury (i) which is elevated further in ILS of CP knockout (CP−/−) (ii) or APP knockout (APP−/−) (iii) mice. Little iron was detected in CLS sections from all mice (iv-vi). **B.** Hippocampal regions from same mice as in A also show elevated iron in ILS sections from all mice (i-iii), but the iron stain was exaggerated in ILS sections from CP−/− (ii) and APP−/− (iii) mice after injury. By morphological identification, cell types appear neuronal and iron deposition is punctate in APP−/−. Insert images in A & B show representative iron staining in neurons. **C.** Infarct size 24 hours after injury was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining in alternate sections to those used for iron detection from the series through the site of injury. A significant increase in volume of the infarct was identified in CP−/− and APP−/− compared to their respective wild-type littermates. Data are means ± SEM, N=3, *=p<0.05 as analyzed by two-tailed t test. Scale bar in A = 100µm and B = 50µm.
Highlights

- Pro-oxidant iron elevation occurs in TBI in a temporally distinct pattern.
- Expression of iron exporting proteins APP and CP is induced following TBI.
- Loss of APP or CP exaggerates iron deposition and consequent lesion severity in TBI

Keywords: Traumatic brain injury; iron; mouse models; amyloid precursor protein; ceruloplasmin
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