

# Accumulation of Non-Transferrin-Bound Iron by Neurons, Astrocytes, and Microglia

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**Abstract** Neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, and hemorrhagic stroke are associated with increased levels of non-transferrin-bound iron (NTBI) in the brain, which can promote Fenton chemistry. While all types of brain cells can take up NTBI, their efficiency of accumulation and capacity to withstand iron-mediated toxicity has not been directly compared. The present study assessed NTBI accumulation in cultures enriched in neurons, astrocytes, or microglia after exposure to ferric ammonium citrate (FAC). Microglia were found to be the most efficient in accumulating iron, followed by astrocytes, and then neurons. Exposure to 100  $\mu$ M FAC for 24 h increased the specific iron content of cultured neurons, astrocytes, and microglial cells by 30-, 80-, and 100-fold, respectively. All cell types accumulated iron against the concentration gradient, resulting in intracellular iron concentrations that were several orders of magnitude higher than the extracellular iron concentrations. Accumulation of these large amounts of iron did not affect the viability of the cell cultures, indicating a high resistance to iron-mediated toxicity. These findings show that neurons, astrocytes and microglia cultured from neonatal mice all have the capacity to accumulate and safely store large

quantities of iron, but that glial cells do this more efficiently than neurons. It is concluded that neurodegenerative conditions involving iron-mediated toxicity may be due to a failure of iron transport or storage mechanisms, rather than to the presence of high levels of NTBI.

**Keywords** NTBI · Neurodegeneration · Iron overload · FAC · Oxidative stress

## Introduction

Iron is an essential trace element required by brain cells for processes such as energy production, myelination and neurotransmitter synthesis (Connor and Menzies 1996). Many of these essential functions utilize the redox capacity of iron, yet this capacity can make iron cytotoxic (Halliwell and Gutteridge 2007). Once iron has been taken up from the extracellular space, its presence in the cytosol can promote Fenton chemistry and cause oxidative stress until the iron is safely stored within ferritin (Halliwell and Gutteridge 2007; Hoepken et al. 2004). A number of neuropathological conditions are associated with increased iron content in the brain, including Alzheimer's disease, Parkinson's disease, aceruloplasminemia, stroke, and traumatic brain injury (Burdo and Connor 2003; Berg et al. 2001; Connor et al. 1992; Lipscomb et al. 1998; Miyajima et al. 1987). The pathology observed in these disorders has been linked to the presence of abnormally high concentrations of non-transferrin-bound iron (NTBI), which can promote Fenton chemistry (e.g., Bishop et al. 2002; Gerlach et al. 1994; Robinson et al. 2009). The NTBI present within the cerebrospinal fluid is predominantly complexed as ferric citrate (Bradbury 1997), and can be transported into cells as ferric iron or reduced by membrane-bound

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reductases and then transported into cells as ferrous iron (Tulpule et al. 2010).

All types of brain cells are capable of accumulating NTBI, including neurons and microglia (Kress et al. 2002; Oshiro et al. 2000; Oshiro et al. 2008; Takeda et al. 1998). Astrocytes have been reported to possess an especially high capacity for NTBI accumulation (Takeda et al. 1998; Riemer et al. 2004; Swaiman and Machen 1985; Oshiro et al. 1998). Consequently, astrocytes are generally considered to be primarily responsible for scavenging NTBI from the extracellular space of the brain, thereby protecting other neural cells from iron-mediated oxidative stress (e.g., Dringen et al. 2007). This conclusion is consistent with the general role of astrocytes as protectors of neurons, yet it is not based on conclusive data. A limitation in the literature is that studies of NTBI uptake generally focus on a single cell type, so comparisons between cell types are confounded by differences in experimental design. Admittedly Oshiro et al. (2000) did compare iron uptake in mixed glial cultures and neurons, but cultures strongly enriched in astrocytes or microglia were not compared. Similarly, Takeda et al. (1998) compared different types of glial cells, but did not examine neurons. Thus, it is not yet certain that astrocytes do have higher rates of iron uptake than neurons or microglia. A direct comparison may provide insights into the roles of the various cell types in brain iron overload, as well as indicate the most appropriate cells to target for therapeutic interventions.

The present study used a ferrozine-based colorimetric assay to quantify the accumulation of NTBI by cultures enriched in neurons, astrocytes, and microglia. A modified Perls' stain was used to visualize the cellular distribution of iron, allowing a complementary microscopic analysis that demonstrates the cellular compartmentalization of iron. The capacity of neurons, astrocytes, and microglia to resist the toxicity of the accumulated iron was compared by assessing cell viability after the uptake of iron.

## Materials and Methods

### Animals

C57BL/6J mice were obtained from Monash Animal Services. All animal experimentation was approved by the Monash University SPPPM Animal Ethics Committee and met the guidelines of the National Health & Medical Research Council of Australia.

### Reagents

Neurobasal medium, B27 serum-free supplement, fetal calf serum (FCS), Dulbecco's-modified Eagle medium

(DMEM), and penicillin/streptomycin were purchased from Gibco. Paraformaldehyde was from ProSciTech or Aldrich, and potassium ferrocyanide was from ICN. Ferric ammonium citrate (FAC) and all other chemicals were obtained from Sigma or Merck. Antibodies used were: chicken anti-cow microtubule-associated protein-2 (MAP-2; Abcam), rabbit anti-cow glial fibrillary acidic protein (GFAP; Dako), rat anti-mouse CD11b (Chemicon), donkey anti-chicken IgY (Chemicon), donkey anti-rabbit Ig (Amersham), goat anti-rat IgG (Chemicon), and biotinylated-streptavidin horseradish peroxidase (Amersham).

### Primary Neuronal Cell Cultures

Primary cerebellar granule cultures were derived from 6-day-old mice, using a modification of a published protocol (Cheung et al. 1998). The cerebellum was isolated following decapitation and collected into a Preparation Buffer (Hanks buffered salt solution (HBSS) containing 1 mM sodium pyruvate, 7.4 mM glucose, 10 mM HEPES, 1.164 mM MgSO<sub>4</sub> and 3 mg/ml bovine serum albumin). The cerebella were chemically digested by incubation with a trypsin solution (Preparation Buffer containing 0.2 mg/ml trypsin and 0.08 mg/ml DNase I) for 30 min at 37°C, which was then neutralized with a solution of trypsin inhibitor (Preparation Buffer containing 0.52 mg/ml soybean trypsin inhibitor, 0.08 mg/ml DNase I and 1.5 mM MgSO<sub>4</sub>; 4°C). Cells were mechanically dissociated by 10 times trituration and then resuspended in neuronal culture medium (Neurobasal medium containing 2% B27, 0.5 mM glutamine, 25 mM KCl, 3.7 g/l NaHCO<sub>3</sub>, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS). Viable cells were seeded at 600,000 cells per well (in 0.5 ml) in 24-well culture plates that had been coated with poly-D-lysine (50 µg/ml, MW 30,000–70,000). Cultures were supplemented with 10% CO<sub>2</sub> and 95% humidity, and maintained at 37°C. On day 1 in vitro, cytosine β-D-arabinofuranoside (Ara-C) was added to each culture well (final concentration = 10 µM per well) to inhibit glial cell division, while being used at a concentration that is not toxic to cerebellar granule cells cultured with high potassium (Daniels and Brown 2002). On day 5 in vitro, three-quarters of the media was replaced with fresh media that contained 10 µM Ara-C plus the ingredients listed above, except for FCS. For cultures that were processed for immunocytochemistry or Perls' staining, cells were grown in wells that contained 10 mm glass coverslips or 13 mm plastic coverslips, respectively, which had been coated with poly-D-lysine.

### Primary Astrocyte Cell Cultures

Primary astrocyte cell cultures were derived from newborn mice (<24 h old) by the method of Hamprecht and Löffler

(Hamprecht and Loffler 1985). Viable cells were seeded at 300,000 cells per well in 24-well culture plates and incubated in 1 ml astrocyte culture medium (90% DMEM, 10% FCS, 20 U/ml penicillin, 20 µg/ml streptomycin). For cultures that were processed for immunocytochemistry or Perls' staining, viable cells were seeded at 150,000 cells per well in 24-well culture plates that contained 10 mm glass coverslips or 13 mm plastic coverslips, respectively. Cultures were supplemented with 10% CO<sub>2</sub> and 95% humidity, and maintained at 37°C. The culture medium was replaced every 7 days.

### Microglial Cell Cultures

Secondary microglia cultures were harvested from primary astrocyte cultures as described previously (Hirrlinger et al. 2000). Briefly, primary astrocytes were seeded at 30,000,000 cells/flask in 550 ml culture flasks and grown to confluence as described above. Between 14 and 36 days in vitro, microglia were shaken loose from the astrocyte cell layer and seeded at 150,000 cells per well in 24-well culture plates and then incubated with 1 ml of astrocyte-conditioned medium (1:1 ratio of 0.2 µm-filtered supernatant from the culture flasks and astrocyte culture medium). For immunocytochemistry or Perls' staining, cells were grown in wells that contained 10 mm glass coverslips or 13 mm plastic coverslips, respectively. Cultures were supplemented with 10% CO<sub>2</sub> and 95% humidity, and maintained at 37°C.

### Immunocytochemical Staining

To verify the purity of the cell cultures, they were immunocytochemically labeled for MAP-2, GFAP, or CD11b, which are markers for neurons, astrocytes, and microglia, respectively. Following our standard protocol (Edwards and Robinson 2006), cultures were incubated with antibodies against MAP-2 (1:1000), GFAP (1:1000), or CD11b (1:50) for 1 h, and then incubated for 1 h with the respective secondary antibody, anti-chicken IgY (1:500), anti-rabbit Ig (1:300), or anti-rat IgG (1:500). All cultures were then incubated with biotinylated-streptavidin horseradish peroxidase (1:300) for 1 h and then incubated with the chromagen 3',3'-diaminobenzidine tetrahydrochloride-nickel sulfate. Cells were examined using light microscopy and photomicrographs were taken with an Olympus BX-50 microscope with a DP-50 digital camera. Micrographs were adjusted for contrast, brightness, sharpness and color balance using Adobe Photoshop 8.0.

### Incubation of Cultures with FAC

Neuron, astrocyte and microglia cultures were used for experiments after 8 days, 2 weeks, or 3 days in vitro,

respectively, and the culture media was replaced at least 24 h prior to experimentation. Cultures were washed twice with pre-warmed (37°C) media and then incubated with media containing 0–100 µM FAC, as indicated in the figure legends. Neurons were incubated in a volume of 0.5 ml Neurobasal medium that contained 0.5 mM glutamine, 25 mM KCl, 3.7 g/l NaHCO<sub>3</sub>, and 10 µM Ara-C. The medium used for astrocytes and microglia was DMEM, and these cultures were incubated in a volume of 1 ml or 0.5 ml, respectively. Following the incubation, the cells and media were processed as indicated in the figure legends or text.

### Quantification of Iron and Protein Contents

The iron content of the cells or the media was determined using a ferrozine-based colorimetric assay as described previously (Riemer et al. 2004). The cellular iron content was standardized to the protein content of each culture, determined by the Lowry method (Lowry et al. 1951).

### Cell Viability

For neurons and astrocytes, cell viability was assessed by measuring the activity of cytosolic lactate dehydrogenase (LDH) released into the media as previously described (Dringen et al. 1998). Due to the low number of microglial cells per culture, the LDH assay could not be used for these cultures; instead, cell viability was assessed by measuring the cellular protein content of the cultures according to the Lowry method (Lowry et al. 1951).

### Visualization of Iron with a Modified Perls' Stain

To visualize cellular iron, cells were cytochemically stained with a modified Perls' stain, as previously described (Dang et al. 2010). Cells were examined using light microscopy and photomicrographs were taken as described above for immunocytochemical staining.

### Statistical Analysis

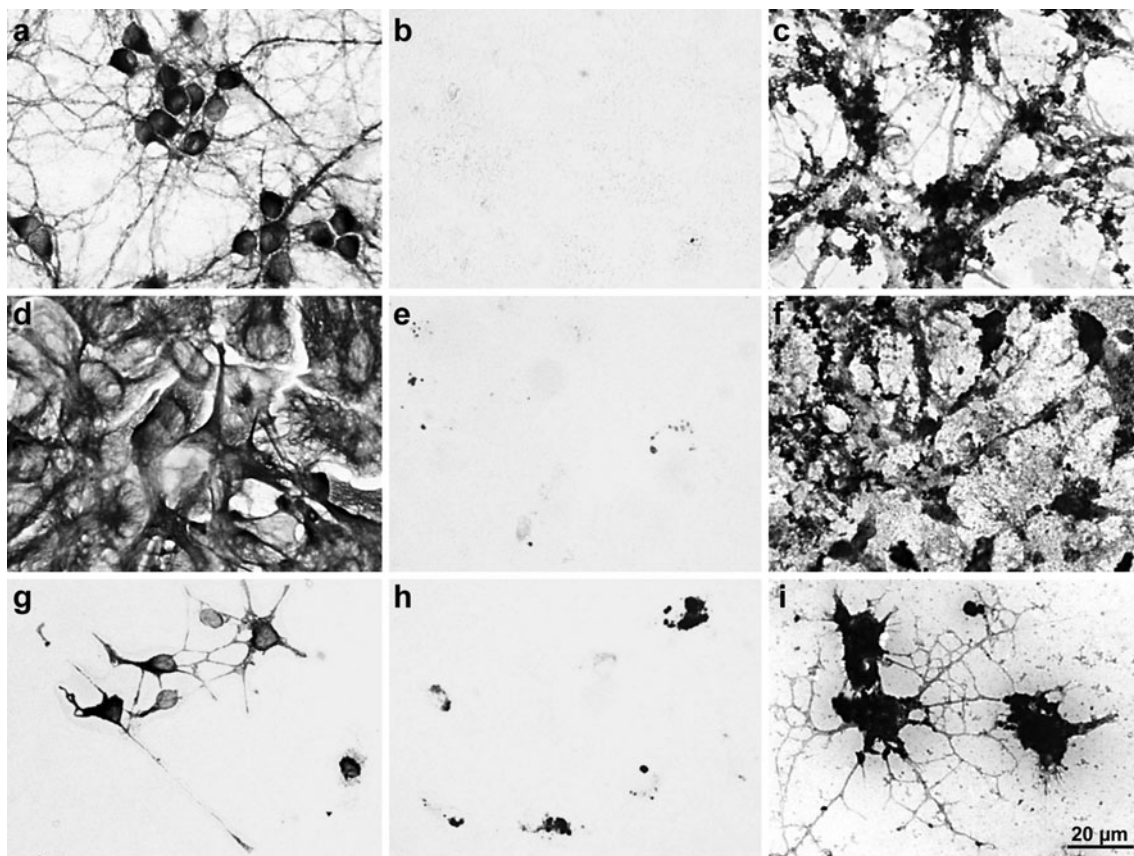
All experiments were performed on a minimum of three independent cultures, with triplicate samples within each culture. The data are presented as mean ± SD. Statistical analysis was performed by independent samples *t*-tests, with statistical significance set at  $\alpha = 0.05$ . When appropriate, *P* values were adjusted using the False Discovery Rate (FDR) for multiple comparisons (Benjamini and Hochberg 1995).

## Results

To verify the purity of the cell cultures, representative sample wells from each culture type were immunocytochemically labeled for cell-specific markers. The cells in neuronal cultures expressed MAP-2 and the labeling was concentrated in the somata, with clear labeling also present within the neurites that extended from the cell bodies (Fig. 1a). Cells in astrocyte cultures expressed GFAP, with strong labeling in their somata and processes, a typical astrocytic morphology was evident, with a high level of confluence throughout the cultures (Fig. 1d). Cells in microglial cultures expressed CD11b and generally displayed a ramified morphology (Fig. 1g). Labeling of the cultures with antibodies directed against the other cell types demonstrated a numerical purity of >95% for astrocyte cultures and >99% for neuronal and microglial cultures (data not shown).

Using a ferrozine-based colorimetric assay, the cellular iron content of untreated cultures was determined. These control cultures of neurons, astrocytes, and microglia contained similar amounts of iron, as indicated by their

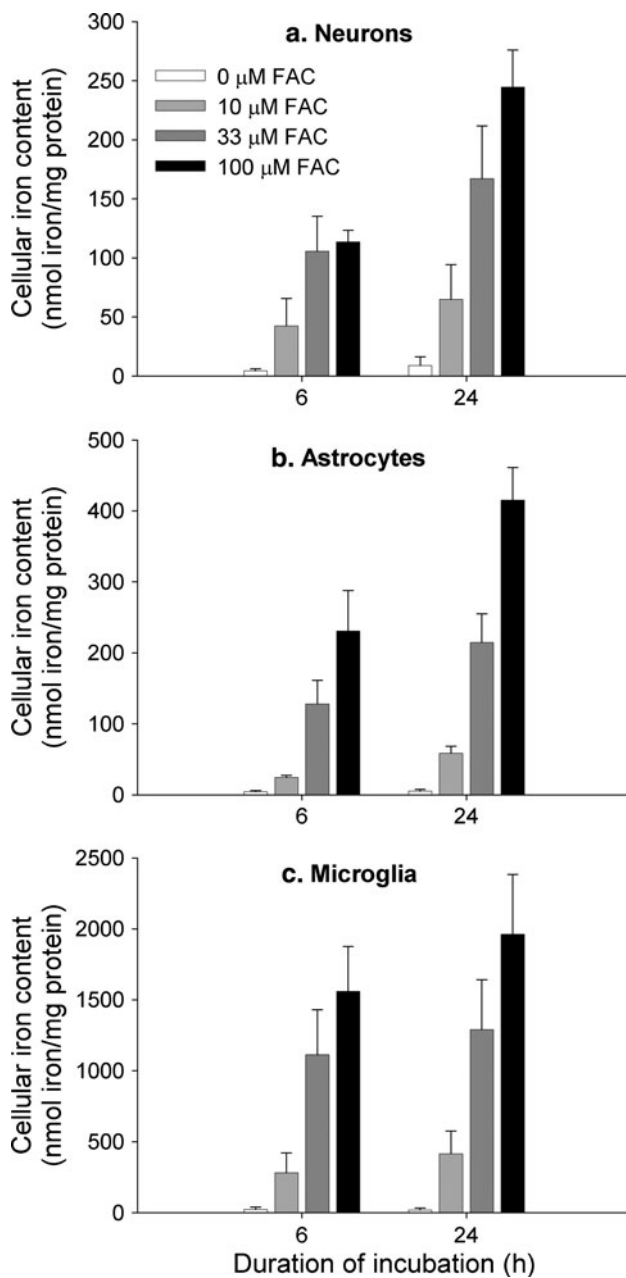
specific iron contents of  $4.4 \pm 4.4$  ( $n = 4$  cultures),  $4.4 \pm 1.0$  ( $n = 4$  cultures), and  $9.3 \pm 6.3$  ( $n = 3$  cultures) nmol iron/mg protein, respectively. To determine the capacity of these cells to accumulate NTBI, cultures of neurons, astrocytes, or microglia were incubated for 6 or 24 h with 0–100  $\mu\text{M}$  FAC. All three types of cell culture were found to accumulate iron in a concentration- and time-dependent manner (Fig. 2). Following 6 h incubation with 100  $\mu\text{M}$  FAC, neurons had accumulated  $113.5 \pm 9.7$  nmol iron/mg protein, astrocytes had accumulated twice as much ( $230.5 \pm 57.4$  nmol iron/mg protein;  $P < 0.05$ ), and microglia had accumulated 13.7-fold as much ( $1559.3 \pm 316.6$  nmol iron/mg protein;  $P < 0.05$ ). A similar difference was observed following 24 h incubation with 100  $\mu\text{M}$  FAC, such that neurons had accumulated  $244.6 \pm 31.4$  nmol iron/mg protein, astrocytes had accumulated  $415.4 \pm 46.1$  nmol iron/mg protein, and microglia had accumulated  $1961.5 \pm 423.1$  nmol iron/mg protein. At this point, astrocytes had accumulated 1.7-fold more iron than neurons ( $P < 0.05$ ), while microglia accumulated 8.0-fold more iron than neurons ( $P < 0.05$ ) and 4.7-fold more iron than astrocytes ( $P < 0.05$ ).



**Fig. 1** Immunocytochemical labeling and Perls' staining of neural cell cultures. Neuron cultures were positively labeled for MAP-2 (a), astrocyte cultures were positively labeled for GFAP (d), and microglial cultures were positively labeled for CD11b (g). Perls'

staining of neurons (b, c), astrocytes (e, f), or microglia (h, i) that had been incubated for 24 h with culture media only (0  $\mu\text{M}$  FAC; b, e, h) or 100  $\mu\text{M}$  FAC (c, f, i)



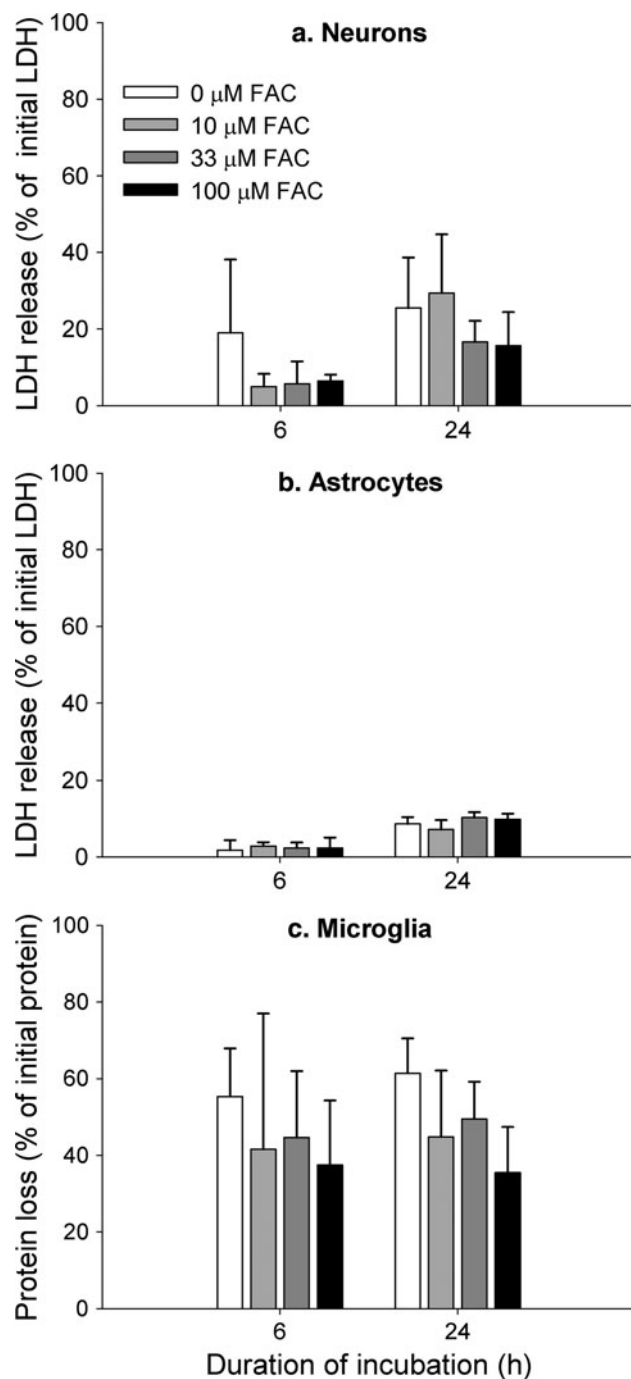


**Fig. 2** Accumulation of iron by neural cell cultures from FAC. Cultures of neurons (a), astrocytes (b), or microglia (c) were incubated for 6 or 24 h with 0–100  $\mu\text{M}$  FAC, and the iron content within the cells was determined and then standardized using the protein content of the culture. Untreated neuron cultures contained  $66.5 \pm 7.1$   $\mu\text{g}$  protein per well, astrocyte cultures contained  $123.8 \pm 20.8$   $\mu\text{g}$  protein per well and microglial cultures contained  $11.3 \pm 0.5$   $\mu\text{g}$  protein per well

Perls' staining was used to visualize the iron within the cultured cells, and to compare the subcellular distribution of accumulated iron. Cultures of neurons or astrocytes that had been incubated for 24 h without FAC displayed very little Perls' reaction (Fig. 1b, e), demonstrating that the low iron content present in cultured cells, while detectable by a

colorimetric assay (Fig. 2), is not detectable by Perls' staining. In comparison, in microglial cultures that had been incubated for 24 h without FAC, a proportion of cells displayed punctuate labeling in the perinuclear cytoplasm (Fig. 1h). When cultures of neurons, astrocytes, or microglia were incubated for 24 h with 100  $\mu\text{M}$  FAC, there was a strong increase in the intensity of Perls' staining (Fig. 1c, f, i), however, there were subtle differences in the distribution of iron in the various cultures. The Perls' reaction in cultured neurons was located within the somata and the neurites of some of the cells in the culture (Fig. 1c). Counterstaining with methylene blue showed that a large proportion of the neurons did not show a positive Perls' reaction, and that there was some non-specific label that was likely due to the poly-D-lysine coating on the coverslips which is essential for neuronal adherence (data not shown). In astrocytes by contrast, the Perls' stain was concentrated in the perinuclear cytoplasm of the somata, with some label also present in the primary processes (Fig. 1f). Counterstaining with methylene blue showed that most, but not all, of the astrocytes were labeled (data not shown). Microglial cultures showed a similar pattern to astrocytes, such that the strongest reaction was present in the somata (Fig. 1i), with an intensity that exceeded both neurons and astrocytes. Microglial cells that had become ramified displayed Perls' reaction in their numerous processes. Methylene blue counterstaining showed that all of the cells in the microglial cultures were intensely stained (data not shown), and the somata were considerably enlarged compared to cultures that had not been treated with FAC. There was a tendency for microglia to cluster following FAC incubation, sometimes giving the impression of a single very large cell.

To determine whether the accumulation of NTBI affects cell viability, cultures of neurons, astrocytes or microglia were incubated for 6 or 24 h with 0–100  $\mu\text{M}$  FAC. The viability of cultured neurons and astrocytes was determined by comparing the amount of LDH activity in the media following the incubation, to that present in the cells prior to the incubation. While the LDH assay provides a more precise estimate of cell viability than protein assays, microglial cultures had a cell density that was too low to permit accurate detection by the LDH assay, so their viability was estimated with the Lowry protein assay. The cellular accumulation of iron was found not to decrease the viability of any cell type (Fig. 3). Incubation with FAC did not increase the release of LDH into the media, when compared to 0  $\mu\text{M}$  FAC (control condition), for cultures of neurons (Fig. 3a) or astrocytes (Fig. 3b). Incubation of microglial cultures with 0  $\mu\text{M}$  FAC caused a substantial decrease in protein content (Fig. 3c), most likely due to a loss of cellular adherence to the culture wells as the cells adopted a motile phenotype. The extent of the protein loss



**Fig. 3** Viability of neural cell cultures following incubation with FAC. Cell viability following 6 or 24 h incubation with 0–100 μM FAC was assessed using the LDH assay for cultures of neurons (a) or astrocytes (b), and by the Lowry protein assay for microglial cultures (c)

was similar after both the 6 and the 24 h incubation, and thus may have occurred in the initial stages of the incubation. Incubation with FAC resulted in a tendency for the protein loss in microglial cultures to decrease at both time points, however, statistical analysis using independent-samples *t*-tests, followed by FDR correction, found that

there was no significant difference in protein loss between control culture wells (incubated without FAC) and those incubated with 100 μM FAC.

## Discussion

The present study compared the capacity of different neural cell types to accumulate NTBI, and examined the consequences of this iron accumulation for cell viability. It was found that neurons, astrocytes, and microglia have the capacity to accumulate NTBI, although the extent of the accumulation differs substantially between these cell types. It was also observed that the accumulation of large quantities of NTBI does not alter cell viability, regardless of cell type. These findings, and their implications for neurological disorders, are discussed below.

The uptake of transferrin-bound iron is principally mediated by the cellular requirement for iron (e.g., Qian and Tang 1995), whereas the uptake of NTBI is largely associated with pathological situations, in which cells remove excess iron from the extracellular space to prevent extracellular Fenton chemistry. In the brain, astrocytes have been considered to be primarily responsible for the uptake of NTBI (Dringen et al. 2007). This view was challenged by the present study in which neurons, astrocytes and microglia were all found to accumulate large amounts of iron from FAC. The fact that Perls' staining was observed within cultured neurons confirms that neurons are able to accumulate NTBI, and are not wholly reliant on glia for defense against iron dyshomeostasis. It should be noted, however, that Perls' staining revealed the presence of many NTBI deposits adherent to the poly-D-lysine coating of the wells used for the neuronal cultures. Such deposits would have substantially inflated the quantitative estimates of neuronal iron content in the current study. It is noteworthy that many of the neurons and some of the astrocytes within the cultures did not display a Perls' reaction after incubation with FAC, whereas others were strongly labeled. This heterogeneity may indicate that some cells accumulate NTBI less rapidly than other cells. Alternatively, the lack of label may be related to the fact that the Perls' stain has a relatively high threshold for the detection of intracellular iron, and hence the unlabeled cells may have contained substantial amounts of iron that nonetheless were below the limit of detection.

Neurons are evidently capable of accumulating NTBI, yet the capacity of astrocytes and microglia is superior, supporting previous suggestions that glial cells play a key role in iron metabolism, and are probably the main sites of NTBI uptake in the brain (Dringen et al. 2007; Koeppen 1995). After 24 h, microglial cells had accumulated approximately fivefold more iron than astrocytes and

eightfold more than neurons. These data indicate that microglia are more effective than astrocytes at accumulating NTBI, and presumably play an important role in defending the brain from pathological conditions that involve the release of NTBI into the extracellular space. Interestingly, microglia were particularly efficient at accumulating iron from low concentrations of FAC, whereas astrocytes were efficient at all concentrations. The direct comparison of uptake rates can be misleading, however, because the opportunity to take up NTBI is likely to be the principal factor that determines the contribution of the various cell types *in vivo*. In this context, it should be noted that astrocytes account for 17.3–20.2% of the total number of brain cells in the neocortex (Pelvig et al. 2008), and they line the blood–brain barrier (Nedergaard et al. 2003), whereas microglia account for only 2.9–6.5% of the total number of brain cells in the human neocortex (Pelvig et al. 2008; Mittelbronn et al. 2001). Furthermore, as individual astrocytes are considerably larger than microglia, astrocytes must occupy much more volume within the cerebral cortex than microglia. Thus, astrocytes will have more immediate access to NTBI during acute iron overload, and their greater cytosolic volume will enable astrocytes to play a more substantial role than microglia in the uptake of NTBI from the extracellular space. Although astrocytes proliferate after cortical injury, microglia do so more readily, and they can be recruited to the site of injury from other locations (Rock et al. 2004). These considerations suggest a division of labor, whereby astrocytes provide most of the protection from NTBI in the critical 24 h following a stroke or traumatic brain injury, and thereafter this role is increasingly assumed by microglia.

The average cytosolic volume of cells in astrocyte-rich cultures has been estimated by the 3-*O*-methylglucose method to be  $4.1 \pm 0.1$   $\mu\text{l}/\text{mg}$  protein (Dringen and Hamprecht 1998). Based on this value, control cultures of astrocytes (not incubated with FAC) had an intracellular iron concentration of  $1.2 \pm 0.6$  mM iron, which is comparable to estimates of total iron in normal mouse cerebrum (approximately 0.43 mM iron; 24  $\mu\text{g}/\text{g}$  wet weight (Malecki et al. 2002)), and of normal human parietal cortex, which vary from approximately 0.54 mM (30.2  $\mu\text{g}/\text{g}$  wet weight (Griffiths et al. 1999)) to 0.68 mM (38.1  $\mu\text{g}/\text{g}$  wet weight (Hallgren and Sourander 1958)). Following 24 h incubation with 100  $\mu\text{M}$  FAC, the intracellular iron concentration of astrocytes increased to  $97.9 \pm 10.5$  mM, while there was  $16.1 \pm 4.0$   $\mu\text{M}$  iron remaining in the culture medium. Thus, astrocytes increased their iron content 80-fold to reach an intracellular iron concentration that was approximately 6,000-fold higher than in the culture media. Such high intracellular concentrations of iron, if present in low molecular weight forms that are redox-active, would cause severe osmotic and oxidative stress. However, the lack of any

detectable loss in viability, despite the high intracellular iron concentration, suggests that the accumulated iron is either bound in a redox-inactive form to intracellular chaperones, or is stored within ferritin. Indeed, it has been reported that cellular iron accumulation by astrocytes from FAC is accompanied by a strong upregulation of ferritin synthesis (e.g., Hoepken et al. 2004), allowing the storage of iron in a redox-inactive form to prevent Fenton chemistry (Halliwell and Gutteridge 2007).

Since the cytosolic volumes of cultured neurons and microglia are not known, the intracellular iron concentration cannot be calculated for these cells. Regardless of this limitation, it is clear that all cell types strongly accumulated NTBI against the concentration gradient over a 24 h period, indicating that NTBI accumulation by neural cells is an active process, which would be ideal for scavenging iron from the interstitial space during pathological conditions associated with iron overload.

The concentration of intracellular iron achieved by astrocytes following 24 h incubation with 100  $\mu\text{M}$  FAC is in considerable excess to that observed in some neurodegenerative conditions associated with pathological iron overload. For instance, in Alzheimer's disease the concentration of iron found in  $\beta$ -amyloid plaques is 0.94 mM (52.5  $\mu\text{g}/\text{g}$  tissue), while in the surrounding neuropil it is 0.69 mM (Lovell et al. 1998). In Parkinson's disease, the total iron content of the substantia nigra is 5.0 mM (280.9  $\mu\text{g}/\text{g}$  wet weight), which is an increase of twofold compared to non-diseased brains (Griffiths et al. 1999). However, in hemorrhagic stroke, the iron content may be much higher since the concentration of iron in whole human blood is on average 10 mM, and a large proportion of this will become unbound as the red blood cells lyse and their hemoglobin degrades (see Robinson et al. 2009; Bishop and Robinson 2001).

Given the very high concentrations of iron achieved by neural cell cultures in the present study, it was surprising that neurons, astrocytes and microglia did not die from this accumulated iron. Our findings are consistent with reports that astrocytes and microglia have considerable resistance to iron toxicity (e.g., Hoepken et al. 2004; Kress et al. 2002; Oshiro et al. 2000; Oshiro et al. 2008), but do not support suggestions that neurons are susceptible to iron-mediated toxicity (e.g., Kress et al. 2002; Oshiro et al. 2008). This difference does not seem to be due to the duration of the incubation period, since we have observed that extending the incubation period to 72 h does not lower the viability of neuronal cultures in response to 100  $\mu\text{M}$  FAC (data not shown). A potential reason for this discrepancy is that the present study examined cerebellar granule neurons, whereas other studies have examined iron toxicity in cultures of hippocampal or cortical neurons. If this is the reason, it would mean that different populations

of neurons have differential susceptibility to iron toxicity, which may be significant given that diseases of iron overload are frequently associated with neurodegeneration in forebrain structures rather than in the cerebellum.

The present study has found that neural cells accumulate NTBI very rapidly and can accumulate iron to levels that are 30- to 100-fold higher than those in untreated cells. This accumulation of iron does not appear to compromise cell viability, indicating that neural cells have a high resistance to the toxicity of iron, at least during the first 3 days after exposure to iron. These data suggest that the iron-mediated toxicity associated with many neurodegenerative conditions could be due to an impairment of iron transport or storage mechanisms, rather than to the presence of elevated levels of NTBI.

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