Modest Amyloid Deposition is Associated with Iron Dysregulation, Microglial Activation, and Oxidative Stress

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Abstract. There is a well-established literature indicating a relationship between iron in brain tissue and Alzheimer's disease (AD). More recently, it has become clear that AD is associated with neuroinflammatory and oxidative changes which probably result from microglial activation. In this study, we investigated the correlative changes in microglial activation, oxidative stress, and iron dysregulation in a mouse model of AD which exhibits early-stage amyloid deposition. Microfocus X-ray absorption spectroscopy analysis of intact brain tissue sections prepared from A β PP/PS1 transgenic mice revealed the presence of magnetite, a mixed-valence iron oxide, and local elevations in iron levels in tissue associated with amyloid- β -containing plaques. The evidence indicates that the expression of markers of microglial activation, CD11b and CD68, and astrocytic activation, GFAP, were increased, and were histochemically determined to be adjacent to amyloid- β -containing plaques. These findings support the contention that, in addition to glial activation and oxidative stress, iron dysregulation is an early event in AD pathology.

Keywords: Alzheimer's disease, iron, microglia, oxidative stress, spectrometry, X-ray fluorescence

INTRODUCTION

Iron plays a pivotal role in many physiological processes, for example in the transport of oxygen, electron transport, and in the synthesis of certain neurotransmitters [1]. Intracellular iron is stored in the cytosolic protein ferritin, a 12 nm diameter 24-subunit protein shell encasing a hollow interior capable of containing

a maximum of approximately 4,500 iron atoms. Iron is normally taken up from the redox active Fe²⁺ state and converted to the less reactive ferric (Fe³⁺) valence state in a ferrihydrite-like hydrated iron oxide [2]. Non-heme iron circulates in the blood mainly in a tight, but reversible, bond with the glycoprotein, transferrin. Blood brain barrier endothelial cells express a specific transferrin receptor (TfR) which facilitates cellular internalization of iron [3]. Ferritin, brain endogenous transferrin and TfR are heterogeneously expressed in different brain cell types and brain regions which further complicates a currently poor understanding of iron release into the brain and its subsequent regulation

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[4]. Recent work has identified that alterations in iron metabolism, due to mutations in genes encoding iron metabolism-related proteins in disorders such as Friedreich's ataxia and neuroferritinopathy, lead to a wide range of debilitating behavioral deficits which are associated with neuronal degeneration [5, 6].

Considerable evidence supports the role of amyloid- β (A β) in the pathogenesis of Alzheimer's disease (AD) [7] and more recent evidence has identified soluble forms of A β as the potential progenitor of several pathological aspects of AD [8]; these include mitochondrial abnormalities [9], deficits in axonal transport [10], alterations in synaptic density [11] and, most interestingly in the context of this study, widespread oxidative stress [12, 13] and robust, persistent microglial activation [14, 15]. Several reports propose that oxidative stress is one of the earliest changes in AD pathogenesis [16, 17]. Complementary work indicating that soluble A β triggers microglial activation [18], and that activated microglia which stain positively for inflammatory cytokines [19] are recruited to newly formed A β plaques [20], suggest these two processes may be integral to subsequent pathology progression.

A long-standing link between aberrant accumulation of iron in the central nervous system (CNS) and neurodegenerative disorders such as AD exists [21]. Reports identifying the presence of iron, copper, and zinc in A β -containing plaques [22–24], in addition to alterations in histochemically-identified patterns of ferritin and transferrin proximal to plaques [25] in end-stage human AD brain tissue, suggest iron accumulation and/or dysregulation may be pathogenic in AD. Recent work has indicated that iron concentrations and redox-active iron were elevated in postmortem brain tissue from patients diagnosed with mild cognitive impairment (MCI), the probable clinical precursor to AD, which is suggestive of dysregulation [26].

The predominant iron oxide in human brain tissue is ferrihydrite-like as found in ferritin cores; in addition there is some evidence of iron oxide deposition as haemosiderin, where the mineral form in tissues is often observed to be goethite-like. Experimental work has demonstrated the presence of another form of iron oxide, biogenic magnetite (Fe₃O₄), in human brain tissue. This form contains alternating lattices of ferrous and ferric ions and was first isolated from human brain tissue by Kirschvink and co-workers [27]. This finding has been replicated in several subsequent magnetometry studies, utilizing the differences in magnetic properties between magnetite and the iron oxides normally found in brain tissue [28]. Recent advances in

the application of microfocus X-ray absorption spectroscopy (μ XAS) have enabled *in situ* localization and characterization of magnetite deposits in cortical senile-plaque-rich AD brain tissue [29]. Electron microscopy techniques have also confirmed the presence of iron oxides with the crystalline properties of magnetite in isolated A β plaque cores [30, 31], and an increased fraction of magnetite in ferritin from the postmortem brain of individuals with AD and supranuclear palsy [31]. It has been hypothesized that the formation of biogenic magnetite may be associated with elevated levels of Fe²⁺ where the latter may stimulate excess production of tissue-damaging free radicals [32, 33]. Furthermore, it has been shown *in vitro* that in neutral oxygenated buffer, under conditions where Fe²⁺ auto-oxidizes to Fe³⁺, a proportion of the iron is stabilized as Fe²⁺ in the presence of aggregating A β ₄₂ [34].

In this study we investigated the presence of various iron oxide forms, and combined this with analysis of microglial activation and oxidative stress, in tissue prepared from a mouse model of AD which exhibits early-stage amyloid deposition. This mouse is engineered to overexpress the Swedish mutation of human amyloid- β protein precursor (A β PP) and presenilin 1 (PS1). Our findings support the hypothesis that iron dysregulation, as determined by μ XAS analysis of brain tissue prepared from a mouse model of AD, is an early event in AD pathogenesis and is associated with microglial activation, oxidative stress and early-stage A β deposition.

METHODS

Animals

Specific pathogen-free (SPF) C57BL/6 mice were purchased from Harlan UK Ltd. (Bicester, UK). Transgenic animals from an existing colony of A β PP^{swe}/PS1^{dE9} transgenic mice (hereafter referred to as A β PP/PS1 transgenic mice), purchased from The Jackson Laboratory (Maine, USA), were used to form breeding pairs with the C57BL/6 mice and bred in an SPF animal housing facility in the Bioresources Unit, Trinity College, Dublin. Mice were maintained according to the regulations and guidelines provided by the local ethical committee. All animals were housed under a 12-h light-dark cycle at an ambient temperature of 22°C–23°C and were maintained under veterinary supervision throughout. Normal laboratory chow and water were freely available to all animals. Female mice, aged 8–9 months, were used in this study. Animal experimentation was performed under a

license granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act 1876 and the European Community Directive, 86/609/EEC, and every effort was made to minimize animal stress.

Surgical equipment and surface metal-particulate decontamination

Ceramic forceps, ceramic tweezers, and a sapphire cryosectioning blade (Electron Microscopy Services, USA) were acid-washed (3% HCl) and rinsed in de-ionized water. All surfaces were plastic, acid-washed (3% HCl), and rinsed in de-ionized water prior to use. All containers with reagents were placed on NdFeB bar magnets and, to ensure that magnetic contaminants were removed and, as a further precaution to avoid magnetic particulate contamination, reagents were always pipetted from the top of the containers.

Intracardial perfusion

All A β PP/PS1 transgenic and wildtype mice used in this study were anaesthetized with an i.p., injection of sodium pentobarbital (30 μ l, Euthanol, Merial Animal Health, UK); the absence of the toe-pinch reflex was indicative of deep anesthesia. Intra-cardial perfusion was performed for 20 min using 0.1 M sodium cacodylate (Sigma-Aldrich, UK) which avoided the introduction of extraneous phosphates and the possibility that they might chelate trace metals of interest from the tissue. The brain was removed using ceramic tweezers and forceps and processed as required. Following brain dissection the intact brain was cut using a sapphire blade and the right hemisphere used for SQuID magnetometry and subsequent ICP-MS analysis. The left hemisphere was retained for molecular, immunohistochemical, and fluorescence analysis.

Immunohistochemistry

A β -containing plaques were visualized using Congo red staining [35]. Briefly, air-dried coronal cryostat sections (15 μ m), obtained from tissue mounted on cork and flash frozen in OCT (Tissue-Tek, Finland), were fixed in ice-cold ethanol for 10 min, washed twice with phosphate-buffered saline (PBS), incubated in an alkaline NaCl solution for 20 min and incubated in a filtered alkaline Congo red solution for 30 min at room temperature. Sections were rinsed in ddH₂O for 30 s and counterstained for 1 min in a 1% (w/v) methyl-green solution (Sigma-Aldrich, UK). Excess staining solution was removed by washing in ddH₂O for 1 min

and sections were dehydrated through a graded alcohol series (75%, 95%, 100%; Sigma, UK). Sections were air-dried and placed in 3 separate xylene baths for 5 min each. Coverslips were placed onto DPX (RA Lamb, UK) covered sections and left to dry in a fume hood for 24 h. For double staining, Congo red-stained sections were washed 3 times with ddH₂O prior to dehydration (5 min/wash). Congo-red stained A β -plaques were counted on coronal brain sections containing the hippocampus ($n = 4$ sections were counted per animal) to determine the average plaque count per brain section ($n = 4$ mice were assessed).

Immunohistochemical assessment of CD68 expression was performed using the avidin–biotin–peroxidase complex reaction in Tris-buffered saline (TBS, pH 7.5). Non-specific interactions were blocked by incubation in TBS (4% bovine serum albumin (BSA); Sigma, UK, 10% normal goat serum (NGS); Vector, UK), for 30 min at room temperature. Sections were incubated overnight in rabbit anti-CD68 antibody solution (1/200 Santa Cruz Biotechnology, US; in 2% BSA in TBS), washed in TBS, incubated in biotin-conjugated goat anti-rabbit secondary antibody, (Vector, UK; 1/200 in 2% BSA in TBS) for 2 h at room temperature and washed. To block endogenous peroxidases, sections were incubated in 0.3% H₂O₂ in TBS for 15 min and after washing were incubated in a pre-made avidin solution. To develop color, washed sections were incubated in diaminobenzidine (DAB solution, 0.06% H₂O₂; Vector, UK) for 10 min. Sections were washed, dehydrated through a series of graded alcohols (75%, 95%, 100%; Sigma, UK), and cleared by immersion in xylene (Sigma, UK). Coverslips were applied using DPX (RA Lamb, UK) as the mount, and slides were left overnight to set in a fume hood.

Immunoreactivity of 8-hydroxy-2' deoxyguanosine (8-OHdG), which assesses oxidative damage to DNA and RNA, was used as a proxy marker of oxidative stress. Sections were washed in ice-cold methanol, rinsed in TBS buffer (pH 7.4) and incubated sequentially in 4% HCl for 7 min, and in 50 mmol/L Tris base (denaturation and neutralization respectively). Sections were incubated for 30 min at room temperature in 10% normal horse serum (Vector, UK) and 4% BSA (Sigma, UK) in TBS, to block nonspecific interactions and then incubated (overnight at 4°C) in goat anti-8-OHdG antibody (1/1000 in 2% BSA; Abcam, UK). Sections were rinsed, and incubated in biotin-conjugated horse anti-goat antibody (1/200 in 2% BSA in TBS; (Vector, UK)), rinsed, incubated in 0.3% H₂O₂ in TBS for 15 min and washed. Sections

were incubated in avidin-biotinylated peroxidase complex solution (ABC kit, Vector, UK) for 1 h and DAB solution (Vector, UK; 2 μ l H₂O₂/mL) was applied for 10 min. Sections were rinsed in distilled water, dehydrated through a series of graded alcohols (75%, 95%, 100%; Sigma, UK), cleared in xylene, and mounted in DPX (RA Lamb, UK) and left to set overnight in a fumehood.

Air-dried cryostat sections (15 μ m) prepared from the brains of A β PP/PS1 transgenic and wildtype mice were assessed for A β immunoreactivity. To permeabilize the tissue, sections were fixed in ice-cold methanol for 10 min, washed in PHEM, PHEM containing 0.1% Triton (Sigma-Aldrich, UK) and again in PHEM. Non-specific binding was blocked by incubating samples in 4% BSA containing 10% NGS for 2 h at room temperature. Sections were incubated in the presence of the primary antibody (1 : 1000, Pan-Amyloid (A β ₄₂) antibody, Calbiochem, US) in 2% BSA containing 5% NGS overnight at 4°C, washed in PHEM and incubated in the presence of the secondary antibody (1 : 4000 in 2% BSA containing 5% NGS; 90 min at room temperature; ALEXA 488, Invitrogen, US). The sections were washed, mounted with DAPI-containing mounting medium (Vectashield, Vector Labs, USA), dried for 24 h and visualized by confocal microscopy (Axioplan 2, Zeiss, Germany). In some cases, A β -stained sections were washed and the staining process repeated with a primary antibody for MHC II (1 : 1000, MHC II antibody, Calbiochem, US) as previously described [36].

mRNA analysis (CD11b, CD68, IL-1 β , and GFAP mRNA)

RNA was extracted from CNS tissue, encompassing the hippocampus and cortex, using a NucleoSpin RNAII isolation kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. RNA concentrations were equalized to 1 μ g before cDNA synthesis using a High Capacity cDNA RT Kit (Applied Biosystems, Germany) according to the manufacturer's protocol. Equal concentrations of cDNA were used for Real-time (RT)-PCR amplification. RT-PCR primers were delivered as "Taqman® Gene Expression Assays" as previously described [37], and the assay IDs for the genes assessed were: CD11b (Mm01271263_m1), CD68 (Mm03047343_m1), IL-1 β (Mm00434288_m1), and GFAP (Mm01253033_m1). Target gene expression was calculated relative to the endogenous control (β -actin) to give a relative quantification (RQ) value ($2^{-\Delta\Delta CT}$, where CT is threshold cycle).

SQuID magnetometry

All equipment and surfaces used in this procedure were treated to remove metal particulate contamination as outlined above. Tissue was prepared for SQuID magnetometry as previously described [38]. Following freeze-drying, a single wildtype sample was removed from the study as it exhibited a 45% decrease in weight, compared with $77 \pm 5.2\%$ for all other samples. Samples were fragmented using a sapphire blade and tightly packed into the centre of a SQuID sample straw (Quantum Design, CA, USA) using custom-made non-metallic presses and assessed using a MPMS 7 SQuID magnetometer (Quantum Design, CA, USA). A sample degaussing sequence was carried out at 300 K, and then the isothermal remanent magnetization (IRM) was measured at 5 K and 150 K following exposure to a field of 1 Tesla; this field is sufficient to saturate magnetite ensuring the IRM at 150 K is dominated by magnetite as ferrihydrite contributes to IRM at 5 K only. Eight measurements of the IRM were obtained directly after the field had been removed, and the last five measurements (where the initial decay had stabilized) were averaged to obtain the IRM value for each sample. A negative control containing no sample was prepared, assessed using an identical protocol, and used for blank subtraction purposes.

ICP-MS

The samples analyzed using SQuID magnetometry were assessed for metal content using an Varian 820 ICP-MS (Varian, USA). A known weight of each sample was digested in a 7 : 1 ratio of 69% HNO₃ and 35% H₂O₂ heated to 200°C at 1000 W for 20 min (EZ digester; Milestone Scientific, US) and brought to 50 ml with ultrapure water (Sigma-Aldrich, UK). Data were acquired in peak-hopping mode with 15 scans per replicate and 3 non-consecutive replicates per sample. A sample containing only the digested reagents was used for blank subtraction purposes.

X-ray Absorption Spectroscopy analysis

Tissue preparation

All equipment and surfaces used in this procedure were treated to remove metal particulate contamination as outlined above. Following brain dissection, coronal cryosections (50 μ m) from an A β PP/PS1 transgenic and wildtype mouse brain were taken using a sapphire cryomicrotome blade (Electron Microscopy Services, USA) on a cryostat (Leica CM1900, Leica Microsystems Inc, USA) that was not used for 2 days beforehand

to minimize air-borne dust particles. A single section from each animal was placed onto a high quality quartz microscope slide (H Baumbach & Co. Ltd., UK) and stored at -80°C in a parafilm wrapped slide box. Air-dried cryostat sections were mounted for room temperature microfocus X-Ray Fluorescence (μXRF) and microfocus X-ray Absorption Near Edge Spectroscopy (μXANES) analysis. A layer of kapton film ($25\text{ }\mu\text{m}$ thick; Electron Microscopy Services, USA) covered the tissue sections during measurements with an airtight epoxy seal joining the kapton film to the quartz slide.

Microfocus X-Ray Fluorescence (μXRF) acquisition and analysis

High resolution μXRF mapping was carried out on the I18 beamline at Diamond Light Source [39]. Sections were screened for signal from a range of metal elements by measuring μXRF intensity above the K-edge for iron (7112 keV), using a primary beam of 10 keV . μXRF spectra were captured at a rate of one second per pixel, following stepped sample rastering on an x-y stage, with fluorescence signal detected using a high rate fluorescence 9 element Ge solid state detector system. Large area maps were obtained at $60\text{ }\mu\text{m}$ resolution and off-site analysis was carried out in the PyMCA software program [40] where data were normalized to incoming beam intensity. Linear combination fitting of the individual fluorescence peaks in each spectrum enabled the contributions from major peaks, including Fe, Cu, and Zn to be calculated, and the contributions from smaller peaks (e.g., Mn) to be identified. Intensity maps for each element of interest were computed from the fitted spectra, determining signal intensity (and the standard deviation obtained from the fit quality), for the chemical elements in each pixel. In order to allow comparison of relative concentrations of elements between selected areas in a tissue section, the intensity spectrum was normalized to the number of pixels in each selection. Slide regions without tissue were used to obtain the background spectrum, so that this could be subtracted from the main spectra to obtain the true signal from the tissue. Potassium concentration within the brain region under investigation was used to give an approximate indication of sample thickness, as the distribution was more uniform than for the other elements observed, and any potassium in the quartz slide and coverslip was below detection levels. The potassium maps were useful in image analysis and correlation with the light microscopy images, as they allowed features such as folds, tears, and voids in the tissue sections to be clearly identified. Subsequent staining of the sections

with Congo red made it possible to compare relative metal ion concentrations in those regions with extensive A β deposition, and those lacking Congo-red-positive material. During the same experiment, areas exhibiting high iron fluorescence were subjected to μXRF -mapping at a higher resolution of $5\text{ }\mu\text{m}$ (these data were processed as outlined above) and sites within these areas were chosen for μXANES analysis (these areas were not viable for Congo-red staining).

Microfocus X-ray Absorption Near Edge Spectroscopy (μXANES) acquisition and analysis

μXRF -mapping ($5\text{ }\mu\text{m}$) of areas exhibiting high iron fluorescence on the $60\text{ }\mu\text{m}$ XRF maps enabled identification of sites exhibiting focal increases in iron fluorescence. These were chosen for μXANES characterization and data were obtained through the K-edge from 6960 to 7090 eV in steps of 5 keV with a dwell time of 3 s , from 7090 to 7145 eV in steps of 0.5 keV with a dwell time of 7 s and from 7145 to 7450 eV in gradually increasing steps of 1.0 to 3.0 keV with a dwell time of 3 s . A single spectrum was taken at each site identified for XANES analysis, with spectra taking approximately 30 min to acquire. Previous studies utilizing the same experimental protocol and setup have identified unchanged XANES spectra from sites subjected to repeated acquisition; and dispersed nanoparticle iron oxide standards with a variety of redox states have been successfully characterized: in this context, beam induced redox changes are thought to be negligible. A reference trace from an iron foil (positioned well away from the sample) was obtained in parallel with each XANES measurement in order to provide confirmation of the edge energy. μXANES data were analyzed using the IFEFFIT Athena software package [38]. Briefly, fluorescence intensity was first normalized to the incident beam intensity at each measured point. Background removal was performed following the standard procedure in Athena, with line regression to the pre-edge range (-100 to -20 eV), and polynomial regression to the post-edge range (50 to 350 eV). The components of each μXANES spectrum were fit with a set of standards measured under the same experimental conditions (including ferrihydrite-containing ferritin, haematite, dispersed magnetite nanoparticles, and dispersed maghemite nanoparticles), using Athena's linear combination fitting routine that enables the relative proportions of fitted standards to be compared between spectra [41]. Representative examples of μXANES data are presented in Fig. 5, with a subset of the energy range assessed plotted (7080 – 7180 keV). Subsequent Congo red staining of the areas subjected to μXANES and μXRF -mapping

at a resolution of 5 μm was not possible due to tissue fragility.

μXAS statistical analysis and sampling

A single coronal brain section, at approximately -2.30 mm Bregma, prepared from an 8-9 month old A β PP/PS1 transgenic mouse was assessed using 60 μm -resolution XRF-mapping; tissue subjected to only this analysis was viable for subsequent Congo red staining. Colocalization of areas of iron deposition identified on the 60 μm XRF map and congo-red positive staining were identified in the cortex ($n=3$) and the hippocampus ($n=2$). These areas were assessed for the relative increase in iron, copper, and zinc by comparison to adjacent regions. Thus normalizing the data to concentration variability due to brain region and cell type; Fig. 4a–d contains a representative example of colocalization in the hippocampus while Fig. 4e presents all colocalization data. Within the same brain section 2 areas (hippocampal CA3 subfield and dorsal retrosplenial cortical regions) were XRF-mapped at a 5 μm resolution identifying 4 areas which were then subjected to μXANES analysis (these areas were not viable for Congo red staining). To maintain consistency with the 60 μm XRF maps obtained from the hippocampus provided in Fig. 4, the data presented in Fig. 5 were obtained from the hippocampal CA3 subfield.

RESULTS

The total iron concentration and IRM of a single brain hemisphere is not altered by the amyloid deposition associated with 8-9 month old A β PP/PS1 transgenic mice

Immunohistochemical assessment of brain sections prepared from 8-9 month old A β PP/PS1 transgenic and wildtype mice using Congo red confirmed the presence of A β -containing plaques (Fig. 1a, b); no plaques were observed in brain sections prepared from wildtype mice. Average plaque number per coronal brain section prepared from transgenic mice was 14.45 ± 1.6 per section. This is in line with previous studies at the same timepoint and is modest relative to the extensive levels of deposition reported in older mouse models of AD [42–44]. No significant change in brain tissue iron concentration was observed in A β PP/PS1 transgenic, compared with wildtype, mice (Fig. 1c); copper and zinc concentration were also similar in both groups (data not shown). Analysis of IRM data, obtained from brain tissue prepared from A β PP/PS1 transgenic and

wildtype mice, revealed no significant differences at 5 K or 150 K (Fig. 1d). The signal at 5 K is predominantly associated with ferrihydrite-like ferritin cores which are usually not blocked above 20–30 K, and the trace signal at 150 K is only slightly greater than that from the measured blank. The iron concentration and IRM data are in agreement, and no difference between the A β PP/PS1 and wildtype mice can be determined on the basis of these bulk measurements.

Modest amyloid deposition is associated with an increase in markers of microglial and astrocytic activation and oxidative stress

Markers of microglial and astrocytic activation were assessed using RT-PCR and the data indicate that the expression of CD11b, CD68, IL-1 β , and GFAP mRNA was significantly increased in tissue prepared from A β PP/PS1 transgenic, compared with, wildtype mice ($**p < 0.01$; $***p < 0.001$; Student's t -test for independent means, Fig. 2). Immunohistochemical analysis confirmed microglial activation by revealing increases in MHC II (Fig. 3b) and CD68 (Fig. 3c–e) in tissue prepared from A β PP/PS1 transgenic mice compared with wildtype mice. Staining for both markers was enhanced proximal to plaques. Increased 8-OHdG immunoreactivity proximal to A β -containing plaques was also observed (Fig. 3f); this indicates oxidative damage to DNA and/or RNA and is therefore a marker of oxidative stress. These data demonstrate that A β PP/PS1 transgenic mice exhibit neuroinflammatory and oxidative changes at a relatively early age and that these accompany the development of A β -containing plaques.

Iron levels are increased in tissue associated with A β -containing plaques

μXAS spectral analysis was combined with immunohistochemical analysis to establish whether areas of high iron deposition were spatially associated with A β -containing plaques;¹ this analysis was performed on 60 μm resolution XRF maps only. The

¹ A small number of isolated pixels included highly localized intense concentrations of calcium. As similar intensity of calcium inclusions were observed when mapping background regions, these pixels were omitted from the analysis. It has since been confirmed with further XRF mapping at the same beamline that although no XRF-detectable trace metals are present in the quartz slides, the Kapton coverslips contain a small number of calcium inclusions. Although areas that coincided with calcium inclusions were automatically omitted from the analysis as a precaution, no other trace metals were detectable in or on the Kapton.

Fig. 1. Brain tissue prepared from AβPP/PS1 transgenic and wildtype mice display no difference in total iron content or IRM signal. Representative micrographs confirm Congo red-positive Aβ-containing plaques in brain sections prepared from AβPP/PS1 transgenic mice in the hippocampus (a) and cortex (b). No plaques were observed in tissue prepared from wildtype mice (insets; $\times 10$ magnification; scale bar = 250 μ m). c) Total iron concentration was similar in tissue prepared from AβPP/PS1 transgenic and wildtype mice. IRM measured at 5 K and 150 K (d) in tissue prepared from AβPP/PS1 transgenic and wildtype mice, following exposure to a magnetic field of 1 Tesla, was similar (IRM data are expressed as μ emu per grams of iron per mg of brain tissue (dry weight)). Data are expressed as means $\pm$ SEM ($n = 4$ or 5).

computed iron map of the CA2 field of the hippocampus is presented (Fig. 4a) with the highlighted area enlarged (b) to allow a comparison with Congo red staining of the same tissue sample (Fig. 4c). The elemental composition in the area co-localized to Aβ-containing plaques (labeled 1 in (b) and (c)) was compared with background levels in the tissue (dotted boxed area) and a notable increase in iron ($112 \pm \%$), but not copper or zinc, was observed (Fig. 4d). μ XRF spectra and peak analysis of all areas of Aβ-containing plaque deposition analyzed in the brain section, including CA1, CA2, and auditory cortex (number of areas sampled = 5; Fig. 4e), indicated that elevated concentrations of iron in these regions did not consistently co-localize with elevations or decreases in copper and zinc relative to background tissue levels (Fig. 4e). Potassium was found to be a good general indicator of

section thickness, and allowed us to confirm that local variations in iron concentration were not attributable to significant variations in sample thickness.

Mixed-valence iron oxides are present in brain tissue associated with high iron deposition

μ XRF microfocus maps identified areas of high iron deposition which were subjected to μ XANES analysis to determine which iron oxide forms were present. These data were collected in the same session and prior to any histological analysis of the tissue. However, histological analysis of areas subjected to μ XRF mapping at 5 μ m and XANES analysis was not possible. An analysis of two areas in the CA2 subfield of the hippocampus is presented (Fig. 5a, b). μ XANES spectra from the ferritin and magnetite standards are plotted

Fig. 2. Markers of microglial and astrocytic activation are increased in brain tissue prepared from AβPP/PS1 transgenic, compared with wildtype, mice. CD11b, CD68, IL-1β, and GFAP mRNA expression was significantly increased in brain tissue prepared from AβPP/PS1 transgenic, compared with wildtype, mice (** $p < 0.01$; *** $p < 0.001$; Student's t -test for independent means; CD11b, t -value = 4.929, $df = 7$; CD68, t -value = 10.06, $df = 7$; IL-1β, t -value = 4.512, $df = 7$; GFAP, t -value = 4.583, $df = 7$). Data are expressed as a ratio of CD11b, CD68, IL-1β, GFAP mRNA:β-actin mRNA, and are means $\pm$ SEM ($n = 4$ or 5).

of maghaemite or hematite-like oxides. The component proportions indicated by the fit (in this instance, of ferritin and magnetite) enable comparisons of relative, but not absolute, iron oxide concentrations to be made between sites. Identical analysis performed on area of high iron deposition in the dorsal retrosplenial cortex obtained similar results (data not shown). These findings indicate that magnetite may be co-localizing with ferritin in areas of high iron deposition in brain tissue prepared from an AβPP/PS1 transgenic mouse. It cannot be confirmed if the regions characterized by μ XANES co-localized with Aβ-containing plaques as these regions were rendered fragile by 5 μ m resolution μ XRF-mapping and μ XANES analysis, and subsequent staining of these local tissue regions was not possible.

DISCUSSION

We provide evidence indicating the presence of mixed-valence iron oxides, activated microglia, and oxidative stress in a mouse model of AD at a timepoint where amyloid deposition was modest, suggesting these changes occur relatively early in the time-course of the AD-like pathology replicated by this mouse model.

This is the first demonstration of the presence of magnetite in a mouse model of AD and it was seen to co-localize with areas of high iron deposition, although it was not possible to determine if these deposits were intracellular or extracellular. The findings indicate that areas of high iron deposition were spatially associated with Aβ-containing plaques and this suggests that magnetite was also associated with plaques. However we were unable to directly demonstrate co-localization of magnetite with Aβ deposition due to localized tissue fragility resulting from extended high-resolution μ XAS analysis. The present data are in agreement with previous findings which identified the presence of magnetite in areas displaying high iron concentration and within Aβ plaque cores in human AD tissue [29, 30] and, interestingly, redox-active iron deposits have been identified by immunohistochemistry [45] in brain tissue obtained from MCI patients [26]. Optical microscopy accuracy enabled identification of these deposits as being spatially associated with GFAP-positive immunoreactivity, with intracellular and extracellular deposits being observed. Inspection of the published micrographs suggests that their average size is between 5 and 20 μ m which correlates with the deposits observed in the present study. These

beside Area 2 to enable visual comparison of the spectra (Fig. 5c). μ XANES spectra from areas 1 and 2 were linear-combination-fitted with the full set of standards to look for evidence of iron oxide inclusions other than ferritin [29]. Results are presented as a percentage weighting and can be considered a good indication of relative proportions to $\pm 5\%$. A plot of the linear combination fit data generated for Area 2 is provided for inspection (Fig. 5d). The μ XANES spectra from areas 1 and 2 fit well with a combination of magnetite (16% and 26%, respectively) and ferritin (84% and 74%, respectively; Fig. 4e). The analyses indicate which specific iron oxides are most consistent with the measured XANES. In the traces obtained from the AβPP/PS1 tissue, there was no evidence for significant components

Fig. 3. Increase in markers of microglial activation and oxidative stress proximal to A β -containing plaques. (a) and (b) A β -containing immunofluorescent plaques (green), in a brain section prepared from an A β PP/PS1 transgenic mouse at 2 magnifications (a; $\times 40$; b $\times 63$; scale bar = 50 μ m), are encircled by cell nuclei (stained blue with DAPI). MHC II immunofluorescence (red) is expressed in cells proximal to the plaques. c-e) CD68 staining is markedly increased in a representative brain section prepared from an A β PP/PS1 transgenic, (d), and the enlargement of the region of interest, (e), compared with a wildtype, mouse (c; magnification $\times 40$). f) Increased 8-OHdG immunoreactivity (brown staining) is identified proximal to a Congo red-positive A β -containing plaque (pink) in a brain section prepared from an A β PP/PS1 transgenic mouse (magnification $\times 40$).

deposits may precipitate plaque formation; however, the reported association with astrocytes is puzzling considering the low levels of ferritin observed in astrocytes [1, 26]. It would be of value in the future to extend this analysis to larger cohorts, in order to establish the extent to which mixed valence iron oxide is formed in regions of amyloid deposition in AD mouse

models. This is significant not only for testing the degree to which isolated *in vitro* mechanisms are relevant *in vivo* [34], but also for establishing the extent to which related observations in human *ex vivo* AD tissue are replicated in mouse models of AD [30].

Iron dysregulation is considered to be an early event in the pathogenesis of AD [26] and some groups have

Fig. 4. Areas of high iron concentrations co-localize with Aβ-containing plaques. mXRF spectral analysis identifies iron deposition in the hippocampus of a brain section prepared from an AβPP/PS1 transgenic mouse. An μXRF iron map (a; scale bar = 1200 μm, resolution = 60 × 60 μm) identifies the area under investigation, an enlarged image (b; scale bar = 300 μm) of the inset from (a) identifies an area of high iron deposition (labeled 1) and an area in the CA2 subfield of the hippocampus (boxed area, dotted line). The pixel intensity represents iron fluorescence (normalized to incident beam) and is proportional to total iron present. The white pixels are those exhibiting strong elevations of calcium typical of those identified in the Kapton cover, and are therefore discounted. c) Subsequent Congo red staining of the sample identified Aβ-containing plaques and the area marked 1 corresponds with that marked 1 in (b), showing spatial correlation of Aβ-containing plaques with high iron concentration (×10 magnification; scale bar = 200 μm). To aid effective comparison between (b) and (c) the lateral blade of the dentate gyrus (DG) and the pyramidal layer of the CA3 field (CA3) are identified on both. d) The μXRF spectra analysis displays the change in the fluorescence intensity of several elements in area 1 relative to the boxed area. Spectra associated with the K_α edge for Iron (Fe), Copper (Cu), and Zinc (Zn) are shown (with the K_β edge for iron also identified). e) The fluorescence intensity change (relative to a local background) for all areas of tissue, with Aβ-containing plaque deposition, analyzed; Fe, Cu, and Zn changes are shown ($n = 5$; areas analyzed include hippocampal (CA2 and CA3 subfield areas) and cortical (Cx1, Cx2 and Cx3) amyloid deposits). A magnified version of the Cu and Zn portion of the radar plot is also provided. The radar plot center represents a 100% decrease in fluorescence intensity, with the radar plotlines away from the center indicating a 0–500% increase in fluorescence in 100% steps (relative to a local background in all instances).

Fig. 5. XANES analysis indicates the presence of mixed valence iron oxides in areas of high iron deposition. Microfocus XAS analysis was undertaken in areas of high iron deposition in the CA2 subfield of the hippocampus of a brain section prepared from an A β PP/PS1 transgenic mouse. Composite Congo red and toluidine blue light microscopy (a), microfocus iron μ XRF map (b), XANES spectra for Area 2 and magnetite and ferritin XANES standards (c), XANES spectra linear combination plot for Area 2 are presented (d) and results for Area 1 and 2 shown in (e). The microfocus iron map (b) location is identified in (a) and the areas chosen for XANES analysis labeled in (b). a) Light micrographs in composite image were taken at $\times 2.5$ magnification, scale bar = 1200 μ m. b) Microfocus map pixel resolution = 5 μ m; scale bar = 50 μ m. The pixel intensity represents iron fluorescence (normalized to incident beam) and is proportional to total iron present. (inset; micrograph taken at $\times 20$ magnification, scale bar = 100 μ m).

suggested that it may actually trigger aspects of AD pathology [13, 46, 47]. The evidence indicates that deficits in iron regulation may lead to mitochondrial dysfunction which is identified as an early change in AD [48] and it is interesting that expression of the haemochromatosis protein, which is a hallmark of iron-overload disorders [49], is upregulated in reactive astrocytes and neurons in the AD brain [50].

Whereas our data show that iron was associated with A β -containing plaques, no association for copper or zinc was observed in this study of a transgenic mouse model, however, several groups have reported

increases in copper and zinc levels in A β -plaques in human tissue from end-stage AD patients [22, 23]. No difference in brain tissue concentrations of iron, copper, or zinc were found in the present study. However at 18 months of age, a female mouse model of AD was reported to display a reduction in copper, an increase in iron, and no change in zinc, suggesting that metal ion concentrations may change as pathology progresses [51]. More recent studies have identified a link between zinc transporter proteins and AD; aberrant patterns have been identified in MCI brain tissue [52] and the presence of zinc transporter proteins has been

identified in A β -containing plaques in postmortem brain tissue prepared from AD patients [53]. Although changes in metal concentration may occur in late-stage pathology [54], the evidence from the literature indicates that the dysregulation of iron, and possibly zinc, precede such changes [26, 55]. Although in this study there are no clear differences in the bulk iron concentration and magnetic properties of the wild type and A β PP/PS1 brain tissue, the synchrotron μ XAS analysis has enabled the location and identification of magnetite in regions exhibiting focal increases in iron deposition, highlighting the advantage of examining iron dysregulation at a microscopic scale.

The presence of magnetite suggests dysregulation of normal iron storage and we show that 8-OHdG immunoreactivity, which is indicative of oxidative stress, was increased in tissue proximal to A β -containing plaques. There are several reports of lipid peroxidation occurring prior to amyloid plaque formation [56], while several others confirm oxidative stress damage is associated with plaques [57]. Oxidative stress is also known to upregulate A β PP and β - and γ -secretases [58] and the possibility exists that iron may directly influence plaque formation by modulating A β PP processing. Previous reports indicating magnetite association with A β -containing plaques has led some groups to suggest A β redox chemistry may be ongoing within the plaque itself [30]. Interestingly iron, but not zinc, accelerates A β ₄₂ beta-pleated sheet formation *in vitro* [59] and the presence of aggregating A β ₄₂ has been shown *in vitro* to stabilize a proportion of reactive ferrous iron that in the absence of the A β ₄₂ would auto-oxidize to the more stable ferric form, creating an environment that may lead to the generation of excess radical species [34]. It has also been suggested that redox active iron may mediate the neurotoxicity of A β [60], and several reports suggest interactions between iron and A β may ultimately be neuroprotective if they lead to redox-silencing of iron [61–63]. We observed that the increase in oxidative stress was associated with evidence of activated microglia, identified by increases in expression of CD11b and CD68 mRNA levels and increased CD68 and MHC II immunoreactivity. These findings are consistent with the well-accepted view that activated microglia are primarily responsible for producing oxygen radicals [64] and while the mixed-valence iron oxide deposits may be indicative of disrupted iron handling, and locally increased concentrations of ferrous iron, there is no direct evidence to link the resulting magnetite deposits identified in this study with oxidative damage. The increase in CD68, which is a marker of lysosomal

activity may indicate that microglia are phagocytic [65] but current literature suggests that minimal phagocytosis of plaques occurs [20]. It is possible the CD68-positive cells are involved in the phagocytosis of cellular debris adjacent to A β -containing plaques or perhaps smaller A β deposits.

Activated microglia are considered to be the primary source of inflammatory cytokines, although release from astrocytes also occurs [66]. Here we show that the increase in markers of microglial activation is accompanied by increased GFAP mRNA expression, which is an indicator of astrocytosis, and with a marked increase in IL-1 β mRNA in tissue prepared from A β PP/PS1 transgenic mice. This supports previous work identifying astrocyte activation in mouse models of AD [44] and indicating a close relationship between proinflammatory cytokines and plaque deposition [67]. However the role of IL-1 β in AD pathology is complex. For example, it has been reported that overexpression of IL-1 β leads to reduced plaque deposition in a mouse model of AD [68] but that IL-1 β increases A β PP synthesis and processing [69] and increases in proinflammatory markers, including IL-1 β , are found in the AD brain in patients from different disease stages [70].

We set out to assess whether the early stages of A β plaque deposition were associated with evidence of changes in the handling of iron by cells. Our findings suggest the presence of deposits of a mixed-valence iron oxide proximal to A β -containing plaques in A β PP/PS1 transgenic mice. The co-localization of magnetite and deposits containing normal ferrihydrite-like ferritin cores is consistent with former studies in human tissues indicating altered iron storage in AD ferritin, AD plaques, and plaque-rich AD tissues. Interestingly this observation in the A β PP/PS1 transgenic mouse was coupled with evidence of microglial activation accompanied by evidence of oxidative stress. We propose that this may contribute to the dysregulation in iron handling and may lead to progression of pathology.

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