Impact of autophagy and ageing on iron load and ferritin in *Drosophila* brain

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Abstract

Biometals such as iron, copper, potassium and zinc are essential regulatory elements of several biological processes. The homeostasis of biometals is often affected in age-related pathologies. Notably, impaired iron metabolism has been linked to several neurodegenerative disorders. Autophagy, an intracellular degradative process dependent on the lysosomes, is involved in the regulation of ferritin and iron levels. Impaired autophagy has been associated with normal, pathological ageing and neurodegeneration. Non-mammalian model organisms such as *Drosophila* have proven to be appropriate for the investigation of age-related pathologies. Here, we show that ferritin is expressed in adult *Drosophila* brain and that iron and holoferritin accumulate with ageing to the same extent in both autophagy-deficient and wild-type fly brains. To further investigate the level and state of iron in the brain, we made use of synchrotron X-ray fluorescence imaging (μXRF) and spectroscopy (μXANES). The synchrotron analysis confirmed age-dependent accumulation of iron in wild-type and autophagy-deficient *Drosophila* brain, alongside evidence of an additional iron phase in autophagy-deficient fly brains which may arise from altered mitochondrial iron homeostasis.

1 Introduction

Iron is an essential biometal, widely used as a cofactor by a variety of proteins. Imbalance in iron metabolism, either a deficiency or excess of iron may have harmful effects, and impaired iron metabolism may be a modulator of neurodegeneration in several genetic or sporadic neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis (Ward et al., 2014;Angelova and Brown, 2015;Biasiotto et al., 2016).

Ferritins are iron storage proteins. Two types of subunits, the heavy (H) and light (L) chains, assemble in different ratios into 24-subunit heteropolymers, in which iron can be stored in a mineralized form. Expression of both H and L ferritin chains are closely related to iron bio-availability (Gray and Hentze, 1994). Like in mammals, *Drosophila* genome encodes two types of subunits, known as heavy-chain homologue (Fer1HCH) and light-chain homologue (Fer2LCH) (Georgieva et al., 2002;Nichol et al., 2002;Hamburger et al., 2005). A mitochondrial ferritin subunit was lately identified in both mammals and insects (Levi et al., 2001;Missirlis et al., 2006). On the contrary to other ferritins, mitochondrial ferritin assembles as homopolymers (Levi et al., 2001). When cells exhibit an iron deficiency, iron can be released from the ferritin. However, an excess of free iron may cause substantial damage to lipids, DNA and proteins through the generation of highly reactive hydroxyl radicals (Zecca et al., 2004b;Angelova and Brown, 2015). Therefore, strict regulation of iron storage is essential to maintain cellular homeostasis and integrity. *Drosophila* has been successfully used as a model to evaluate the impact of iron storage deregulation on cell physiology and animal behaviour. Notably, iron metabolism has been linked to circadian rhythms (Freeman et al., 2013;Rudisill et al., 2019), the autosomal recessive disease Friedreich's ataxia (Navarro et al., 2015;Soriano et al., 2016), neurodegenerative diseases and age-associated defects (Xun et al., 2008;Rival et al., 2009;Kosmidis et al., 2011;Tang and Zhou, 2013;Kosmidis et al., 2014).

Cells use two main cytosolic degradative processes: the ubiquitin-proteasome pathway (UPS) and the autophagy-lysosomal pathway. While the UPS is specialized in the degradation of monomeric, short-lived proteins; autophagy has the potency to degrade large protein complexes and organelles (Korolchuk et al., 2010;Nam et al., 2017). Autophagy is divided into three different processes that differ by the way substrates are being delivered to the lysosome for degradation. Chaperone-mediated autophagy and microautophagy are defined by their ability to transfer proteins directly to the lysosomes through pores or membrane invagination respectively (Tekirdag and Cuervo, 2017). However, macroautophagy (referred to as autophagy) requires the isolation of cytoplasmic content into double-membraned autophagosomes that eventually fuse with the lysosomes (Yin et al., 2016). The molecular components involved in autophagy progression are highly conserved among Eukaryotes and most of these proteins have orthologues in *Drosophila* (Mulakkal et al., 2014;Bhattacharjee et al., 2019). The complexes of Atg (Autophagy) proteins regulating the formation of autophagosomes are well conserved and characterised. One essential component of this machinery is the protein Atg8a (LC3 in mammals), which is cleaved and lipidated before anchoring into the autophagosomal membrane (Nagy et al., 2015). Atg8a is essential to the recruitment of other components of the autophagic machinery, as well as for the selection of receptors and their cargoes for selective degradation (Alemu et al., 2012;Wild et al., 2014;Schaaf et al., 2016). The best known selective cargo receptor in *Drosophila* is Ref(2)P (homologous to mammalian p62/SQSTM1) (Nezis et al., 2008;de Castro et al., 2013;Bartlett et al., 2014;Nagy et al., 2014). Selective autophagy can also contribute to the regulation of ferritin turnover (Hou et al., 2016;Gatica et al., 2018). The selective degradation of ferritin by autophagy is referred to as ferritinophagy and requires the cargo receptor NCOA4 in mammals (Mancias et al., 2014;Mancias et al., 2015); no homologous receptor has been yet identified in *Drosophila*.

It has been extensively shown that autophagy declines during ageing. Indeed, essential autophagy genes are transcriptionally down-regulated during healthy ageing (Lipinski et al., 2010;Schultz et al., 2013;Omata et al., 2014). Accumulation of damaged proteins and organelles also constitutes a hallmark of numerous age-associated neurodegenerative disorders (Nixon, 2017;Colacurcio et al., 2018). Alteration of autophagy has been identified as an early onset in Alzheimer's disease-affected neurons (Zare-Shahabadi et al., 2015). However, the interplay between autophagy, iron and neurodegeneration is poorly understood.

In the present study, we used the model organism *Drosophila melanogaster* to investigate the effect of ageing and autophagy disruption on the load of iron in the brain. We show that iron and holoferritin (where ferritin – Fer1HCH and Fer2LCH heteropolymer – protein surrounds an iron oxide core) accumulate in the brain from old flies regardless of their autophagy status, suggesting that autophagy is not essential to regulate total iron levels in the *Drosophila* brain. The spectrum of iron phases present is unchanged within the limits of detection for wild-type as a function of ageing, but there is evidence of a distinct iron fraction in the autophagy-deficient fly brain, consistent with a proportional elevation in an iron-sulphur phase. This may, in turn, indicate disrupted mitochondrial iron homeostasis (Rouault and Tong, 2005).

2 Materials and methods

2.1 *Drosophila* stocks and maintenance

Flies were maintained on standard yeast-cornmeal medium at 25ºC, 70% humidity with a 12 hours light-dark cycle. The following fly strains were used: wild-type w^{1118} (BDRC #3605), Atg8a-deficient $Atg8a^{KG07569}$ (gift from Dr Gabor Juhasz), Atg7-deficient $Atg7^{\Delta 77}$ and $Atg7^{\Delta 14}/CyO$ (Juhasz et al., 2007) (gift from Dr Gabor Juhasz), $Fer1HCH^{G188}/TM3$ (DGRC #110-620; this line encodes a GFP-tagged version of the Fer1HCH subunit) (Missirlis et al., 2007), *hml*(delta)-*GAL4 UAS-eGFP* (BDRC # 30140) and *hml*(delta)-*GAL4 UAS-eGFP UAS-hid/CyO* (gift from Dr François Leulier). For the generation of Atg7-deficient flies, virgin females $Atg7^{\Delta 14}/CyO$ were crossed with males $Atg7^{\Delta 77}$ and the progeny lacking balancer chromosome was collected after hatching. Stocks were backcrossed to w^{1118} to homogenise the genetic background.

2.2 Ageing and lifespan measurement

For all experiments, age-matched adult male flies were used. Flies were collected within 24 hours of hatching and aged in cohorts of 20 individuals. Flies were transferred every 2-3 days on fresh medium until collection after 1 week, 1 month or 2 months. Because of their shorter lifespan, old autophagy-deficient flies were collected at 1 month.

2.3 Generation of hemizygous GFP-Fer1HCH expressing flies

Homozygous virgin females wild-type (w^{1118}) or autophagy-deficient ($Atg8a^{KG07569}$) were crossed with males $Fer1HCH^{G188}/TM3$. From the progeny, only hemizygous males w^{1118}/Y; $Fer1HCH^{G188}/+$ and $Atg8a^{KG07569}/Y$; $Fer1HCH^{G188}/+$ were collected, and aged or fed as mentioned were appropriate in the figure legends.

2.4 Feeding with iron or bortezomib-supplemented diets

Adult males were selected within 24 hours from hatching and placed onto Nutri-Fly Instant *Drosophila* Medium (Genesee Scientific, 66–117) prepared in water supplemented with 1 mM FAC (ferric ammonium citrate) or 20 mM bortezomib in DMSO (#2204 Cell Signaling Technology). Flies were flipped onto freshly made food every day for 5 days. The same diet without FAC or with 0.002% DMSO were used as respective control for regular diets.

2.5 Protein extraction from adult *Drosophila* heads and bodies

Age-matched adult males were flash frozen in liquid nitrogen. Flies were decapitated by short burst vortexing in 15 mL tubes. Heads, bodies and appendices were separated using sieves (no. 25 and no. 40) chilled with liquid nitrogen beforehand. Heads were collected in microcentrifuge tubes and homogenized in ice-cold lysis buffer (20 mM Tris pH 7.5, 137 mM NaCl, 1 % Triton X-100, 1 % glycerol) supplemented with complete protease inhibitor cocktail (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail; Sigma-Aldrich, 04693159001 Roche) and 50 mM N-ethylmaleimide (Sigma-Aldrich, E3876). Protein concentrations were determined using Bradford assay.

2.6 Haemocyte ablation

The cell-specific ablation of mature haemocytes was performed by crossing virgin females *Fer1HCH^{G188}/TM3* with males *hml*(delta)-*GAL4 UAS-eGFP UAS-hid/CyO*. As a control, virgin females *Fer1HCH^{G188}/TM3* were crossed with males *hml*(delta)-*GAL4 UAS-eGFP/CyO*. From the progeny, adult flies lacking the balancer chromosomes were selected and aged for 5 days onto regular diet before collection of their haemolymph.

2.7 Haemolymph collection

Immediately before haemolymph collection, anaesthetized flies were surface sterilized by dipping them briefly in 70% ethanol. Excess ethanol was blotted off on filter paper. Flies were punctured with a tungsten needle in their thorax and immediately placed in a collection tube on ice. Collection tubes were made by piercing through the bottom of a 0.5 mL centrifuge tube with a 25G needle and placing it into a 1.5 mL centrifuge tube. A total of 40 punctured flies per genotype were pooled per collection tube. Haemolymph was isolated by centrifugation at 5000 rpm for 5 minutes at 4ºC. Collected haemolymph samples were then diluted in Laemmli loading buffer and heated for 5 minutes at 95ºC.

2.8 In-gel iron staining

Protein extracts were prepared in 2x concentrated non-denaturing/non-reducing loading buffer (62.5 mM Tris-HCl pH 6.8, 25 % glycerol, 1 % bromophenol blue). Protein concentrations were determined using Bradford assay; 20 µg of total protein for each sample was separated on 6 % native-PAGE gel in ice-cold running buffer (25 mM Tris, 192 mM glycine) after pre-run of the gel for 30 minutes at 100 V. Following protein separation, the gel was stained for 48 hours with Prussian blue staining solution (10 % $K_4Fe(CN)_6$, 350 mM HCl) at room temperature with gentle agitation. After washes in ultrapure water, holoferritin was visible as blue bands. All the glassware and tanks were acid-rinsed (1% HCl in ultrapure water) and let to air dry before use.

2.9 Western blotting and antibodies

Protein extracts were prepared in Laemmli loading buffer containing 2.5% beta-mercaptoethanol (except for experiment in non-reducing condition where no beta-mercaptoethanol was added) and heated for 5 minutes at 95 ºC before separation of 20 µg total proteins on 8 or 12 % SDS-PAGE gels. Separated proteins were transferred onto nitrocellulose or PVDF membranes. The membranes were blocked in TBS (Tris-buffered saline; 50 mM Tris-Cl, pH 7.6, 150 mM NaCl), 0.1 % Tween-20, 5 % non-fat milk. The following antibodies were used: anti-GFP (Santa Cruz sc-9996, 1:1,000), anti-GABARAP/Atg8a (Cell Signalling Technology no.13733, 1:2,000), anti-Ref(2)P (Abcam ab178440, 1:1,000), anti-β actin (Abcam ab8227, 1:2,000), anti-α tubulin (Sigma-Aldrich T5168, 1:40,000), HRP-coupled secondary antibodies anti-rabbit and anti-mouse (Thermo scientific no. 31460 and 31450, 1:10,000). Signals were developed using the ECL detection reagents (Amersham, RPN2209).

2.10 Immunocytochemistry

Dissected brains from adult males *Fer1HCH*^{G188} were fixed for 30 minutes in 4 % paraformaldehyde in 1x PBS (phosphate buffered saline; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4). The brains were permeabilized for 1 hour in permeabilization buffer (0.1 % Triton X-100, 0.3 % BSA in PBS) before incubation overnight at 4ºC with anti-Brp (DSHB, nc82 supernatant; 1:10 in permeabilization buffer) (Wagh et al., 2006) or anti-Elav (DSHB, Elav-9F8A9 supernatant; 1:100 in permeabilization buffer) (O'Neill et al., 1994). Subsequent incubation with an Alexa568-coupled secondary antibody (Sigma no SAB4600082, 1:500) was conducted in permeabilization buffer for 2 hours at room temperature. Nuclei were stained with Hoechst 33342 (1 µg/mL in PBS). All washes were performed with 0.1 % Triton X-100 in PBS. Images were captured with a Zeiss LSM880 confocal microscope.

2.11 X-ray fluorescence imaging and spectroscopy on isolated brains

Whole dissected brain from flies at the desired age were dissected using tungsten-coated titanium tweezers in ultrapure deionized water. Dissected brains were mounted onto ultralene film and allow to air dry for a minimum of 2 hours (nine brains per slide: three brains per genotype/age).

Specimens were analysed at the I18 Microfocus Spectroscopy beamline at the Diamond Light Source in Oxford, UK, using a pair of opposing Si detectors to maximize recovery of the fluorescence emitted from the ultralene-mounted samples. The focused beam was tuned to 10.5keV, with a beam spot diameter of 60µm for initial surveys with microfocus X-ray Fluorescence (µXRF), and 20µm for mapping over the area of each intact brain. The method used here is not as precise in determining absolute concentration as mass spectroscopy imaging, but it is non-destructive and highly sensitive to relative differences in concentration between samples (Collingwood and Adams, 2017). Three to nine intact brains were imaged at room temperature for each group of flies. Detector position and acquisition times were kept consistent throughout the experiment to facilitate the subsequent comparative analysis. Subsequently, site-specific X-ray Absorption Near Edge Spectroscopy with a microfocussed beam (µXANES) analysis was performed at the iron K-edge, using the 20 µm diameter beam to acquire a spectrum from the iron-richest region in the central brain from each fly, where the region of interest was confirmed using the µXRF intensity image for iron. The absorption edge position in energy was calibrated with reference to an iron foil spectrum obtained during the same experiment, with alignment and removal of background being performed using the established workflow in the IFEFFIT Athena software package for XAFS analysis (Ravel and Newville, 2005).

2.12 Genomic DNA extraction and PCR

Genomic DNA (gDNA) from 15 flies per genotype was extracted using DNeasy Blood and Tissues Kit (Qiagen 69504). PCR amplifications were conducted on 100 ng of gDNA with DreamTaq Green PCR Master Mix (ThermoScientific K1081) in a Bio-Rad T100 thermal cycler. Samples were loaded on 1% (w/v) agarose gel in 1x TAE (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA pH 8.0). GelRed (VWR 41003) was used to stain nuclei acids. Primer sequences are listed in Table 1.

2.13 RNA extraction and real time (RT)-qPCR

Total RNA extraction was performed on adult males using the PureLinkTM RNA Mini kit (Life Technologies Ambion) according to the manufacturer protocol. For all subsequent steps, 1 µg of RNA was used for each condition. Genomic DNA were digested out using DNase I (ThermoScientific K1622). Synthesis of cDNA was done using the RevertAid Kit (ThermoScientific K1622). Relative quantitation of gene expression was performed in an Agilent MxPro4005P qPCR system using the GoTaq qPCR Master Mix (Promega A6002). Primer sequences are listed in Table 1.

3 Results

3.1 Ferritin is expressed in adult *Drosophila* brain.

It has been previously shown that ferritin is expressed in various tissues in *Drosophila*. The protein expression of a GFP-knock-in mutant for *fer1hch* has been used to show the localisation of Fer1HCH protein in different tissues and organs, including the larval brain where it accumulates primarily in the optic lobes and notochord (Georgieva et al., 2002;Mehta et al., 2009). However, no information is readily available concerning the localisation of the GFP-Fer1HCH in adult fly brain. To evaluate the expression of GFP-Fer1HCH in the head of adult flies, we performed western blot on lysates from isolated heads and bodies from a fly strain expressing GFP-tagged Fer1HCH due to the genomic insertion of GFP between the first and second exons of the gene (*Fer1HCH*^{G188} flies) (Missirlis et al., 2007). Wild-type flies were used as a negative control. As expected from previous studies, GFP-Fer1HCH was detected in samples from both heads and bodies (Figure 1A).

Drosophila ferritin is known to be secreted in the haemolymph, which is analogous to vertebrates' blood, and remains in direct contact with tissues. In order to evaluate whether the GFP-Fer1HCH protein we detected in *Drosophila* heads corresponds to secreted or cytosolic ferritin, we extracted the haemolymph from adult *Fer1HCH*^{G188} flies. As expected, GFP-Fer1HCH strongly accumulates in the haemolymph, but also remains present in the bodies after draining their haemolymph (Figure 1B). It was shown that GFP-Fer1HCH accumulates in haemocytes (Gonzalez-Morales et al., 2015). To make the distinction between ferritin which is secreted in the haemolymph and expressed in the haemocytes, we compare the level of GFP-Fer1HCH protein in the haemolymph from flies lacking mature haemocytes (phagocytes) and flies with a normal haemocytes pool (Figure 1C, D). Tubulin was used as a marker for the successful ablation of the phagocytes (Figure 1C) and the presence of the relevant transgenes was validated by PCR on gDNA (Figure 1D). We observed a moderate reduction of GFP-Fer1HCH in the haemolymph from the flies lacking phagocytes, meaning that most of the ferritin in the haemolymph corresponds to secreted ferritin (Figure 1C).

To confirm that ferritin detected in the adult *Drosophila* head comes mainly from the tissues rather than the surrounding haemolymph, we used fluorescence microscopy to establish the distribution of ferritin in *Drosophila* adult brain. We observed that GFP-Fer1HCH is expressed in the cell bodies surrounding the neuropil, marked using an antibody against the presynaptic protein Bruchpilot (Brp) (Wagh et al., 2006) (Figure 1D). The localisation of GFP-Fer1HCH also matches the expression of Elav, a neuron-specific protein (Figure 1F).

Taken together, the above results show that ferritin heavy chain is expressed in the brain of adult *Drosophila* fly and accumulates in the neuronal cell bodies.

3.2 Decline of autophagy induces accumulation of high molecular weight Ferritin heavy chain.

Various studies have demonstrated that autophagy, which declines with age, is implicated in the degradation of ferritin and iron turnover (Asano et al., 2011;Mancias et al., 2014;Ott et al., 2016). To evaluate whether autophagy and ageing affect the level of ferritin in adult *Drosophila* heads, we made use of the GFP-Fer1HCH expressing flies. First, extracts from adult heads of wild-type or Atg8a mutant males heterozygous for the *Fer1HCH*^{G188} allele were analysed. Atg8a mutant fly heads were used as a negative control for GFP-Fer1HCH expression. Western blots probed with anti-GFP antibody revealed the presence of a band at the expected size of 50kDa consistent with the fusion of the 27 kDa GFP protein to the 23 kDa Fer1HCH chain (Figure 2A). No noticeable difference was observed between wild-type and autophagy mutant. However, 1-week old Atg8a mutant fly head samples, but not young age-matched wild-type, exhibited the accumulation of a higher molecular weight band around 120 kDa (Figure 2A). Similarly, we noted the presence of higher molecular weight bands in old (2-months old) *Fer1HCH*^{G188} male fly heads that were not detected in young (1-week old) wild-type flies (Figure 2B).

To test whether this high molecular weight ferritin in Atg8a mutant and aged wild-type fly heads corresponds to aggregates, we performed a differential detergent protein extraction (Nezis et al., 2008;Simonsen et al., 2008;Jacomin and Nezis, 2019). Soluble proteins from fly heads were first extracted in a 0.1% Triton X-100 lysis buffer. The pellets, containing aggregated proteins, were then broken down by sonication in a 2% SDS lysis buffer. Samples were reduced and denaturated before separation by SDS-PAGE. We observed that 50 kDa GFP-Fer1HCH is predominantly located in the soluble fraction (Triton). High molecular weight ferritin heavy chains were solely detected in the soluble fraction and were excluded entirely from the insoluble fraction (SDS) (Figure 2C). Some proteins can form oligomers that can be identified using reduced and non-reduced lysis condition. We compared the effect of reducing agent on the formation of higher molecular weight GFP-Fer1HCH. Lysis of wild-type and Atg8a mutant fly heads was performed in lysis buffer supplemented with 50 mM N-ethylmaleimide to prevent the formation of new disulphide bond during the lysis procedures. Loading samples were then prepared by boiling in SDS-loading buffer in the presence (reduced) or absence (non-reduced) of 2.5% β-mercaptoethanol (Figure 2D). The preparation of the samples from in non-reduced condition had no effect on the accumulation of the high molecular weight GFP-Fer1HCH which was consistently observed in 1-week old autophagy mutant (but not age-matched wild-type) fly heads samples.

Taken together, these results show that ferritin does not form aggregates in *Drosophila* head when autophagy is impaired.

3.3 Holoferritin is not affected in autophagy mutant fly heads.

It has been well-documented that iron bioavailability and ferritin levels are correlated. We have verified that we can detect changes in holoferritin in wild-type and autophagy-deficient *Drosophila* by feeding adult flies on either normal diet or on a diet supplemented in iron in the form of FAC. Flies lacking either Atg8a or Atg7, two major regulators of autophagy, were used as autophagy-deficient *Drosophila*. We performed Prussian blue staining for holoferritin in protein samples from whole adult flies separated on native-PAGE. As expected, holoferritin accumulated in flies fed on iron-supplemented diet (Figure 3B, C) and correlated with an increase in the mRNA expression level of *fer1hch* and *fer2lch*, while Atg8a and Atg7 remained unchanged (Figure 3D).

We then performed in-gel Prussian blue staining on protein lysates prepared from wild-type or autophagy-deficient fly heads collected from age-matched flies at 1-week, 1-month or 2-months old. Because of their significantly shorter lifespan, samples from old Atg8a and Atg7 mutant flies were collected at 1-month old only, solely wild-type samples were collected at 2-month old (Figure 3A). We

observed that holoferritin accumulated in protein samples from heads isolated from old flies, regardless of their autophagy-deficiency status. No difference was observed in heads from young autophagy-deficient flies when compared to age-matched wild-type (Figure 3E, F). The quantity of holoferritin was normalized against α-tubulin, used as a loading control, from the same samples separated by SDS-PAGE after denaturation and reduction of the samples in Laemmli loading buffer and boiling at 95ºC. Gene expression of *fer1hch* and *fer2lch*, as well as *Atg8a* and *Atg7*, was accessed by RT-qPCR. Wild-type and Atg7 mutant flies showed an increased level in *fer1hch* and *fer2lch* as a result of their respective ageing, which correlated with the accumulation of holoferritin, while no significant change was observed in Atg8a mutants (Figure 3G). As expected, Atg8a expressing is reduced in old wild-type flies (Simonsen et al., 2008;Omata et al., 2014).

Altogether, these results suggest that the accumulation of holoferritin during the course of ageing in *Drosophila* head is independent of autophagy.

3.4 Ferric iron accumulates in the brain of aged flies.

X-ray fluorescence microscopy was previously demonstrated to be a robust way to image and quantify biometals in the non-mammalian model organisms, *Drosophila* and *Caenorhabditis elegans* (Lye et al., 2011;Jones et al., 2015;Ganio et al., 2016). Therefore, we used synchrotron μXRF imaging to measure the concentration of iron in the brain of *Drosophila*. Elemental maps were collected from whole brains dissected from young and old wild-type flies and young autophagy-deficient flies (*Atg8a* mutant). Three to nine entire brains per fly group were imaged at 20μm resolution, providing an excellent signal to noise for the elements of interest. The data collected were used to quantify and compare iron concentrations (ppm) between each group. The quantification was done by first defining the region of interest (ROI) encompassing each brain based on the iron distribution map, and calculating the mass fraction for elements of interest after subtraction of the background signal (accounting for any background scatter reaching the detector, including any signal from the ultralene). As the present study focuses on iron, the other elements simultaneously acquired in the XRF imaging merit further investigation and will be the subject of future work. We observed that iron accumulated as a function of ageing in the wild-type and autophagy-deficient brains (Figure 4A). Checking the relationship between the area mapped and the total metal ion signal confirmed that there was no correlation between the size of the brain and the amount of iron as shown by the Spearman's rank correlation coefficient ρ=0.3311 (Figure 4B).

In addition to determining the elemental distribution of iron by μXRF, we performed site-specific microfocus X-ray Absorption Near Edge Structure (μXANES) spectroscopy analysis to obtain information about the dominant iron phase(s) present in the central brain region where iron deposition was the highest for each of the three groups. The μXANES spectra from wild-type and autophagy deficient flies incorporate the sum of contributions from the iron phases present at the iron-richest region in the central brain, including signal from the mineralized iron in holoferritin, typically a ferrihydrite-like hydrated iron oxide; (Collingwood and Telling, 2016); the spectrum from the iron in purified horse spleen ferritin is included for comparison (Figure 4C). The μXANES spectra from these iron-rich sites in the 1-week and 2-months old wild-type flies are indistinguishable in this experiment (Figure 4D), whereas comparison of 1-week old wild-type and Atg8a-deficient reveals an additional feature in the autophagy-deficient fly at 7138 eV (Figure 4E). Linear combination fitting was undertaken for the 1-week old Atg8a-deficient fly spectrum using the 1-week wild-type spectrum and a range of reference standards (including those measured recently at the same beamline: iron metal reference foil, ferric sulphate, ferric and ferrous chloride, ferric citrate, horse spleen ferritin, previously-acquired iron nitride, and iron oxide standards including ferrihydrite, magnetite, and wustite). The best

fitting result indicated that the feature can be well-accounted for by including a ferric sulphate reference standard (Figure 4F), suggesting that approximately 1/3 of the signal might be attributed to iron-sulphur complexes, and 2/3 attributed to the spectrum of iron phases found in the central brain of the wild-type fly.

4 Discussion

During the course of ageing, the load of iron in the brain increases significantly, possibly due to decreased efficiency of the iron homeostasis system. Neurodegenerative pathologies associated with ageing, such as Parkinson's or Alzheimer's diseases, have previously been associated with changes in iron homeostasis (Zecca et al., 2004a;Ward et al., 2014).

Our data corroborate previous studies of iron accumulation in the brain during ageing in *Drosophila* (Massie et al., 1985;1993). However, it was surprising to observe that neither iron nor ferritin heavy-chain levels are affected in autophagy-deficient flies. Indeed, an increase in iron in those flies was anticipated because of the recent studies pinpointing at the impact of autophagy on iron mobilization and recycling (Kurz et al., 2011;Mancias et al., 2014;Ott et al., 2016). The lack of accumulation of iron and holoferritin in autophagy-deficient flies at any age, matched to the age of wildtype individuals, suggests that autophagy is either not required or plays a minor role in the turnover of ferritin and iron during the course of ageing in *Drosophila* brain. A recent study showed that lysosomal trafficking of ferritin could be independent of macroautophagy (Goodwin et al., 2017). Nonetheless, we noticed the presence of supernumerary GFP-Fer1HCH bands in the heads of young Atg8a-deficient flies while the main GFP-Fer1HCH band remained unchanged in age-match wild-type fly heads. This high molecular weight GFP-Fer1HCH probably corresponds to a non-functional form of ferritin heavy-chain as there is no accumulation of holoferritin in these flies as demonstrated by Prussian Blue in-gel staining and μXRF. Autophagy has been extensively described for its role in the degradation of protein aggregates, and ferritin has been shown to be degraded by autophagy (Hyttinen et al., 2014;Mancias et al., 2014). However, using a differential-detergent protein fractionation protocol, we have observed that high molecular weight GFP-Fer1HCH bands do not correspond to insoluble aggregates. A shift in protein molecular weight could be associated with posttranslational modifications. A study has shown that both ferritin subunits are ubiquitinated in muscles from a rat model of Amyotrophic Lateral Sclerosis (Halon et al., 2010). Ferritin has also been detected as being pupylated (prokaryotic homolog of ubiquitination) in the bacterium *Corynebacterium glutamicum* (Kuberl et al., 2016). Ferritin is also known to be glycosylated in mammals and insects; notably, secreted ferritin L has been shown to be N-glycosylated in culture hepatocytes (Cragg et al., 1981;Ketola-Pirie, 1990;Ghosh et al., 2004;Cohen et al., 2010). Therefore, it is possible that high molecular weight Fer1HCH corresponds to a modified, soluble form of the protein.

It was previously shown that iron storage increases significantly with age in both mammals and insects (Massie et al., 1985;Zecca et al., 2001). The control of ferritin subunit synthesis frequently occurs at the translational level. Ferritin mRNAs contain an iron-responsive element (IRE) in 5' UTR that can be recognized by iron regulatory proteins (IRP). Depending on iron availability, the translation of ferritin subunits is modulated by the binding or releasing of the IRPs to the mRNA (Gray and Hentze, 1994;Lind et al., 1998;Missirlis et al., 2007). Interestingly, no noticeable increase in the quantity of GFP-Fer1HCH with age was observed in the fly brain while the iron levels and holoferritin levels were significantly increased. Most of the studies aiming at elucidating the IRE/IRP-dependent regulation of ferritin are based on supplementation of animal food with iron or chelators. It is possible that such

changes in the diet have more drastic effects on the iron uptake by the cells than that which would occur under physiological conditions. It is also possible that intestinal ferritin is more prone to transcriptional regulation as the gut is the first organ to be affected by dietary iron.

The evidence for an additional minor peak at 7138 eV in the iron absorption spectrum for autophagy deficient flies, but not in wild-type, is consistent with signal contribution from an iron-sulphur-rich material. The good fit achieved with the inclusion of ferric sulphate does not exclude other possibilities; we note that iron-phosphorus-containing material can also exhibit a peak in this energy region. However, examination of the XRF signal at the sites where the XANES spectra were acquired indicate that sulphur was significantly more abundant than phosphorus, and that while iron and sulphur levels at sites of XANES acquisition were equivalent for wild-type and autophagy mutant, the phosphorus level at the XANES site in the autophagy deficient fly was lower (approximately $1/5^{th}$) of that measured in wild-type. Therefore, it is more likely that the additional feature in the autophagy mutant is associated with iron-sulphur than with iron-phosphorus. Iron-sulphur clusters might account for this signal, a ubiquitous class of metalloproteins involved in many regulatory processes, including mitochondrial iron homeostasis (Rouault and Tong, 2005).

In summary, we have shown that holoferritin accumulates in the brain from old flies but not young Atg8a-deficient flies, suggesting that macroautophagy is not a dominant process in ferritin and iron turnover in the *Drosophila* adult head. The origin of increased iron in the brain during the course of ageing in *Drosophila* brain remains unclear but appears not to be related to, or sufficient to induce the synthesis of, ferritin heavy-chain. In addition, our work provides evidence of the feasibility to accurately detect variation in biometal levels and distributions in intact isolated adult *Drosophila* brain, thus opening new fields of investigation for normal and pathological ageing. Most neurodegenerative diseases are linked to altered metabolism of biometals, including iron, in the brain. *Drosophila* has been successfully used as model for a broad range of neuropathologies. Further studies combining those readily-available model strains with synchrotron spectromicroscopy methods should contribute to uncovering the relationships between disrupted metabolism of biometals and neurodegeneration.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

AJ, KG, JB, VT and JC performed experiments and analysed data. KG, JB, VT and JC provided material, expertise, and technical help with synchrotron X-Ray Fluorescence microscopy data acquisition and analysis. AJ, IN and JC designed the study. AJ wrote the manuscript and analysed the data. All authors read and contributed to the manuscript.

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10 Figure legends

Figure 1. Ferritin heavy chain is expressed in adult *Drosophila* brain.

(A-C) Western blot analysis of GFP-Fer1HCH in reduced/denaturated protein samples extracted from (A) fly heads or bodies; (B) haemolymph (Haemo) or bodies drained from their haemolymph (Bodies); (C) haemolymph from flies depleted (–) or not (+) of their phagocytes. (D) Gel electrophoresis on PCR products from gDNA extracted from transgenic (transg.) flies used in (C), gDNA from w^{1118} wild-type flies was used as negative control. (E-F) Confocal imaging of an adult *Drosophila* brain expressing GFP-Fer1HCH (left panel, green in merged panel) and stained for either (E) Bruchpilot (Brp) or (F) Elav (middle panel, red in merged channel). For all the experiments, 1-week-old adult males were used.

Figure 2. Soluble high molecular weight heavy-chain ferritin accumulates in autophagy-deficient fly heads.

(A, B) Western blot analysis of GFP-Fer1HCH after samples reduction/denaturation in total protein lysates from (A) 1-week old wild-type (WT) and Atg8a mutant flies or (B) 1-week or 2-months old wild-type flies. (C) Western blot analysis of GFP-Fer1HCH in soluble (Triton) and insoluble/aggregated (SDS) proteins fraction from 1-week old wild-type (WT) and Atg8a mutant fly heads. (D) Western blot analysis of GFP-Fer1HCH in total protein lysates from 1-week old wild-type (WT) and Atg8a mutant fly heads prepared in either reduced or non-reduced conditions before SDS-PAGE and western blotting. (E) Western blot analysis of GFP-Fer1HCH in total protein lysates from Fer1HCHG188 fly heads after feeding for 6 days on 10 µM bortezomib or vehicles. Membranes were probed for GFP, Atg8a (A), ubiquitinated proteins (E); β-actin (A, C) or α-tubulin (B, D, E) were used as loading control. Arrowheads show high molecular weight bands of GFP-Fer1HCH.
'short exp.' And 'long exp.' refer to the duration of film exposure on the membrane before developing. A longer exposure (long exp.) was necessary for the observation of the high molecular weight GFP-Fer1HCH which are less abundant than 50 kDa GFP-Fer1HCH.

Figure 3. Holoferritin accumulation in fly head.

(A) Lifespan of a hundred wild-type and Atg8a and Atg7 mutant flies reared in standard conditions. (B) In-gel staining for holoferritin on protein lysates from wild-type and Atg8a and Atg7 mutant flies fed on either normal diet or diet supplemented with 1 mM FAC. (C) Relative quantity of holoferritin normalized to loading control. (D) Analysis by RT-qPCR of mRNA level for *fer1hch*, *fer2lch*, *Atg8a* and *Atg7* in flies fed on either normal or FAC-supplemented diet. (E) In-gel staining for holoferritin on protein lysates from wild-type (WT) Atg8a (8a) and Atg7 (7) mutant fly heads at 1-week, 1-month and 2-month old. (F) Relative quantity of holoferritin normalized to loading control. (G) Analysis by RT-qPCR of mRNA level for *fer1hch*, *fer2lch*, *Atg8a* and *Atg7* in aged flies. The bar charts show mean ± s.d. Statistical significance was determined using One-way ANOVA, *P < 0.05, **P < 0.01. Western

651 blots on denaturated samples were probed for Ref(2)P as an autophagy-deficiency control and α-
652 tubulin as a loading control.

653

654 **Figure 4. Quantification of iron in isolated *Drosophila* brain.**

655 (A) The total quantity of iron per whole fly brain was measured from complete brain images obtained
656 using μXRF at 20 μm resolution. Individual biological samples are shown as circles. Open circle data
657 were acquired during a different experiment than close circle data. Bar represents mean ± s.d. Statistical
658 significance was determined using One-way ANOVA; significant values are indicated above the bars,
659 ***P < 0.001. (B) Correlation between the area of the brain in pixels (px) and the quantity of iron.
660 Spearman's rank correlation coefficient ρ=0.3311. (C) Fe K-edge XANES from *Drosophila* and
661 ferritin standard, with spectra vertically offset for clarity. (D-E) Overlay of spectra shown in (C),
662 focussing on the region where the spectra from Atg8a and wild-type differ (arrowhead). (F) Linear
663 combination fitting of Atg8a spectrum. The Atg8 spectrum was shown to be consistent with that of the
664 wild-type model + Fe(III) sulphate ($Fe_2(SO_4)_3$), with a $\chi 2$ value of 0.01 obtained for the fit. All XANES
665 spectra were subjected to an edge-step normalisation and flattened using Athena fitting software prior
666 to fitting.

667

Table 1. Primer sequences used in this study

PCR primers		
HmlΔ forward	CCAACAATTTCCGATTAGCCTAAC	
GAL4 reverse	CGATACAGTCAACTGTCTTTGACC	
pUAST3'	AACCAAGTAAATCAACTGC	
Hid reverse	GAATGGTGTGGCATCATGTGC	
EGFP reverse	CTTGTAGTTGCCGTCGTCCTTGAA	
RT-qPCR	**Forward primer sequences**	**Reverse primer sequences**
fer1hch	TCTGATCAATGTGCCGACTG	TGGTAGTGGTTGTAGGGCTTG
fer2lch	GCCAGAACACTGTAATCACCG	GGCTCAATATGGTCAATGCCA
Atg8a	GGTCAGTTCTACTTCCTCATTCG	GATGTTCCTGGTACAGGGAGC
Atg7	TCGTGGGCTGGGAGCTAAATA	GGTTTACAGAGTTCTCAGCGAG
rp49	AAGAAGTTCCTGGTGCACAACGTG	AATCTCCTTGCGCTTCTTGGAGGA