Published in final edited form as:

Neurobiol Dis. 2011 July ; 43(1): 213–219. doi:10.1016/j.nbd.2011.03.013.

Ferritin overexpression in *Drosophila* glia leads to iron deposition in the optic lobes and late-onset behavioural defects

Stylianos Kosmidis¹, Jose A. Botella², Konstantinos Mandilaras³, Stephan Schneuwly², Efthimios M.C. Skoulakis¹, Tracey A. Rouault⁴, and Fanis Missirlis³

¹ Institute of Cellular and Developmental Biology, BSRC “Alexander Fleming”, Vari, 16672, Greece

² Institute of Zoology, University of Regensburg, Regensburg, D-93040, Germany

³ School of Biological and Chemical Sciences, Queen Mary University of London, London, E1 4NS, UK

⁴ Molecular Medicine Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD 20892, USA

Abstract

Cellular and organismal iron storage depends on the function of the ferritin protein complex in insects and mammals alike. In the central nervous system of insects, the distribution and relevance of ferritin remains unclear, though ferritin has been implicated in *Drosophila* models of Alzheimers’ and Parkinsons’ disease and in Aluminum-induced neurodegeneration. Here we show that transgene-derived expression of ferritin subunits in glial cells of *Drosophila melanogaster* causes a late-onset behavioural decline, characterized by loss of circadian rhythms in constant darkness and impairment of elicited locomotor responses. Anatomical analysis of the affected brains revealed crystalline inclusions of iron-loaded ferritin in a subpopulation of glial cells but not significant neurodegeneration. Although transgene-induced glial ferritin expression was well tolerated throughout development and in young flies, it turned disadvantageous at older age. The flies we characterize in this report contribute to the study of ferritin in the *Drosophila* brain and can be used to assess the contribution of glial iron metabolism in neurodegenerative models of disease.

Keywords

optic lamina; inclusions; metals; circadian clock; restless legs; hyperferritinemia

Introduction

Iron is implicated in the pathologies of disorders such as Alzheimer’s and Parkinson’s disease potentially via the function of numerous iron-containing enzymes, but also in its labile form by catalyzing the formation of reactive oxygen species and the propagation of

© 2011 Elsevier Inc. All rights reserved.

Corresponding author: Dr. Fanis Missirlis, Queen Mary University of London, School of Biological and Chemical Sciences, Mile End Road, E1 4NS, London, UK, f.missirlis@qmul.ac.uk, tel. +44 207 882 3044, fax +44 208 983 0973.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

lipid peroxidation reactions (Zecca et al., 2004). Furthermore, defects in several human iron metabolism genes lead to heritable neurodegenerative pathologies (Gregory et al., 2009; McNeill et al., 2008; Rouault and Tong, 2008). Mutations in ferritin light chain gene cause a dominant adult-onset basal ganglia disease, termed neuroferritinopathy (Curtis et al., 2001; Levi et al., 2005; Vidal et al., 2008). In addition, mutation in the iron responsive element (Hentze et al., 1987) of the ferritin light chain gene lead to crystalline inclusions of ferritin in the human lens causing cataracts (Beaumont et al., 1995). Neuronal ferritin overexpression has been associated with beneficial effects attributed to either protection from metal-induced oxidative stress (Zhu et al., 2009), or participation in intracellular trafficking by modulating endogenous opioid signalling (Sengupta et al., 2009), but also with detrimental phenotypes in mammalian models of neurodegeneration (Kaur et al., 2009; LaVaute et al., 2001). Maintenance of metal homeostasis in the brain is essential for neuronal health and a critical component in the development of therapeutic strategies against neurodegenerative disease (Crouch et al., 2009; Ghosh et al., 2008; Madsen and Gitlin, 2007; Molina-Holgado et al., 2007).

Numerous *Drosophila* models of human neurodegenerative disorders were developed in recent years because they can be subjected to genetic (Clark et al., 2006; Fernandez-Funez et al., 2000; Mollereau, 2009; Park et al., 2006) and pharmacological analysis (Apostol et al., 2003; Outeiro et al., 2007; Rana et al.; Tain et al., 2009). Oxidative stress was shown to mediate toxicity in fly models of Parkinson's disease (Faust et al., 2009; Meulener et al., 2006; Wang et al., 2006; Whitworth et al., 2005; Yang et al., 2005), Alzheimer's disease (Dias-Santagata et al., 2007; Rival et al., 2009), lysosomal storage disease (Sweeney, 2008; Venkatachalam et al., 2008), and in the fly model of Friedreich's ataxia, a condition associated with mitochondrial iron overload (Anderson et al., 2008; Llorens et al., 2007; Navarro et al., 2010; Runko et al., 2008). The fly model of Pantothenate kinase associated neurodegeneration (Bosveld et al., 2008; Rana et al., 2010; Wu et al., 2009), a human disorder resulting in iron accumulation in the globus pallidus (Zhou et al., 2001), has not yet been used to address why and how iron accumulates predominantly in specific brain regions, but was shown instead to accumulate high amounts of zinc (Gutierrez et al., 2010).

Indeed, the effect of transition metals in *Drosophila* models of neurodegeneration has received little attention, despite numerous indications implicating ferritin in neuronal pathology and oxidative stress response. For example, proteomic analysis of adult heads from a *Drosophila* model of Parkinson's disease identified Ferritin Light Chain Homologue-2 (Fer2LCH) as 1 of 5 proteins upregulated relative to controls (Xun et al., 2008). Fer2LCH was recently shown to accumulate with age in flies raised at 28 C (Robinson et al., 2010). Similarly, the *Fer2LCH* gene was induced in photoreceptors under endoplasmic reticulum stress and protected them from retinal degeneration (Mendes et al., 2009). Furthermore, when flies were exposed to conditions of hyperoxia that caused marked degeneration in dopaminergic neurons, both *Ferritin Heavy Chain Homologue-1* (*Fer1HCH*) and *Fer2LCH* were induced (Girardot et al., 2004; Gruenewald et al., 2009). In the only functional studies to date, ferritin suppressed β -amyloid toxicity in *Drosophila* models of Alzheimer's disease (Rival et al., 2009) and it also suppressed Aluminum toxicity (Wu et al., 2010). Specifically, transgene-derived neuronal overexpression of either Fer1HCH or Fer2LCH alone was sufficient to rescue the reduced survival of flies expressing a mutant form of the β -amyloid peptide (Arctic A β ₁₋₄₂; Rival et al., 2009). In contrast, transgene-derived ubiquitous overexpression of either Fer1HCH or Fer2LCH was not sufficient to rescue from Aluminum-based toxicity, but simultaneous overexpression of both subunits did (Wu et al., 2010).

Recent findings highlight the role of glia in a fly model of Friedreich's ataxia (Navarro et al., 2010) and in the control of circadian behaviour (Jackson, 2010). Null mutants of the

ebony gene exhibit arrhythmic patterns of locomotor activity because of elimination of a rhythmic glia-specific enzyme (Suh and Jackson, 2007). The proximity of Ebony-containing glial cells to aminergic neurons of several types (histamine, serotonin, dopamine) within the fly optic lobe and central nervous system suggests that the rhythm phenotype of mutants may be due to defective amine recycling. Consistent with a role for dopamine in the phenotype, the *ebony*¹ mutation suppresses the hyperactivity associated with a *Drosophila* dopamine transporter allele (Jackson, 2010; Suh and Jackson, 2007). To evaluate if *Drosophila* ferritin has a role in glia and to assess a potential role of iron in normal circadian behaviour, we interfered with normal ferritin expression in this cell type. Since ferritin is a heteropolymer of two subunits and resides in the secretory pathway of cells and in the hemolymph (Georgieva et al., 2002; Missirlis et al., 2007), we used subunit-specific antibodies to monitor each ferritin subunit individually in the adult brain. We tested flies overexpressing ferritin subunits for behavioural alterations in elicited locomotion at different times during their lifespan and checked their ability to maintain circadian rhythms in the absence of environmental inputs. We describe alterations in brain iron homeostasis, accompanied by a late-onset behavioural syndrome in these flies.

Materials and Methods

Fly maintenance and Western blotting

The pan-glial drivers *Repo-Gal4/TM3*, *Sb* and *Nrv2-Gal4* were obtained from the Bloomington stock centre. Transgenic stocks *UAS-Fer1HCH* and *UAS-Fer2LCH* have been described and characterized in detail elsewhere (Missirlis et al., 2007). SDS PAGE followed by Western blotting was performed under non-reducing conditions to detect assembled holoferritin complexes or in the presence of β -Mercaptoethanol to detect individual ferritin subunits as described previously (Missirlis et al., 2006; Missirlis et al., 2007).

Iron staining

For detection of ferric iron, paraffin embedded sections were incubated for 45 minutes in the dark with Prussian blue stain (10% $K_4Fe(CN)_6$; 20% HCl, 1:1) prior to de-paraffinization. Three washes with water followed and staining was enhanced using DAB following standard procedures. Then sections were de-paraffinized using ascending gradients of ethanol to xylene and preparations were mounted in Glycergel mounting medium (DAKO) and imaged on a Nikon microscope.

Immunohistochemistry and confocal imaging

Immunohistochemistry on paraffin sections was performed as previously described (Kosmidis et al., 2010). Rabbit anti-Fer1HCH (1:100) or rabbit anti-Fer2LCH (1:50) primary antibody incubations were followed by fluorescent secondary antibodies at 1:1000 (Invitrogen) and mounted in DAKO Fluorescent Mounting medium (antibodies described in Missirlis et al., 2006; Missirlis et al., 2007). Sections incubated without the primary antibodies were used to control for autofluorescence. Imaging was performed on a Zeiss confocal microscope.

Electron Microscopy

Adult brains were prepared, cut, and stained as described (Kretzschmar et al., 1997). Ultrathin Epon plastic sections were post stained with 2% uranyl acetate, followed by Reynolds' lead citrate and stabilized for transmission electron microscopy by carbon coating. Examination was done with a Zeiss EM10C/VR (Oberkochen, Germany) electron microscope at 40–80 kV.

Behavioural assays

Male adult flies collected 0–2 days after eclosion were kept throughout their lifetime at 25 C in groups of 20 individuals per vial. For elicited-escape response tests flies were adapted for at least 10 min in an environment of 25 C and 70–80% humidity illuminated by red light. They were then placed in 14mL polystyrene Falcon tubes individually and each fly was vortexed at the highest speed for 2–3 sec and was tested twice in negative geotaxis and horizontal escape assays (Wittmann et al., 2001). A minimum 20 flies per genotype were tested for each experimental datapoint and the data analyzed parametrically for statistical significance using the JMP software. Locomotor activity rhythms were recorded automatically using the *Drosophila* Activity Monitoring system (Trikinetics, Waltham, MA). Flies were raised in light-dark cycles (12 hr: 12 hr) at a constant temperature of 25 C and flipped to fresh food every 3–4 days during ageing to avoid microbial infections. They were then released to constant darkness for the measurements. To quantify the phenotypes we used the rhythmicity statistic value of 1 as the threshold to classify flies as rhythmic (Levine et al., 2002) and present the average period and the average R.S. value for the pool of flies deemed rhythmic. Life span determinations have been described previously (Missirlis et al., 2003).

Results and Discussion

Altered brain iron homeostasis causes a late-onset behavioural decline

To investigate whether increasing levels of ferritin in *Drosophila* resulted in functional compromise of the CNS revealed as aberrant behaviours, we assessed the consequence of ferritin overexpression in behavioural tests throughout the life span of *Drosophila melanogaster* (Figure 1). Ferritin transgenes were driven in all adult brain glia with the *Repo-Gal4* driver. Two different methods were used to quantify the deficits in the fly's innate escape responses. We measured the elicited escape responses of control and transgenic flies in the horizontal (walking) and vertical (climbing) axes. Statistically significant differences in escape response between ferritin overexpression and control flies (Figure 1A, B) were not uncovered in the first four weeks. This indicates that experimental manipulation of ferritin levels in glia does not detectably alter this core behaviour for a significant portion of the life span. However, flies simultaneously accumulating both ferritin subunits in glia exhibited an abrupt and pronounced deficit in escape responses in both horizontal and vertical directions at five and six weeks of age (Figure 1A, B). Ferritin overexpression by means of the Gal4/UAS binary expression system was shown previously to require induction of both ferritin subunits to achieve quantifiable accumulation of assembled holoferritin polymers in adult flies (Missirlis et al., 2007), which could explain why the phenotype strongly manifested only when both subunits were induced.

As glia have been suggested to function in the fly circadian clock by interacting with neuro-aminergic pathways (Jackson, 2010), we tested whether overexpression of ferritin could impact brain iron homeostasis causing circadian disturbances in *Drosophila*. We monitored activity patterns in six-week old flies in complete darkness and quantified individual outputs (Figure 1C and Table 1). Quantification of rhythmic patterns (Levine et al., 2002) showed that 57 % of six-week old individuals overexpressing both ferritin subunits in glia remained rhythmic in constant darkness (Table 1), albeit with a reduced value for rhythmicity statistic (1.7 ± 0.7), suggesting behavioural disturbances even in the flies categorized as rhythmic. 85% of control flies carrying the transgenes without the driver were rhythmic with typical R.S. values greater than 2.5 (R.S.=1 indicates rhythmic behaviour at the 95% confidence interval; see Table 1). Three-week old flies overexpressing both chains in glia showed robust rhythms in 69% of the individuals tested. We repeated these experiments with an independent Gal4-driver specific for glia cells, *Nrv2-Gal4* (Pereanu et al., 2005).

Consistently, 63% of 6-week old *Nrv2-Gal4/UAS-Fer1HCH*, *UAS-Fer2LCH* flies were rhythmic compared to 91% for similar aged *Nrv2-Gal4/+* control flies (Table 1). Flies overexpressing *Fer1HCH* in glia were less affected (75% and 80% rhythmicity when expression was driven with *Repo-Gal4* or *Nrv2-Gal4*, respectively) but flies overexpressing *Fer2LCH* with *Repo-Gal4* showed a circadian phenotype (54% rhythmic and $R.S. = 1.9 \pm 1.2$). Overall, the results were consistent with the milder defects described in the elicited escape responses of individuals from these genotypes. The age-dependence of the phenotype may be related to the gradual decline in cellular degradation pathways, shown in *Drosophila* for both the proteasomal and the autophagic/lysosomal degradative pathways (Girardot et al., 2006; Pandey et al., 2007; Tonoki et al., 2009; Vernace et al., 2007), to a reduced output of mitochondrial activity due to oxidative stress (Hao et al., 2010), or to both physiological functions being affected in parallel. Disturbance of circadian rhythmicity suggests that these flies can be used to study the relevance of brain iron trafficking to the function of the circadian clock.

Changes in the distribution of ferritin subunits upon formation of assembled ferritin

We used antibodies capable of distinguishing individual ferritin subunits (Missirlis et al., 2007) to follow ferritin accumulation in glial cells when expression was induced with the pan-glial *Repo-Gal4* from the respective transgenes. Adult brains were dissected and Western blots performed following SDS-PAGE (Figure 2A,B). Under reducing conditions, ferritin subunits run in monomeric form, and our blots indicated that each subunit accumulated when transgenic expression was driven either alone or in combination with the alternate subunit (Figure 2A). Non-reducing SDS-PAGE was used to visualize assembled ferritin using an antibody against *Fer2LCH* (Figure 2B). This assay confirmed the requirement of both chains to form stable assembled ferritin as previously proposed (Missirlis et al., 2007). Induction of assembled ferritin was only observed when both *Fer1HCH* and *Fer2LCH* transgenes were simultaneously activated by *Repo-Gal4*, but smaller aggregates of *Fer2LCH* were also identifiable in dissected brains from *Repo-Gal4/UAS-Fer2LCH* flies. These results suggest that overexpression of a single ferritin subunit in *Drosophila* glia does not lead to a significant increase in the formation of additional functional ferritin molecules.

We then used the antibodies in immunohistochemical experiments to monitor changes in ferritin accumulation within the central brain of the same flies. We present images from sagittal sections through the optic lobes, where the most striking staining patterns emerged (Figure 2C–J). *Repo-Gal4* induction of single ferritin subunits resulted in independent accumulation of the respective subunits in cell bodies of all glial subtypes present in the optic lobes (Figure 5K for *Fer1HCH* and 5N for *Fer2LCH*). Upon simultaneous expression of both subunits with *Repo-Gal4*, *Fer1HCH* and *Fer2LCH* accumulation was greatly concentrated in satellite, epithelial, marginal and medulla glia but not in neuropil medulla glia or glia from the lobula complex (Figure 2I, J; for identification of glial subtypes see Chotard and Salecker, 2007). These results indicated that glia in the lamina and the frontal region of the medulla could accumulate large amounts of assembled holoferritin, whereas glia in other parts of the optic system and the central brain did not accumulate assembled holoferritin to the same degree. They suggest that some qualifications may be required when interpreting highly interesting and robust phenotypes from experiments using single subunit overexpression (Rival et al., 2009).

Ferritin-iron inclusions in the brain optic lobes

We next asked whether ferritin overexpression in glia was associated with morphological abnormalities of the adult central nervous system or alterations in brain iron accumulation. For this purpose, paraffin-embedded sections of transgenic adult brains from ferritin

overexpressors were stained for iron (Figure 3A–H). Strong staining for iron was specifically detected in the lamina of optic lobes from *Repo-Gal4* driven *UAS-Fer1HCH*, *UAS-Fer2LCH* (Figure 3G, H). In contrast, iron accumulation was neither detected in control flies (Figure 3A, B), nor in flies with single subunit overexpression (Figure 3C–F). We therefore suggest that this increased iron staining is the result of iron-loaded ferritin accumulation in the lamina. Plastic embedded sections of adult brains were also prepared to assess how increased ferritin and iron may impact brain morphology (Figure 3I–L). Interestingly, inclusions were clearly visible with toluidine staining, but the overall brain neuroanatomy of the affected flies was well preserved even at six weeks of age (Figure 3L).

The inclusions were also identifiable at higher resolution using electron microscopy due to their electron dense, rodular shaped nature (Figure 4). They only appeared in the lamina from flies simultaneously overexpressing both ferritin subunits in all glia (Figure 4A, B). Closer examination revealed that the ferritin-iron inclusions have a crystal-like (i.e. symmetric) morphology (Figure 4C, inset) and are surrounded by an intracellular membrane, indicating that they are likely trapped in the secretory system of the lamina glia (Figure 4C, black arrows). Electron density of these inclusions is also suggestive of iron accumulation and indeed similar structures have been observed before in iron-loaded cells of the *Calpodes ethlius* intestine (Locke and Leung, 1984). The inclusions described here are different from those found in patients with hyperferritinemia-cataract syndrome, in that they are intracellular, iron-loaded and composed from both ferritin subunits. In contrast, ferritin crystals in patients are secreted in the eye lens, are iron-poor and form from ferritin light chains only (Beaumont et al., 1995). Nevertheless, a screen for genetic or pharmacological suppressors of crystal formation in flies could also test if the underlying biology leading to ferritin inclusions would be relevant to the human condition.

Overexpression of both subunits in glia lead to enhanced assembly of ferritin evident both in western blots (Figure 2A, B), immunohistochemical sections (Figure 2I, J) iron and toluidine blue staining of brain sections (Figure 3G, H, L), and electron micrographs (Figure 4). Lack of apparent neurodegeneration in these flies was corroborated by lifespan determinations showing no significant differences between the different genotypes (Figure 4D). Further confirmation of the behavioural and anatomical phenotypes presented so far was obtained by repeating experiments with a different glial *Gal4* driver, *Nrv2-Gal4*, and also by using independent *UAS-Fer1HCH* and *UAS-Fer2LCH* transgenes and finding similar results as presented above (data not shown). Strikingly, using either glial *Gal4* driver, increased ferritin concentrations were not distributed evenly among the entire glial population, but accumulated in the optic lobes and more precisely in the outermost layer of the medulla and in the lamina, where they formed intracellular inclusions. We interpret this finding to mean either that degradative pathways operate with different efficiencies in the various glial subtypes or that secretion of ferritin is somehow affected in the lamina glia. It is interesting to note that iron trafficking has been described in mammalian retina (Hadziahmetovic et al.; Hahn et al., 2004), whereas the pathways securing an adequate supply of iron to the *Drosophila* retina, and whether ferritin has a role to play (Mehta et al., 2009), have not been elucidated to date.

A link between brain iron homeostasis and the circadian clock in the *Drosophila* model

The accumulation of iron-loaded ferritin in glia when both ferritin subunits were overexpressed simultaneously suggests that mis-regulation of brain iron homeostasis may result in failure to maintain circadian rhythms and an overall behavioural decline at late age. The anatomical location of the ferritin inclusions raises the intriguing possibility that the function of the Hofbauer-Buchner eyelet, previously shown to participate in the synchronization and entrainment of natural rhythms (Veleri et al., 2007), might be affected as a consequence of ferritin aggregation. Indeed, the eyelet sends projections through the

chiasma between the lamina and medulla (Helfrich-Förster *et al.*, 2002) and might be physically or metabolically constrained by the presence of crystalline formations of iron-loaded ferritin in surrounding glia. Experiments in this study support a previously suggested role of glia in the maintenance of the circadian clock (Jackson, 2010). We propose that *Drosophila* is a useful model to elucidate interactions between iron homeostasis and the endogenous clock.

A poorly understood connection between brain iron metabolism and the dopaminergic neural network is sometimes invoked in explanations of the Restless legs syndrome (RLS) pathology (Clemens *et al.*, 2006; Dowling *et al.*, 2011; Earley *et al.*, 2006; Patrick, 2007; Salas *et al.*, 2010). RLS is a condition characterized by circadian timing of movement disorder. Some patients with RLS respond well to dietary iron supplementation (Patrick, 2007; Wang *et al.*, 2009). Tyrosine hydroxylase, an iron-dependent enzyme in the dopamine biosynthetic pathway, may be induced despite (or as a consequence of) systemic iron deficiency (Connor *et al.*, 2009), a finding that remains poorly understood. As iron deficiency is a serious risk factor for developing RLS (Mizuno *et al.*, 2005, Salas *et al.*, 2010), it will be interesting to investigate whether the iron accumulation in glial ferritin inclusions, upon ferritin overexpression, may deplete bioavailable iron for other cellular functions. Future experiments aimed to uncover links between ferritin, the circadian clock and neuroaminergic outputs in the fly brain will hopefully enhance our understanding of the role of iron in the numerous human neurological disorders it has been already implicated.

Acknowledgments

We thank Manos Mavrikakis, Anuja Mehta, Boris Dunkov and Ralf Stanewsky for their critical comments on earlier drafts of the manuscript and funding from the Marie Curie International Reintegration Grant “DrosoFela” (F.M.), the EPAN-YB13 grant from the Greek General Secretariat of Research and Technology (S.K.) and the intramural programme of the National Institute of Child Health and Human Development (F.M. and T.A.R.).

References

- Anderson PR, *et al.* Hydrogen peroxide scavenging rescues frataxin deficiency in a *Drosophila* model of Friedreich's ataxia. *Proc Natl Acad Sci U S A.* 2008; 105:611–6. [PubMed: 18184803]
- Apostol BL, *et al.* A cell-based assay for aggregation inhibitors as therapeutics of polyglutamine-repeat disease and validation in *Drosophila*. *Proc Natl Acad Sci U S A.* 2003; 100:5950–5. [PubMed: 12730384]
- Beaumont C, *et al.* Mutation in the iron responsive element of the L ferritin mRNA in a family with dominant hyperferritinaemia and cataract. *Nat Genet.* 1995; 11:444–6. [PubMed: 7493028]
- Bosveld F, *et al.* De novo CoA biosynthesis is required to maintain DNA integrity during development of the *Drosophila* nervous system. *Hum Mol Genet.* 2008; 17:2058–69. [PubMed: 18407920]
- Chotard C, Salecker I. Glial cell development and function in the *Drosophila* visual system. *Neuron Glia Biol.* 2007; 3:17–25. [PubMed: 18333286]
- Clark IE, *et al.* *Drosophila* pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature.* 2006; 441:1162–6. [PubMed: 16672981]
- Clemens S, *et al.* Restless legs syndrome: revisiting the dopamine hypothesis from the spinal cord perspective. *Neurology.* 2006; 67:125–30. [PubMed: 16832090]
- Connor JR, *et al.* Altered dopaminergic profile in the putamen and substantia nigra in restless leg syndrome. *Brain.* 2009; 132:2403–12. [PubMed: 19467991]
- Crouch PJ, *et al.* Increasing Cu bioavailability inhibits Abeta oligomers and tau phosphorylation. *Proc Natl Acad Sci U S A.* 2009; 106:381–6. [PubMed: 19122148]
- Curtis AR, *et al.* Mutation in the gene encoding ferritin light polypeptide causes dominant adult-onset basal ganglia disease. *Nat Genet.* 2001; 28:350–4. [PubMed: 11438811]
- Dias-Santagata D, *et al.* Oxidative stress mediates tau-induced neurodegeneration in *Drosophila*. *J Clin Invest.* 2007; 117:236–45. [PubMed: 17173140]

- Dowling P, et al. Dopamine D₃ receptor specifically modulates motor and sensory symptoms in iron-deficient mice. *J Neurosci*. 2011; 31:70–7. [PubMed: 21209191]
- Earley CJ, et al. MRI-determined regional brain iron concentration in early- and late-onset restless legs syndrome. *Sleep Med*. 2006; 7:395–6. [PubMed: 16815752]
- Faust K, et al. Neuroprotective effects of compounds with antioxidant and anti-inflammatory properties in a *Drosophila* model of Parkinson's disease. *BMC Neurosci*. 2009; 10:109. [PubMed: 19723328]
- Fernandez-Funez P, et al. Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature*. 2000; 408:101–6. [PubMed: 11081516]
- Georgieva T, et al. *Drosophila melanogaster* ferritin: cDNA encoding a light chain homologue, temporal and tissue specific expression of both subunit types. *Insect Biochem Mol Biol*. 2002; 32:295–302. [PubMed: 11804801]
- Ghosh MC, et al. Tempol-mediated activation of latent iron regulatory protein activity prevents symptoms of neurodegenerative disease in IRP2 knockout mice. *Proc Natl Acad Sci U S A*. 2008; 105:12028–33. [PubMed: 18685102]
- Girardot F, et al. Specific age-related signatures in *Drosophila* body parts transcriptome. *BMC Genomics*. 2006; 7:69. [PubMed: 16584578]
- Girardot F, et al. Genome wide analysis of common and specific stress responses in adult *drosophila melanogaster*. *BMC Genomics*. 2004; 5:74. [PubMed: 15458575]
- Gregory A, et al. Clinical and genetic delineation of neurodegeneration with brain iron accumulation. *J Med Genet*. 2009; 46:73–80. [PubMed: 18981035]
- Gruenewald C, et al. Hyperoxia-induced neurodegeneration as a tool to identify neuroprotective genes in *Drosophila melanogaster*. *Free Radic Biol Med*. 2009; 46:1668–76. [PubMed: 19345730]
- Gutierrez L, et al. Zinc accumulation in heterozygous mutants of fumble, the pantothenate kinase homologue of *Drosophila*. *FEBS Lett*. 584:2942–6. [PubMed: 20493851]
- Hadziahmetovic M, et al. Age-dependent retinal iron accumulation and degeneration in Hpcidin knockout mice. *Invest Ophthalmol Vis Sci*.
- Hahn P, et al. Disruption of ceruloplasmin and hephaestin in mice causes retinal iron overload and retinal degeneration with features of age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2004; 101:13850–5. [PubMed: 15365174]
- Hamblen MJ, et al. Molecular and behavioral analysis of four period mutants in *Drosophila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. *Genetics*. 1998; 149:165–78. [PubMed: 9584094]
- Hao LY, et al. DJ-1 is critical for mitochondrial function and rescues PINK1 loss of function. *Proc Natl Acad Sci U S A*. 107:9747–52. [PubMed: 20457924]
- Helfrich-Förster C, et al. The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function. *J Neurosci*. 2002; 22:9255–66. [PubMed: 12417651]
- Hentze MW, et al. Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. *Science*. 1987; 238:1570–3. [PubMed: 3685996]
- Jackson FR. Glial cell modulation of circadian rhythms. *Glia* n/a. 201010.1002/glia.21097
- Kaur D, et al. Chronic expression of H-ferritin in dopaminergic midbrain neurons results in an age-related expansion of the labile iron pool and subsequent neurodegeneration: implications for Parkinson's disease. *Brain Res*. 2009; 1297:17–22. [PubMed: 19699718]
- Kosmidis S, et al. Differential effects of Tau on the integrity and function of neurons essential for learning in *Drosophila*. *J Neurosci*. 2010; 30:464–77. [PubMed: 20071510]
- Kretzschmar D, et al. The swiss cheese mutant causes glial hyperwrapping and brain degeneration in *Drosophila*. *J Neurosci*. 1997; 17:7425–32. [PubMed: 9295388]
- LaVaute T, et al. Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. *Nat Genet*. 2001; 27:209–14. [PubMed: 11175792]
- Levi S, et al. Neuroferritinopathy: a neurodegenerative disorder associated with L-ferritin mutation. *Best Pract Res Clin Haematol*. 2005; 18:265–76. [PubMed: 15737889]

- Levine JD, et al. Advanced analysis of a cryptochrome mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. *BMC Neurosci*. 2002; 3:5. [PubMed: 11960556]
- Llorens JV, et al. Causative role of oxidative stress in a *Drosophila* model of Friedreich ataxia. *FASEB J*. 2007; 21:333–44. [PubMed: 17167074]
- Locke M, Leung H. The induction and distribution of an insect ferritin—a new function for the endoplasmic reticulum. *Tissue Cell*. 1984; 16:739–66. [PubMed: 6515641]
- Madsen E, Gitlin JD. Copper and iron disorders of the brain. *Annu Rev Neurosci*. 2007; 30:317–37. [PubMed: 17367269]
- McNeill A, et al. T2* and FSE MRI distinguishes four subtypes of neurodegeneration with brain iron accumulation. *Neurology*. 2008; 70:1614–9. [PubMed: 18443312]
- Mehta A, et al. Ferritin accumulation under iron scarcity in *Drosophila* iron cells. *Biochimie*. 2009; 91:1331–4. [PubMed: 19465081]
- Mendes CS, et al. ER stress protects from retinal degeneration. *EMBO J*. 2009; 28:1296–307. [PubMed: 19339992]
- Meulener MC, et al. Mutational analysis of DJ-1 in *Drosophila* implicates functional inactivation by oxidative damage and aging. *Proc Natl Acad Sci U S A*. 2006; 103:12517–22. [PubMed: 16894167]
- Missirlis F, et al. A putative glutathione peroxidase of *Drosophila* encodes a thioredoxin peroxidase that provides resistance against oxidative stress but fails to complement a lack of catalase activity. *Biol Chem*. 2003; 384:463–72. [PubMed: 12715897]
- Missirlis F, et al. Characterization of mitochondrial ferritin in *Drosophila*. *Proc Natl Acad Sci U S A*. 2006; 103:5893–8. [PubMed: 16571656]
- Missirlis F, et al. Homeostatic mechanisms for iron storage revealed by genetic manipulations and live imaging of *Drosophila* ferritin. *Genetics*. 2007; 177:89–100. [PubMed: 17603097]
- Mizuno S, et al. CSF iron, ferritin and transferrin levels in restless legs syndrome. *J Sleep Res*. 2005; 14:43–7. [PubMed: 15743333]
- Molina-Holgado F, et al. Metals ions and neurodegeneration. *Biomaterials*. 2007; 20:639–54. [PubMed: 17294125]
- Mollereau B. Cell death: what can we learn from flies? Editorial for the special review issue on *Drosophila* apoptosis. *Apoptosis*. 2009; 14:929–34. [PubMed: 19629695]
- Navarro JA, et al. Altered lipid metabolism in a *Drosophila* model of Friedreich's ataxia. *Hum Mol Genet*. 2010; 19:2828–40. [PubMed: 20460268]
- Outeiro TF, et al. Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science*. 2007; 317:516–9. [PubMed: 17588900]
- Pandey UB, et al. HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. *Nature*. 2007; 447:859–63. [PubMed: 17568747]
- Park J, et al. Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by parkin. *Nature*. 2006; 441:1157–61. [PubMed: 16672980]
- Patrick LR. Restless legs syndrome: pathophysiology and the role of iron and folate. *Altern Med Rev*. 2007; 12:101–12. [PubMed: 17604457]
- Pereanu W, et al. Morphogenesis and proliferation of the larval brain glia in *Drosophila*. *Dev Biol*. 2005; 283:191–203. [PubMed: 15907832]
- Rana A, et al. Pantethine rescues a *Drosophila* model for pantothenate kinase-associated neurodegeneration. *Proc Natl Acad Sci U S A*. 107:6988–93. [PubMed: 20351285]
- Rival T, et al. Fenton chemistry and oxidative stress mediate the toxicity of the beta-amyloid peptide in a *Drosophila* model of Alzheimer's disease. *Eur J Neurosci*. 2009; 29:1335–47. [PubMed: 19519625]
- Robinson RA, et al. Insights into aging through measurements of the *Drosophila* proteome as a function of temperature. *Mech Ageing Dev*.
- Rouault TA, Tong WH. Iron-sulfur cluster biogenesis and human disease. *Trends Genet*. 2008; 24:398–407. [PubMed: 18606475]

- Runko AP, et al. Overexpression of frataxin in the mitochondria increases resistance to oxidative stress and extends lifespan in *Drosophila*. *FEBS Lett.* 2008; 582:715–9. [PubMed: 18258192]
- Salas RE, et al. Update in restless legs syndrome. *Curr Opin Neurol.* 23:401–6. [PubMed: 20581683]
- Sengupta R, et al. Morphine increases brain levels of ferritin heavy chain leading to inhibition of CXCR4-mediated survival signaling in neurons. *J Neurosci.* 2009; 29:2534–44. [PubMed: 19244528]
- Suh J, Jackson FR. *Drosophila* ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron.* 2007; 55:435–47. [PubMed: 17678856]
- Sweeney ST. Modelling lysosomal storage disease in *Drosophila*. *SEB Exp Biol Ser.* 2008; 60:115–39. [PubMed: 18309790]
- Tain LS, et al. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. *Nat Neurosci.* 2009; 12:1129–35. [PubMed: 19684592]
- Tonoki A, et al. Genetic evidence linking age-dependent attenuation of the 26S proteasome with the aging process. *Mol Cell Biol.* 2009; 29:1095–106. [PubMed: 19075009]
- Veleri S, et al. Hofbauer-Buchner eyelet affects circadian photosensitivity and coordinates TIM and PER expression in *Drosophila* clock neurons. *J Biol Rhythms.* 2007; 22:29–42. [PubMed: 17229923]
- Venkatachalam K, et al. Motor deficit in a *Drosophila* model of mucopolidosis type IV due to defective clearance of apoptotic cells. *Cell.* 2008; 135:838–51. [PubMed: 19041749]
- Vernace VA, et al. Aging perturbs 26S proteasome assembly in *Drosophila melanogaster*. *FASEB J.* 2007; 21:2672–82. [PubMed: 17413001]
- Vidal R, et al. Expression of a mutant form of the ferritin light chain gene induces neurodegeneration and iron overload in transgenic mice. *J Neurosci.* 2008; 28:60–7. [PubMed: 18171923]
- Wang D, et al. Antioxidants protect PINK1-dependent dopaminergic neurons in *Drosophila*. *Proc Natl Acad Sci U S A.* 2006; 103:13520–5. [PubMed: 16938835]
- Wang J, et al. Efficacy of oral iron in patients with restless legs syndrome and a low-normal ferritin: A randomized, double-blind, placebo-controlled study. *Sleep Med.* 2009; 10:973–5. [PubMed: 19230757]
- Whitworth AJ, et al. Increased glutathione S-transferase activity rescues dopaminergic neuron loss in a *Drosophila* model of Parkinson's disease. *Proc Natl Acad Sci U S A.* 2005; 102:8024–9. [PubMed: 15911761]
- Wittmann CW, et al. Tauopathy in *Drosophila*: neurodegeneration without neurofibrillary tangles. *Science.* 2001; 293:711–4. [PubMed: 11408621]
- Wu Z, et al. Aluminum induces neurodegeneration and its toxicity arises from increased iron accumulation and reactive oxygen species (ROS) production. *Neurobiol Aging.*
- Wu Z, et al. Pantothenate kinase-associated neurodegeneration: insights from a *Drosophila* model. *Hum Mol Genet.* 2009; 18:3659–72. [PubMed: 19602483]
- Xun Z, et al. Proteome Response to the Panneural Expression of Human Wild-Type alpha-Synuclein: A *Drosophila* Model of Parkinson's Disease. *J Proteome Res.* 2008; 7:3911–21. [PubMed: 18683964]
- Yang Y, et al. Inactivation of *Drosophila* DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling. *Proc Natl Acad Sci U S A.* 2005; 102:13670–5. [PubMed: 16155123]
- Zecca L, et al. Iron, brain ageing and neurodegenerative disorders. *Nat Rev Neurosci.* 2004; 5:863–73. [PubMed: 15496864]
- Zhou B, et al. A novel pantothenate kinase gene (PANK2) is defective in Hallervorden-Spatz syndrome. *Nat Genet.* 2001; 28:345–9. [PubMed: 11479594]
- Zhu W, et al. Genetic iron chelation protects against proteasome inhibition-induced dopamine neuron degeneration. *Neurobiol Dis.* 2009

Figure 1.

Age dependent behavioural deficits caused by ferritin overexpression in glia. A, B) Elicited escape response of flies was assessed on a weekly basis. The first bar on each graph corresponds to the first day of the first week and so on for six weeks. Control flies (carrying the driver transgene only, i.e. *Repo-Gal4/+*) and flies expressing either the heavy or the light chain alone did not exhibit statistical differences in their escape response until the fifth week. However at 6 weeks post-eclosion their response was significantly different than that of all younger flies (Tukey-Kramer $\alpha=0.01$). In contrast, flies overexpressing both ferritin subunits in glia showed a highly significant impairment at 5 and 6 weeks of age (Tukey-Kramer $\alpha=0.001$). The delay in the vertical direction had an even earlier onset at 4 weeks (Tukey-Kramer $\alpha=0.01$). Planned comparisons of the vertical and horizontal escape response between *Repo-Gal4/+* and flies expressing ferritin subunits at 6 weeks revealed a highly significant difference ($p<0.001$) only with flies expressing simultaneously both subunits. C) Age dependent loss of circadian rhythmicity caused by ferritin overexpression in glia. Locomotor activity was monitored automatically during the third and sixth weeks of ageing male flies. Five representative outputs spanning six days were averaged and plotted (output days 2, 3, 4, 5 are duplicated in plots to help visualize the rhythmic pattern). A significant proportion of transgenic flies with simultaneous overexpression of both ferritin subunits in glia resulted in an arrhythmic behavioural phenotype during the sixth week of adult life span. This phenotype is less common during the third week of life span in sibling flies with the same genotype and in control flies carrying the transgenes in absence of a driver (*UAS-Fer1HCH*, *UAS-Fer2LCH/TM3*, *Sb*). The quantification of results is presented in Table 1.

Figure 2.

Ferritin accumulation differs when single subunits are expressed in all glia, or if both subunits are expressed simultaneously. Ferritin was overexpressed by means of the UAS/Gal4 system. A) Western blots following reducing SDS-PAGE (ferritin chains dissociate) probed with antibodies against both subunits and syntaxin as a loading control. Note that Fer2LCH (which has higher molecular weight in *Drosophila* (Georgieva et al., 2002)) accumulates when overexpressed either singly or in combination with Fer1HCH. Similarly, Fer1HCH accumulates when overexpressed either singly or in combination with Fer2LCH. B) Gel electrophoresis under non-reducing conditions maintained the integrity of ferritin complexes. The Western blot was probed with an antibody against the Fer2LCH subunit. Multiple Fer2LCH complexes were detected in brains overexpressing the single gene. Co-expression of both ferritin genes resulted in significant accumulation of assembled ferritin and absence of low molecular weight bands. C–J) Ferritin immunohistochemistry was performed in sections of the adult optic lobes of flies overexpressing Fer1HCH and Fer2LCH in glia. Flies were sectioned in sagittal orientation; optic retina, lamina and medulla are shown. Note large accumulations of Fer1HCH in glial cells in *UAS-Fer1HCH/+; Repo-Gal4/+* flies (E) but not of Fer2LCH in the same genotype (F) and, conversely, Fer2LCH (but not Fer1HCH) accumulations in *UAS-Fer2LCH/+; Repo-Gal4/+* flies (G, H). Dramatic overexpression of both subunits was seen when both subunits are co-expressed (*UAS-Fer1HCH, UAS-Fer2LCH/+; Repo-Gal4/+*) but holoferritin was primarily concentrated in the lamina (I, J).

Figure 3.

Ferritin iron inclusions accumulate specifically in glial cells of the lamina following overexpression of both ferritin subunits in all glia. A–H) Iron stainings were performed on paraffin-embedded sections of 40-day old flies. Close up views from laminae are juxtaposed to the entire optic lobes. Note that iron accumulation is seen only in laminae of flies overexpressing both ferritin subunits using the *Repo-Gal4* driver line. Iron is detected in a punctate pattern, but larger inclusions are also visible (arrows in G, H). I–L) Plastic embedded sections from 38-day old fly heads stained with toluidine blue. Abnormal inclusions (arrow in L) are seen only in the lamina and only in flies overexpressing both ferritin subunits in glia (*Repo-Gal4/UAS-Fer1HCH, UAS-Fer2LCH*).

Figure 4.

Electron micrographs of the iron-ferritin inclusions (white arrows). Inclusions were only found in the lamina of optic lobes. Transverse (A) and longitudinal (B) views of the rodlike shaped inclusions at 16,000X and 1,000X magnifications are shown, respectively. The electron-dense material showed symmetric properties consistent with a putative crystalline structure and inclusions are found within the endomembrane system (black arrows point to membranes in C; image at 80,000X). D) No significant differences were noted in total or mean life span of flies with targeted overexpression of ferritin subunit(s) in all glia.

Effects of ferritin overexpression on rhythmic behaviour of flies kept in constant darkness. Longer than 24h periods observed in the genotypes are attributed to the *per^{SLH}* allele (Hamblen et al., 1998), present in both transgenic *Gal4* stocks used.

Table 1

Genotype	Age	N	% Rhythmic	Period	R.S.
<i>TM3, Sb/UAS-Fer1HCH, UAS-Fer2LCH</i>	3 wk	16	93	26.2 ± 1.4	2.7 ± 0.7
<i>Repo-Gal4/UAS-Fer1HCH, UAS-Fer2LCH</i>	3 wk	16	69	26.3 ± 1.9	2.6 ± 1.1
<i>TM3, Sb/UAS-Fer1HCH, UAS-Fer2LCH</i>	6 wk	13	84	26.2 ± 2.5	2.4 ± 1.0
<i>Repo-Gal4/UAS-Fer1HCH, UAS-Fer2LCH</i>	6 wk	28	57	25.7 ± 2.6	1.7 ± 0.7
<i>Nrv2-Gal4/UAS-Fer1HCH, UAS-Fer2LCH</i>	6 wk	16	63	26.1 ± 2.6	2.7 ± 1.1
<i>Nrv2-Gal4/+</i>	6 wk	11	91	26.9 ± 2.1	2.5 ± 1.0
<i>TM3, Sb/UAS-Fer1HCH</i>	6 wk	14	85	26.3 ± 0.9	2.6 ± 0.9
<i>Repo-Gal4/UAS-Fer1HCH</i>	6 wk	32	75	26.4 ± 1.9	2.3 ± 1.3
<i>Nrv2-Gal4/UAS-Fer1HCH</i>	6 wk	15	80	26.8 ± 2.1	2.1 ± 0.8
<i>TM3, Sb/UAS-Fer2LCH</i>	6 wk	14	85	26.7 ± 0.4	3.1 ± 1.1
<i>Repo-Gal4/UAS-Fer2LCH</i>	6 wk	24	54	24.9 ± 2.7	1.9 ± 1.2
<i>Nrv2-Gal4/UAS-Fer2LCH</i>	6 wk	15	80	26.1 ± 2.7	2.1 ± 0.8