Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Biol Trace Elem Res. Author manuscript; available in PMC 2020 May 01.

Published in final edited form as:

Biol Trace Elem Res. 2019 May ; 189(1): 241–250. doi:10.1007/s12011-018-1442-7.

Iron deficiency reduces synapse formation in the *Drosophila* clock circuit

Samuel S. Rudisill[1], **Bradley R. Martin**[1], **Kevin M. Mankowski**[2], and **Charles R. Tessier**[*,2]

[1]Department of Biological Sciences, University of Notre Dame, South Bend, IN, United States

[2]Department of Medical and Molecular Genetics, Indiana University School of Medicine-South Bend, South Bend, IN, United States

Abstract

Iron serves as a critical cofactor for proteins involved in a host of biological processes. In most animals, dietary iron is absorbed in enterocytes and then disseminated for use in other tissues in the body. The brain is particularly dependent on iron. Altered iron status correlates with disorders ranging from cognitive dysfunction to disruptions in circadian activity. The exact role iron plays in producing these neurological defects, however, remains unclear. Invertebrates provide an attractive model to study the effects of iron on neuronal development since many of the genes involved in iron metabolism are conserved, and the organisms are amenable to genetic and cytological techniques. We have examined synapse growth specifically under conditions of iron deficiency in the *Drosophila* circadian clock circuit. We show that projections of the small ventrolateral clock neurons to the protocerebrum of the adult *Drosophila* brain are significantly reduced upon chelation of iron from the diet. This growth defect persists even when iron is restored to the diet. Genetic neuronal knockdown of ferritin 1 or ferritin 2, critical components of iron storage and transport, does not affect synapse growth in these cells. Together, these data indicate that dietary iron is necessary for central brain synapse formation in the fly and further validate the use of this model to study the function of iron homeostasis on brain development.

Keywords

iron; BPS; chelation; *Drosophila*; sLNv; PDF; clock; ferritin; neurodevelopment; brain; synapse

INTRODUCTION

Iron is a biological trace element that is vital to the survival of organisms across the evolutionary spectrum. Proteins requiring iron as a cofactor play critical roles in many cellular pathways, including cell proliferation, immune response, oxygen transport, and respiration[1–5]. Due to such diverse and important functions, cells use many mechanisms to tightly control the levels of bioavailable iron. The brain is particularly sensitive to defects in iron homeostasis. Iron-containing enzymes are involved in important neurodevelopmental mechanisms such as axon myelination, neurotransmitter synthesis, and neural

[*]Corresponding Author: Charles R. Tessier, Department of Medical and Molecular Genetics, Indiana University School of Medicine - South Bend, Raclin Carmichael Hall 127, 1234 Notre Dame Avenue, South Bend, IN 46617, Tel: +1 574 631 2519, crtessie@iu.edu.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

transmission[6– 8], In humans, iron deficiency is strongly associated with neurodevelopmental delay and is also a key component of restless leg syndrome whose symptoms can sometimes be significantly reduced by treatment with iron[9–12]. Furthermore, iron deficiency in mammalian models reduces dendritic elaborations, perturbs synaptic function, and results in developmental cognitive defects[13–15]. While the role of iron in brain function has been well studied in vertebrates, the neurological functions of iron in invertebrates such as insects are not well understood.

Drosophila melanogaster serves as an excellent model to study the role of iron in invertebrate brain development and function. Flies express homologs to many of the mammalian genes involved in iron homeostasis, though there are some differences which suggest the mechanisms of iron regulation in invertebrates may be somewhat unique[16,17]. For example, mammals express a transferrin receptor protein which is necessary to transport transferrin-bound iron across the blood brain barrier[18]. The fly genome however, does not seem to contain a transferrin receptor gene. In flies, ferritin is the protein considered to be the primary iron storage and transport molecule[19]. A holoferritin complex consisting of 12 ferritin 1 heavy chain subunits and 12 ferritin 2 light chain subunits is capable of binding over 1000 iron atoms[20]. Ferritin is believed to bind dietary iron in the gut where it is then secreted to disseminate iron throughout the body. Ferritin, however, is also expressed in the fly brain[21]. Indeed, ferritin is required during embryogenesis for proper formation of both the central and peripheral nervous systems[22]. While the extent of ferritin's cellular function is unknown, ferritin is also required to regulate circadian activity. Neuronal knockdown of either ferritin 1 or ferritin 2 leads to arrhythmic behavior of adult animals, though ferritin 2 does seem to be more critical to this process[23]. In addition to ferritin, several other proteins involved in regulating the bioavailability of iron also regulate behavioral rhythmicity. Together, these findings indicate that proper control of iron homeostasis specifically in the brain is necessary for circadian circuit function. It is unknown, however, at what level of neuronal development iron functions in this circuit.

Circadian activity in *Drosophila* is mediated by a well characterized group of pacemaker neurons. In brief, light signals are transmitted from the Hofbauer-Buchner eyelet photoreceptors in the compound eye to large and small ventrolateral neurons in the central brain, which connect the signal to neurons in the dorsal protocerebrum[24,25]. Since deficiencies in iron-binding proteins affect the behavioral output of this circuit, we wanted to determine whether or not the synaptic structure of neurons involved in this neural relay system was affected by manipulation of iron. Defects in the structure of the small ventrolateral neuron projections have been associated with defects in circadian activity[26,27]. We therefore analyzed their synaptic structure during times of iron deficiency. Iron deficiency generated throughout development or acutely during adulthood, produced significant deficits in synapse formation in these primary fly circadian neurons. These defects were unable to be rescued by restoring iron concentrations to normal levels. Synaptic development of these neurons was also not impacted by neuronal knockdown of ferritin 1 or 2, suggesting that the previously characterized effects of knockdown of these proteins on circadian activity may occur via another mechanism. These results indicate an important role for dietary iron in synapse development but suggest that the mechanism of synapse regulation may be multi-fold.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

METHODS

Drosophila stocks

The genetic control strain w^{1118} was used for all iron chelation analyses. Elav-gal4 was crossed to w^{1118} and the heterozygous progeny were used as the control for RNAi experiments. UAS- ferritin 1 RNAi (y^1 v^1; P{TRiP.HMC04808}attP2, stock number: 60000) and UAS-ferritin 2 RNAi (y^1 sc* v^1; P{TRiP.HMS02784}attP40, stock number: 44067) were obtained from the Bloomington Drosophila Stock Center. Each RNAi line was crossed to Elav-gal4 flies so that the construct and the driver were both heterozygous in the experimental animals. All animals were maintained on standard cornmeal molasses agar (except for iron chelation experiments) at 25 °C with 12 h light/dark cycling.

Bathophenanthrolinedisulfonic acid disodium salt hydrate administration

The iron chelator bathophenanthrolinedisulfonic acid (BPS, Alfa Aesar) was made as a 10 mM stock solution and stored in the dark at room temperature until needed. Standard cornmeal molasses agar was liquefied, cooled to 40 °C, and supplemented with final concentrations of 100 μM or 200 μM developed on this food for their complete life cycle. Upon pupal eclosion, animals were transferred to fresh BPS treated food and aged 3–4 days when they were dissected. 2) Adult flies laid eggs directly on normal food. Embryos hatched and developed on this food for their complete life cycle. Upon pupal eclosion, animals were transferred to fresh BPS treated food and aged 3–4 days when they were dissected. 3) Adult flies laid eggs directly on BPS treated food. Embryos hatched and developed on this food for their complete life cycle. Upon pupal eclosion, animals were transferred to fresh normal food and aged 3–4 days when they were dissected.

Immunohistochemistry

To control for potential time of day differences in sLN_v arbor growth, all animals were dissected between *zeitgeber* 3 and 6. Adult *Drosophila* brains were dissected in 1X phosphate buffer saline (PBS) and fixed in a 1X PBS, 4% formaldehyde, 4% sucrose solution for 30 min at 25 °C. Brains were then washed three times in wash buffer (1X PBS; 1% BSA; 0.2% Triton X-100) for 30 minutes each at 25 °C, incubated in anti-PDF antibody (1:5 dilution, Developmental Studies Hybridoma Bank) for 12–16 h at 4 °C, then washed three times in wash buffer for 30 min each before incubation with Alexa Fluor 488 conjugated goat anti-mouse IgG secondary antibody (1:2000 dilution, Jackson Laboratories) for 1–2 h at 25 °C. Brains were then washed three times for 30 min each at 25 °C and mounted on glass slides in Fluoromount G (eBioscience, Inc.).

Confocal Microscopy

A Zeiss LSM 710 confocal microscope was used to acquire all images. Whole brain Z-stack images were obtained using a 10X (1.3 na) air objective and 2X zoom. Z-stacks of the left and right sLN_v arbors were obtained using a 40X (1.3 na) oil objective and 3X zoom. These images were used for analysis of synaptic growth.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Synaptic Growth Analysis

Imaris 8.2.0 (Bitplane, Inc.) was used to analyze development of the small ventrolateral neurons. PDF-positive puncta within the sLN_vs were quantified using the Imaris Spot algorithm. For this program, regions of interest around the dorsal lateral projects were drawn, the estimated XY diameter was set at 1 μm, and background subtraction was turned on. To account for antibody labeling variations, threshold intensities were optimized for each image. Only spots between the point of bifurcation and terminal branches were quantified. Values from the two paired sLN_v arbors for each animal were averaged to produce a single data point.

Quantitative Reverse Transcription PCR

Adult brains were dissected in 1XPBS, transferred to 0.5mL TRIzol reagent (Ambion) and stored at −80°C. Fifteen brains were pooled for each biological replicate. Extraction was done according to TRIzol manufacturer's specifications. Total RNA was treated with Turbo DNase (Invitrogen) and quantified by absorbance at 260nm on a Take3 Micro-Volume Plate Reader (BioTek). 500ng of RNA from each sample were then used to make cDNA using Superscript III Reverse Transcriptase and random hexamer primers (Invitrogen). 2μL of cDNA was used in reactions using Power SYBR Green Master Mix (Applied Biosystems) and qPCR was performed on a BioRad CFX Connect Real Time System thermocycler. Each sample for each primer set was run in triplicate and a melt profile was observed for each primer set to ensure single product amplifications. PCR cycling parameters were as follows: 96°C - 10min (94°C - 30s, 60°C - 30s. 72°C - 30s) × 40 cycles, 72°C - 1min. *fer1HCH* and *fer2LCH* primers were the same as previously used[28]. Primers for *alpha tubulin* were as follows: forward: 5' -ACGTTTGTCAAGCCTCATAGC - 3'; *alpha tubulin* reverse: 5'-GAGATACATTCACGCATATTGAGTT - 3'. ΔΔCt calculations were performed using *alpha tubulin* as the reference to determine fold change of the target transcript.

Statistical Analysis

One-way ANOVAs were performed on all data sets followed by a Tukey's post hoc multiple comparisons test. In all cases, p values are represented as $*0.01 < p < 0.05$, $**0.001 < p < 0.01$.

RESULTS

Iron is an essential nutrient, and while iron deficiency has long been associated with neurological defects including aberrant circadian activity, the role of iron in synapse formation remains uncertain. To determine iron's effects on synapse formation, we exploited *Drosophila* by analyzing the development of the fly's primary circadian activity control circuit in the brain. The small ventrolateral neurons (sLN_vs) are a well characterized group of cells which control sleep and circadian activity in flies and are clearly identified by expression of the neuropeptide Pigment Dispersing Factor (PDF). PDF-expressing cells receive input from the photoreceptors in the compound eye and send contralateral projections across the midline to the protocerebrum where they bifurcate into dorsal and lateral branches (Fig 1). The termini of these branches form punctate PDF-positive

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

structures which are indicative of synapses[26]. The number of these PDF-positive puncta can be quantified to assess synapse formation in this circuit.

BPS is a common iron chelation agent which effectively inhibits uptake of iron into *Drosophila* enterocytes[21]. To investigate the role of iron deficiency on sLN_v synapse development, *Drosophila* were reared on food containing either 100 μM or 200 μM BPS as both of these concentrations have been used to effectively chelate iron from standard fly food[29]. Animals reared on standard fly food lacking any supplements were raised in parallel and used as controls. Adult *Drosophila* were allowed to lay embryos onto these food conditions and experimental animals developed completely on their respective food. As the sLN_v PDF-positive arbor is well formed by eclosion (Online Resource 1), animals were then aged on their conditioned food until 3 to 4 days post-eclosion (Fig 2A). Brains were then dissected, stained with anti-PDF antibodies, and the PDF-positive synaptic puncta of the sLN_vs were quantified (Figs 2B and 2C). Flies fed 200 μM BPS had a statistically significant decrease in PDF-positive sLN_v puncta relative to control untreated samples (control: 40.2 ±1.2, n=29; 200 μM BPS: 33.6 ± 1.1, n=32, p=0.001). 100 μM BPS treatment also appeared to reduce the number of PDF-positive synapses in these cells, but the decrease was not statistically significant (100 μM BPS: 36.6 ± 1.4, n=29, p=0.11). These data demonstrate that iron is necessary for proper synaptic connections in the *Drosophila* circadian circuit and also suggest that there are likely differences in the amount of iron chelated by different concentrations of BPS.

To determine if the decreases in synapse growth caused by iron chelation were dependent on the development of the fly, *Drosophila* were raised on standard fly food and then switched within 1 day of eclosion to food containing BPS (Fig 3A). Flies were kept on this iron deficient food until 3–4 days of age and then the sLN_vs were analyzed as before. This acute exposure to iron chelation in the adult animal similarly reduced the number of PDF-positive synaptic puncta in 200 μM treated animals (Fig 3B. control 51.9 ± 1.6 n=47; 200 μM BPS: 43.5 ± 1.7, n=47, p=0.002), and slightly reduced, though to a lesser extent, the synaptic arbors in animals treated with 100 μM BPS (100 μM BPS: 45.3 ± 1.6, n=50, p=0.017). These findings suggest that iron deficiency even in an adult fly can have a significant impact on synapse formation.

Since sLN_v synaptic arbor development was significantly attenuated under iron deficient conditions, we wanted to determine whether or not restoration of normal iron content could reverse the observed undergrowth. Animals were therefore raised on iron deficient conditions throughout development as before in Fig 2. All animals were then switched within 1 day of eclosion to normal food lacking any supplements (Fig 4A). Animals were maintained on normal food for 3–4 days and then analyzed for PDF-positive synaptic growth. As before, 200 μM BPS treated animals had a significantly reduced number of PDF-positive puncta when treated in this manner (Fig 4B. control: 39.5 ± 1.3, n=23; 200 μM BPS: 34.981 ± 1.028, n=26, p=0.0341) indicating that the synaptic changes caused by iron deficiency were not affected by returning the animals to normal food. The result of this feeding paradigm was similar, though milder, in the 100 μM treated animals where the number of synaptic puncta were nearly significantly reduced relative to controls (100 μM

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

BPS: 35.3 ± 1.4, n=26, p=0.053) (Fig 4). Thus, it is possible that the level of iron chelation may affect the capacity of this circuit to remodel after restoration of the dietary iron supply.

Drosophila ferritin is a well characterized iron storage and transport molecule consisting of 2 subunits, ferritin 1 heavy chain and ferritin 2 light chain[20]. Disruption of either of these two subunits in the fly brain leads to defects in circadian activity[23]. We therefore assayed the effect of neuronal knockdown of each ferritin subunit on PDF-positive synapse formation in sLN_v neurons. UAS-RNAi constructs to either ferritin 1 or ferritin 2 were expressed using the pan-neuronal Elav-gal4 driver. Quantitative RT-PCR was used to determine the level of target transcript knockdown. We determined that both ferritin 1 RNAi and ferritin 2 RNAi reduced their respective target mRNAs by over 80% relative to controls (ferritin 1: 82% ± 2, n=3; ferritin 2: 83% ± 5, n=3). These levels are comparable to those seen previously[28], RNAi-expressing animals were therefore aged 3–4 days post-eclosion, and the sLN_v circuit was quantified as before. Neither the ferritin 1 (control: 51.8 ± 1.6, n=22; FerIRNAi: 54.8 ± 1.9, n=33, p=0.27) nor the ferritin 2 genetic knockdowns (control: 50.4 ± 2.2, n=23; Fer2RNAi: 52.0 ± 1.8, n=24, p=0.57) resulted in any change in the number of PDF-positive puncta relative to controls (Figs 5A and 5B). These results indicate that the aberrant rhythmicity seen in pan-neuronal ferritin knockdown animals is not due to alteration in the structure of these neurons.

DISCUSSION

Iron is an important dietary trace element which must be tightly controlled to avoid cellular damage. The association of excess iron with neurodegeneration and iron deficiency with neurodevelopmental delay highlights the importance of this regulation[10,30]. The specific mechanisms underlying these physiological conditions, and the specific role iron plays in their manifestation, however, remains unclear. Most of the information on the role of iron in neuronal development comes from vertebrate studies, however virtually all organisms on the planet utilize iron for redox biochemistry. Therefore, neuronal analyses in other species are likely to yield important insights into iron's function. We sought to determine the role of iron in invertebrate synapse development. We chose the small ventrolateral neurons of the *Drosophila* circadian circuit as a model for this study given their well characterized synaptic architecture and the established role of iron in affecting circadian activity[23,25]. Since excessive iron is known to lead to the formation of neurodegenerative vacuoles in fly brain, we focused this study instead on the effects of iron deficiency[28].

Iron chelation by adding BPS to *Drosophila* food is an effective means of reducing the levels of bioavailable iron. Both 100 μM and 200 μM concentrations of BPS are able to produce iron-dependent phenotypes in *Drosophila*[29,28]. In this study, 200 μM BPS reduced synaptic elaboration of the sLN_v dorsal arbor whether it was administered throughout development or acutely in adult animals (Figs 2 and 3). Halving the concentration of BPS produced similarly trending results, though they were somewhat less pronounced. While a significant reduction of PDF-positive synapses was detected when 100 μM BPS was administered acutely in adulthood, a small but not significant reduction was seen when this low level of BPS was provided throughout development. These differential results are consistent with 100 μM BPS chelating dietary iron at sub-maximal levels. This is seen too in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

flies fed BPS during development but then returned to iron sufficient food in adulthood (Fig 4). The 100 μM BPS treated animals did show a reduction in PDF-positive synapses, but the reduction in 200 μM treated animals was more significant. In all cases, the addition of BPS was well tolerated by the animals, so future studies of iron deficiency in the brain utilizing higher concentrations are more likely to produce the most robust results.

Reduction of synaptic development in *Drosophila* due to iron chelation is consistent with studies from vertebrates (Figs 2 and 3). In both the rat and mouse hippocampus, for example, reduced levels of iron either through dietary restriction or genetic alterations reduces the growth of dendrites specifically within the CA1 region[14,15]. Cortical neurons are also affected by iron deficiency though changes in branch number as opposed to dendritic length seem to be the prevailing phenotype in that brain region[31]. Similarly, structural changes have also been observed in developing neurons in culture[32]. Therefore, irrespective of the model system, iron depletion results in reduced neuronal development. This is interesting because vertebrates and invertebrates handle at least some parts of iron homeostasis through different mechanisms. For example, the blood brain barrier of vertebrates is different than that of flies, requiring transferrin receptors, which flies lack, to import iron from the blood[33,34]. Flies also lack erythropoiesis, which is the primary iron utilization pathway in vertebrates[35,17]. These are significant differences in iron homeostasis but the finding that each system reduces neuronal elaborations as a consequence of iron depletion suggests a highly conserved involvement of iron in brain development.

Further supporting the conservation of iron's role in brain development is the finding that restoration of iron to the diet does not rescue the sLN_v synaptic defects caused by chelation in developing flies (Fig 4). Persistent effects of early developmental iron deficiency have long been known from experiments in vertebrate models and human patients. Children exposed to iron deficiency pre- or postnatally experience cognitive defects for many years despite iron supplementation[36–38]. Also in mice and rats, learning and memory behavioral defects assayed in a variety of testing paradigms remain defective well after animals have been restored to an iron sufficient diet[39–41,13]. At the structural level, dendrites in the rat hippocampus typically shorten over time but actually over grow when iron deficient animals are placed on iron sufficient food[42]. Similarly, synaptic plasticity as measured by long term potentiation (LTP) declines over time in control rat hippocampal neurons[14]. LTP is normal in young rats which were iron deficient prenatally but ultimately becomes reduced relative to control animals raised completely on iron sufficient food. Furthermore, gene expression analyses in rats and mice under iron deficient conditions show persistent alterations well into adulthood[32,43]. It is therefore clear that in vertebrates, the presence of iron during a defined developmental window is critical for establishing proper neuronal pathways. Whether such a critical period exists in *Drosophila* has yet to be defined. However, failure of an iron sufficient diet to rescue the reduced number of PDF-positive synapses caused by iron deficiency suggests that the developmental program in these neurons is indeed shaped by iron. It will also be important to determine whether the molecular, physiological, and behavioral defects seen in other iron deficient animals persist in these iron deficient flies.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

It is interesting to note, that acute administration of BPS in adult animals also reduces synapse formation (Fig 3). During development, specific circuit formation pathways may be irreversibly altered by low iron as described above, but in adulthood, these mechanisms are less likely to be affected. One mechanism in which acute adult iron chelation may reduce synapse formation in adults, however, is by affecting sLN_v synaptic pruning. sLN_v structure expands and contracts over the 24-hour light-dark period as part of a normal circadian cycling program[44,27]. Both the synaptic arbor itself, and the synaptic contacts made throughout the 24-hour period are dynamic. It is possible that sufficient iron levels are required to maintain this homeostasis, and that iron chelation leads to either over-pruning of the synapses, failed regrowth by either the sLN_v or its synaptic partner, or a combination of both. An hourly examination of circuit growth and analysis under constant light or constant dark conditions could be used to differentiate among these possibilities. Furthermore, behavioral analyses in iron deficient *Drosophila* can be used to directly correlate the sLN_v defects identified here with circadian activity output. A finding, for example, that rhythmicity is affected by only acute iron chelation in adults would suggest that the changes occurring during developmental chelation are different than those that occur in adults. Such a result would indicate that iron functions at multiple levels, and that cells other than the sLN_v's are likely also affected by this treatment.

Circadian activity in flies is sensitive to genetic defects in iron homeostasis. RNAi studies have demonstrated that pan-neuronal knockdown of the iron storage and transport proteins ferritin 1 or 2, can produce arrhythmic activity in *Drosophila[23].* Our data here indicate that neither ferritin 1 nor ferritin 2 knockdown alters synapse development of the sLN_vs (Fig 5). This discrepancy may suggest that the mechanism by which ferritin expression affects circadian behavior is not structural but perhaps functional. Alternatively, it is possible that some other component of the circadian circuit is morphologically disrupted, while the sLN_vs themselves remain intact. Indeed, genetic knockdown of ferritin 2 solely in the PDF-expressing sLN_vs does not result in arrhythmic flies, while knockdown in cells expressing the clock proteins timeless or cryptochrome does produce arrhythmia[23]. Determining whether or not the structures of these other clock circuit neurons are altered by ferritin knockdown may shed more light on the role of ferritin expression on neuronal structure. Such isolated changes in other aspects of the clock circuitry would also be consistent with studies in vertebrates, where dendritic morphological defects in iron deficient animals are region specific, even within the same functional lobe[15,42].

Another interesting observation from the data presented here is that the number of PDFpositive puncta in control treated animals varied from experiment to experiment (Figs. 2–4). The source of this variation is unknown. There is no apparent sexual dimorphism of this circuit though a higher degree of individual structural variation does exist in female flies[44]. Since gender was not controlled for in our studies, it's possible that simply an unequal usage of males and females accounts for the experimental variation. Another possibility, however, is potential discrepancy in the fly food. We have showed that different levels of iron can affect sLN_v growth, so it is possible that not all batches of food contained the same levels of this trace element. Molasses is a well-known contributor of dietary iron, so differences in the amounts of molasses used, or even the batch of molasses supplied from the manufacturer, could account for changes in control animals[45,46]. In addition,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

seemingly minor variations in the amount of each food ingredient could alter the quantities of other trace elements not specifically controlled for here, and those other micronutrients could also contribute to the experimental variation. For example, copper and zinc are well characterized modulators of synaptic function so it is conceivable that changes in concentrations of these elements could also impact the formation of the sLN_V circuit[47,48]. Use of a defined food source as has been advocated for caloric intake experiments could be highly valuable for understanding how these or other micronutrients potentially affect neuronal growth[49].

Clearly many questions about how iron affects brain development in invertebrates remain unanswered. Most vertebrate neurological analyses of iron deficiency have been performed in higher cognitive brain regions due to the strong correlation in humans of iron deficiency with neurodevelopmental delay. Therefore, in order to directly compare the vertebrate and invertebrate consequences of changes in iron homeostasis on brain development, it will be important to investigate analogous higher-ordered circuits in the fly. The fly mushroom body is the central learning and memory circuit of *Drosophila* and is amenable to genetic and cytological analyses. Understanding whether or not mushroom body neurons are affected by iron deficiency and similarly whether or not learning and memory behaviors are altered by this condition, will be necessary for future comparative studies. Genetic analyses using mutations in ferritins or other iron-binding proteins will also further our understanding of the evolutionary conserved components of iron homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by a Research Enhancement Grant from the Indiana Clinical and Translational Sciences Institute and a National Institute of Mental Health grant R03MH107766, both to C.R.T.. This work was also supported by the Indiana University School of Medicine— South Bend Imaging and Flow Cytometry Core Facility. Conflict of Interest: The authors declare that they have no conflicts of interest.

References

1. Manganas P, MacPherson L, Tokatlidis K (2017) Oxidative protein biogenesis and redox regulation in the mitochondrial intermembrane space. Cell Tissue Res 367 (1):43–57. doi:10.1007/s00441-016-2488-5 [PubMed: 27632163]
2. Martins AC, Almeida JI, Lima IS, Kapitao AS, Gozzelino R (2017) Iron Metabolism and the Inflammatory Response. IUBMB Life 69 (6):442–450. doi:10.1002/iub.1635 [PubMed: 28474474]
3. Nairz M, Dichtl S, Schroll A, Haschka D, Tymoszuk P, Theurl I, Weiss G (2018) Iron and innate antimicrobial immunity-Depriving the pathogen, defending the host. J Trace Elem Med Biol 48:118–133. doi:10.1016/j.jtemb.2018.03.007 [PubMed: 29773170]
4. Yu H, Guo P, Xie X, Wang Y, Chen G (2017) Ferroptosis, a new form of cell death, and its relationships with tumourous diseases. J Cell Mol Med 21 (4):648–657. doi:10.1111/jcmm.13008 [PubMed: 27860262]
5. Lui GY, Kovacevic Z, Richardson V, Merlot AM, Kalinowski DS, Richardson DR (2015) Targeting cancer by binding iron: Dissecting cellular signaling pathways. Oncotarget 6 (22):18748–18779. doi:10.18632/oncotarget.4349 [PubMed: 26125440]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

6. Munoz P, Humeres A (2012) Iron deficiency on neuronal function. Biometals 25 (4):825–835. doi: 10.1007/s10534-012-9550-x [PubMed: 22639188]
7. Munoz P, Humeres A, Elgueta C, Kirkwood A, Hidalgo C, Nunez MT (2011) Iron mediates N-methyl-D-aspartate receptor-dependent stimulation of calcium-induced pathways and hippocampal synaptic plasticity. J Biol Chem 286 (15):13382–13392. doi:10.1074/jbc.M110.213785 [PubMed: 21296883]
8. Lu H, Chen J, Huang H, Zhou M, Zhu Q, Yao SQ, Chai Z, Hu Y (2017) Iron modulates the activity of monoamine oxidase B in SH-SY5Y cells. Biometals 30 (4):599–607. doi:10.1007/s10534-017-0030-1 [PubMed: 28685208]
9. Allen RP, Earley CJ (2007) The role of iron in restless legs syndrome. Mov Disord 22 Suppl 18:S440–448. doi:10.1002/mds.21607 [PubMed: 17566122]
10. Georgieff MK, Brunette KE, Tran PV (2015) Early life nutrition and neural plasticity. Dev Psychopathol 27 (2):411–423. doi:10.1017/S0954579415000061 [PubMed: 25997762]
11. Freeman A, Pranski E, Miller RD, Radmard S, Bernhard D, Jinnah HA, Betarbet R, Rye DB, Sanyal S (2012) Sleep fragmentation and motor restlessness in a Drosophila model of Restless Legs Syndrome. Curr Biol 22 (12):1142–1148. doi:10.1016/j.cub.2012.04.027 [PubMed: 22658601]
12. Freeman AA, Mandilaras K, Missirlis F, Sanyal S (2013) An emerging role for Cullin-3 mediated ubiquitination in sleep and circadian rhythm: insights from Drosophila. Fly (Austin) 7 (1):39–43. doi:10.4161/fly.23506 [PubMed: 23455037]
13. Unger EL, Hurst AR, Georgieff MK, Schallert T, Rao R, Connor JR, Kaciroti N, Lozoff B, Felt B (2012) Behavior and monoamine deficits in prenatal and perinatal iron deficiency are not corrected by early postnatal moderate-iron or high-iron diets in rats. J Nutr 142 (11):2040–2049. doi: 10.3945/jn.112.162198 [PubMed: 22990465]
14. Jorgenson LA, Sun M, O'Connor M, Georgieff MK (2005) Fetal iron deficiency disrupts the maturation of synaptic function and efficacy in area CA1 of the developing rat hippocampus. Hippocampus 15 (8):1094–1102. doi:10.1002/hipo.20128 [PubMed: 16187331]
15. Brunette KE, Tran PV, Wobken JD, Carlson ES, Georgieff MK (2010) Gestational and neonatal iron deficiency alters apical dendrite structure of CA1 pyramidal neurons in adult rat hippocampus. Dev Neurosci 32 (3):238–248. doi:10.1159/000314341 [PubMed: 20689287]
16. Tang X, Zhou B (2013) Iron homeostasis in insects: Insights from Drosophila studies. IUBMB Life 65 (10):863–872. doi:10.1002/iub.1211 [PubMed: 24078394]
17. Mandilaras K, Pathmanathan T, Missirlis F (2013) Iron absorption in Drosophila melanogaster. Nutrients 5 (5):1622–1647. doi:10.3390/nu5051622 [PubMed: 23686013]
18. McCarthy RC, Kosman DJ (2015) Iron transport across the blood-brain barrier: development, neurovascular regulation and cerebral amyloid angiopathy. Cell Mol Life Sci 72 (4): 709–727. doi: 10.1007/s00018-014-1771-4 [PubMed: 25355056]
19. Nichol H, Law JH, Winzerling JJ (2002) Iron metabolism in insects. Annu Rev Entomol 47:535–559. doi:10.1146/annurev.ento.47.091201.145237 [PubMed: 11729084]
20. Gutierrez L, Zubow K, Nield J, Gambis A, Mollereau B, Lazaro FJ, Missirlis F (2013) Biophysical and genetic analysis of iron partitioning and ferritin function in Drosophila melanogaster. Metallomics 5 (8):997–1005. doi:10.1039/c3mt00118k [PubMed: 23771129]
21. Mehta A, Deshpande A, Bettedi L, Missirlis F (2009) Ferritin accumulation under iron scarcity in Drosophila iron cells. Biochimie 91 (10):1331–1334. doi:10.1016/j.biochi.2009.05.003 [PubMed: 19465081]
22. Gonzalez-Morales N, Mendoza-Ortiz MA, Blowes LM, Missirlis F, Riesgo-Escovar JR (2015) Ferritin Is Required in Multiple Tissues during Drosophila melanogaster Development. PLoS One 10 (7):e0133499. doi:10.1371/journal.pone.0133499 [PubMed: 26192321]
23. Mandilaras K, Missirlis F (2012) Genes for iron metabolism influence circadian rhythms in Drosophila melanogaster. Metallomics 4 (9):928–936. doi:10.1039/c2mt20065a [PubMed: 22885802]
24. Veleri S, Rieger D, Helfrich-Forster C, Stanewsky R (2007) Hofbauer-Buchner eyelet affects circadian photosensitivity and coordinates TIM and PER expression in Drosophila clock neurons. J Biol Rhythms 22 (1):29–42. doi:10.1177/0748730406295754 [PubMed: 17229923]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

25. Chang DC (2006) Neural circuits underlying circadian behavior in Drosophila melanogaster. Behav Processes 71 (2–3):211–225. doi:10.1016/j.beproc.2005.12.008 [PubMed: 16414209]
26. Gatto CL, Broadie K (2009) Temporal requirements of the fragile x mental retardation protein in modulating circadian clock circuit synaptic architecture. Front Neural Circuits 3:8. doi:10.3389/neuro.04.008.2009 [PubMed: 19738924]
27. Gorostiza EA, Depetris-Chauvin A, Frenkel L, Pirez N, Ceriani MF (2014) Circadian pacemaker neurons change synaptic contacts across the day. Curr Biol 24 (18):2161–2167. doi:10.1016/j.cub.2014.07.063 [PubMed: 25155512]
28. Tang X, Zhou B (2013) Ferritin is the key to dietary iron absorption and tissue iron detoxification in Drosophila melanogaster. FASEB J 27 (1):288–298. doi:10.1096/fj.12-213595 [PubMed: 23064556]
29. Yoon S, Cho B, Shin M, Koranteng F, Cha N, Shim J (2017) Iron Homeostasis Controls Myeloid Blood Cell Differentiation in Drosophila. Mol Cells 40 (12):976–985. doi:10.14348/molcells.2017.0287 [PubMed: 29237257]
30. Rouault TA (2013) Iron metabolism in the CNS: implications for neurodegenerative diseases. Nat Rev Neurosci 14 (8):551–564. doi:10.1038/nrn3453 [PubMed: 23820773]
31. Greminger AR, Lee DL, Shrager P, Mayer-Proschel M (2014) Gestational iron deficiency differentially alters the structure and function of white and gray matter brain regions of developing rats. J Nutr 144 (7):1058–1066. doi:10.3945/jn.113.187732 [PubMed: 24744313]
32. Bastian TW, von Hohenberg WC, Mickelson DJ, Lanier LM, Georgieff MK (2016) Iron Deficiency Impairs Developing Hippocampal Neuron Gene Expression, Energy Metabolism, and Dendrite Complexity. Dev Neurosci 38 (4):264–276. doi:10.1159/000448514 [PubMed: 27669335]
33. Duck KA, Connor JR (2016) Iron uptake and transport across physiological barriers. Biometals 29 (4):573–591. doi:10.1007/s10534-016-9952-2 [PubMed: 27457588]
34. Stork T, Engelen D, Krudewig A, Silies M, Bainton RJ, Klambt C (2008) Organization and function of the blood-brain barrier in Drosophila. J Neurosci 28 (3):587–597. doi:10.1523/JNEUROSCI.4367-07.2008 [PubMed: 18199760]
35. Kafina MD, Paw BH (2017) Intracellular iron and heme trafficking and metabolism in developing erythroblasts. Metallomics 9 (9):1193–1203. doi:10.1039/c7mt00103g [PubMed: 28795723]
36. Congdon EL, Westerlund A, Algarin Cr, Peirano PD, Gregas M, Lozoff B, Nelson CA (2012) Iron deficiency in infancy is associated with altered neural correlates of recognition memory at 10 years. J Pediatr 160 (6):1027–1033. doi:10.1016/j.jpeds.2011.12.011 [PubMed: 22244466]
37. Felt B, Jimenez E, Smith J, Calatroni A, Kaciroti N, Wheatcroft G, Lozoff B (2006) Iron deficiency in infancy predicts altered serum prolactin response 10 years later. Pediatr Res 60 (5):513–517. doi:10.1203/01.PDR.0000242848.45999.7b [PubMed: 16966351]
38. Lozoff B, Beard J, Connor J, Barbara F, Georgieff M, Schallert T (2006) Long-lasting neural and behavioral effects of iron deficiency in infancy. Nutr Rev 64 (5 Pt 2):S34–43; discussion S72–91 [PubMed: 16770951]
39. Carlson ES, Fretham SJ, Unger E, O'Connor M, Petryk A, Schallert T, Rao R, Tkac I, Georgieff MK (2010) Hippocampus specific iron deficiency alters competition and cooperation between developing memory systems. J Neurodev Disord 2 (3):133–143. doi:10.1007/s11689-010-9049-0 [PubMed: 20824191]
40. Carlson ES, Tkac I, Magid R, O'Connor MB, Andrews NC, Schallert T, Gunshin H, Georgieff MK, Petryk A (2009) Iron is essential for neuron development and memory function in mouse hippocampus. J Nutr 139 (4):672–679. doi:10.3945/jn.108.096354 [PubMed: 19211831]
41. Felt BT, Beard JL, Schallert T, Shao J, Aldridge JW, Connor JR, Georgieff MK, Lozoff B (2006) Persistent neurochemical and behavioral abnormalities in adulthood despite early iron supplementation for perinatal iron deficiency anemia in rats. Behav Brain Res 171 (2):261–270. doi:10.1016/j.bbr.2006.04.001 [PubMed: 16713640]
42. Jorgenson LA, Wobken JD, Georgieff MK (2003) Perinatal iron deficiency alters apical dendritic growth in hippocampal CA1 pyramidal neurons. Dev Neurosci 25 (6):412–420. doi:10.1159/000075667 [PubMed: 14966382]
43. Carlson ES, Stead JD, Neal CR, Petryk A, Georgieff MK (2007) Perinatal iron deficiency results in altered developmental expression of genes mediating energy metabolism and neuronal

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

morphogenesis in hippocampus. Hippocampus 17 (8):679–691. doi:10.1002/hipo.20307 [PubMed: 17546681]

44. Fernandez MP, Berni J, Ceriani MF (2008) Circadian remodeling of neuronal circuits involved in rhythmic behavior. PLoS Biol 6 (3):e69. doi:10.1371/journal.pbio.0060069 [PubMed: 18366255]
45. Jain R, Venkatasubramanian P (2017) Sugarcane Molasses - A Potential Dietary Supplement in the Management of Iron Deficiency Anemia. J Diet Suppl 14 (5):589–598. doi: 10.1080/19390211.2016.1269145
46. Rempoulakis P, Afshar N, Osorio B, Barajas-Aceves M, Szular J, Ahmad S, Dammalage T, Tomas US, Nemny-Lavy E, Salomon M, Vreysen MJ, Nestel D, Missirlis F (2014) Conserved metallomics in two insect families evolving separately for a hundred million years. Biometals 27(4):1323–1335. doi:10.1007/s10534-014-9793-9 [PubMed: 25298233]
47. D'Ambrosi N, Rossi L (2015) Copper at synapse: Release, binding and modulation of neurotransmission. Neurochem Int 90:36–45. doi:10.1016/j.neuint.2015.07.006 [PubMed: 26187063]
48. Toth K (2011) Zinc in neurotransmission. Annu Rev Nutr 31:139–153. doi:10.1146/annurev-nutr-072610-145218 [PubMed: 21548772]
49. Lee WC, Micchelli CA (2013) Development and characterization of a chemically defined food for Drosophila. PLoS One 8 (7):e67308. doi:10.1371/journal.pone.0067308 [PubMed: 23844001]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

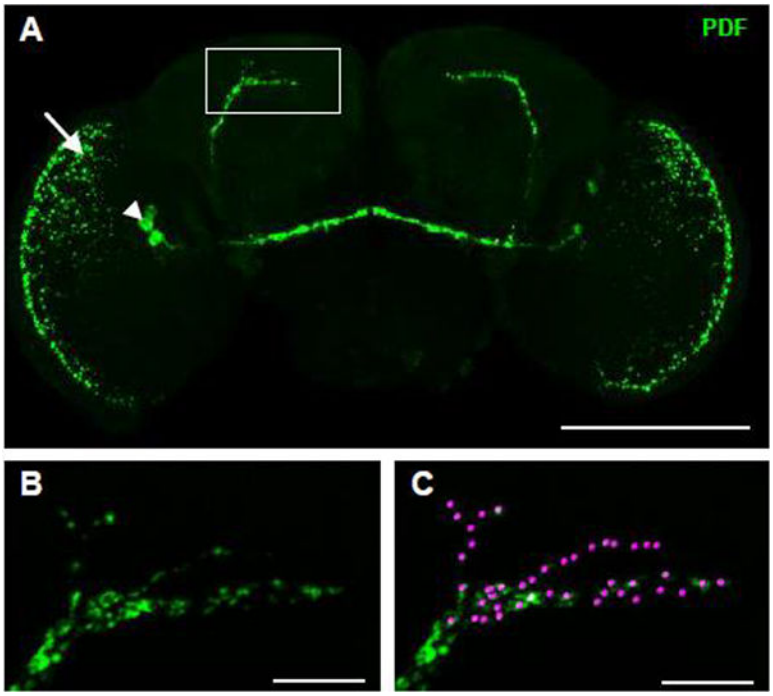

Fig 1. Small ventrolateral neurons are identified by expression of PDF

A) Representative image of a *Drosophila* brain showing PDF-positive small ventrolateral neurons (sLN_v). Input from the optic lobes is sent by contralateral projections to the protocerebrum, where they bifurcate into dorsal and lateral branches (arrow). Cell bodies are marked with arrowhead. Scale bar = 100 μm. B) Boxed inset from (A) shows magnified sLN_v arbor. Scale bar =10 μm. C) The termini of the PDF-positive sLN_v branches form punctate synapses which are identified and quantified using the Imaris Spot algorithm (purple spheres). Scale bar = 10 μm

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

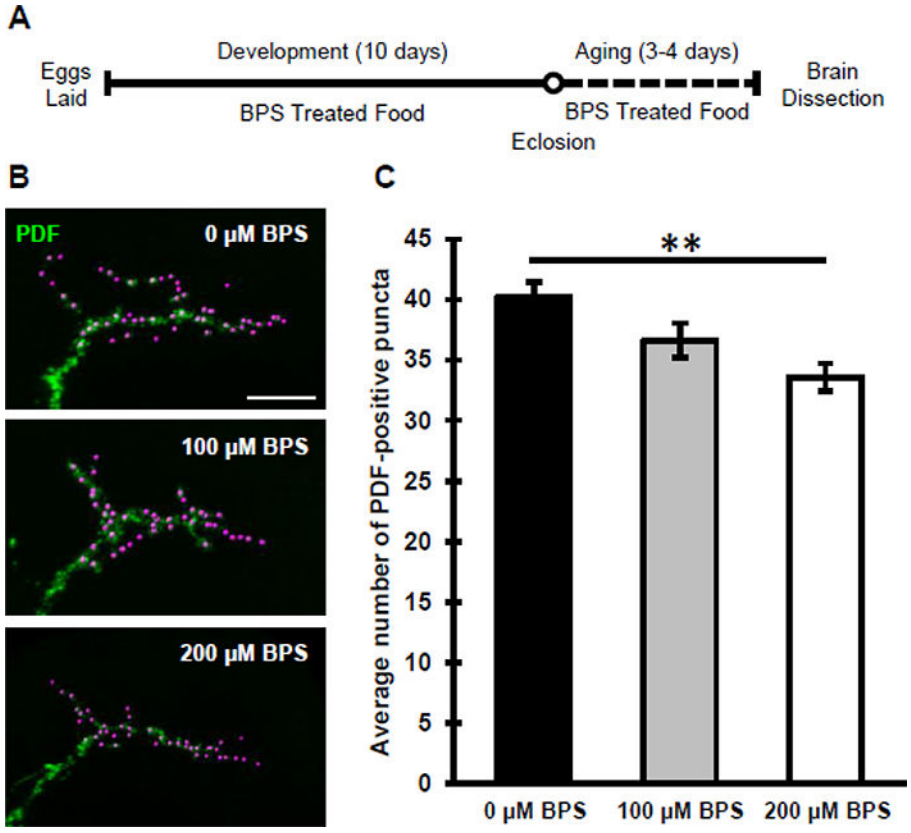

Fig 2. Iron chelation throughout development attenuates sLN_v synaptic development

A) *Drosophila* were fed BPS to chelate iron from the diet throughout development and adulthood according to the outlined paradigm. B) Representative images of PDF-positive synaptic puncta in sLN_vs of animals treated with 0 μM, 100 pM, and 200 μM BPS. Purple spheres generated by the software counting algorithm are overlaid the immunohistochemical anti-PDF stain (green). Scale bars = 10 μm. C) The average number of PDF-positive puncta quantified for each treatment condition. Values are ± SEM. **p<0.01

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

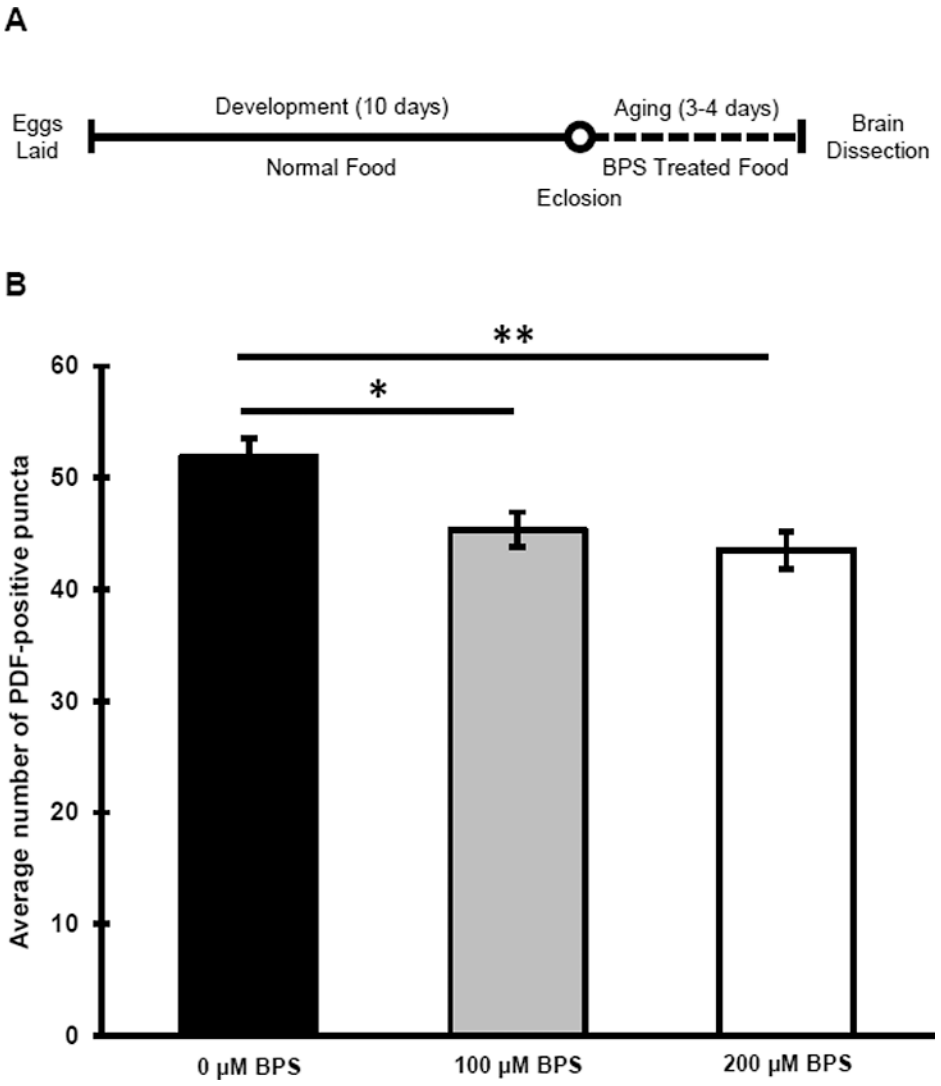

Fig 3. Acute iron chelation in adult animals attenuates sLN_v synaptic development

A) *Drosophila* were fed BPS to chelate iron from the diet only during adulthood according to the outlined paradigm. B) The average number of PDF-positive puncta quantified for each treatment condition in this paradigm. Values are ± SEM. *p<0.05, **p<0.01

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

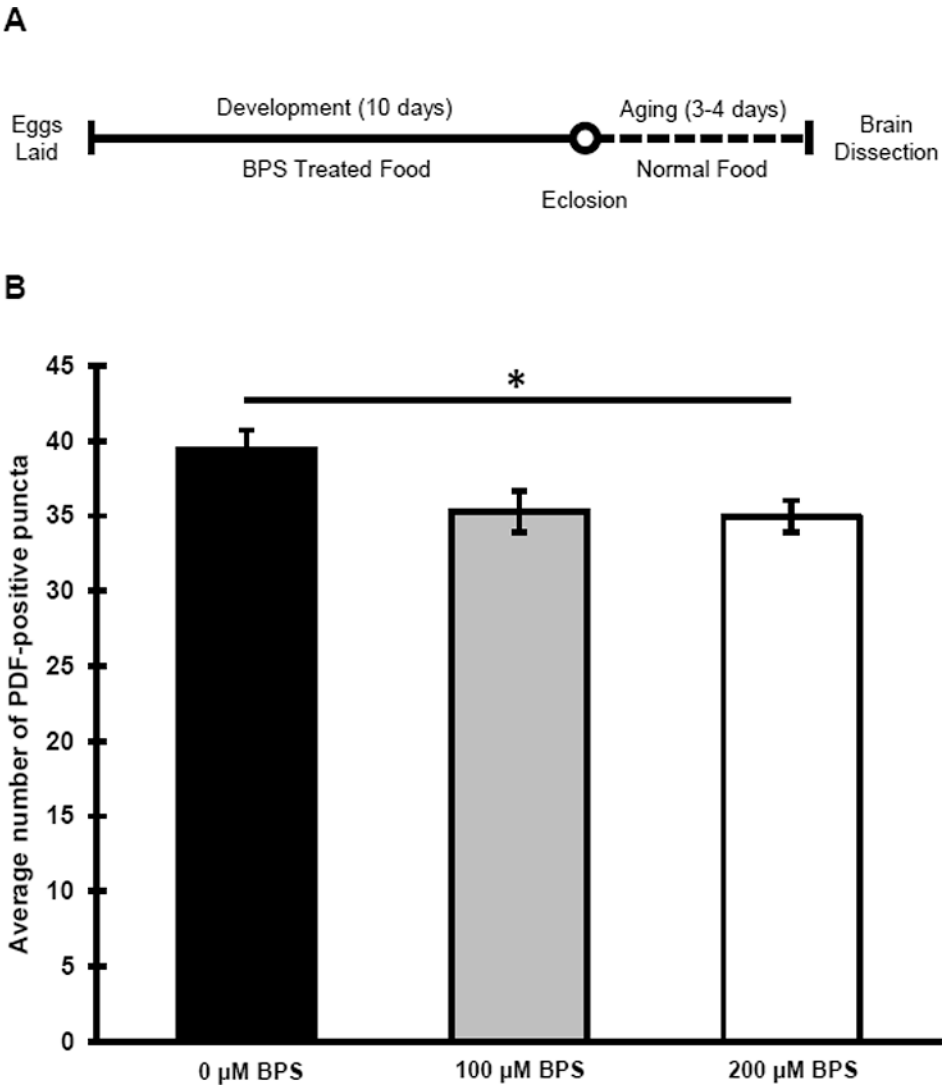

Fig 4. Restoration of iron levels after iron chelation does not rescue defects in sLN_v synapse development

A) *Drosophila* were fed BPS to chelate iron from the diet during development and then switched to normal food according to the outlined paradigm. B) The average number of PDF-positive puncta quantified for each treatment condition in this paradigm. Values are ± SEM. *p<0.05

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

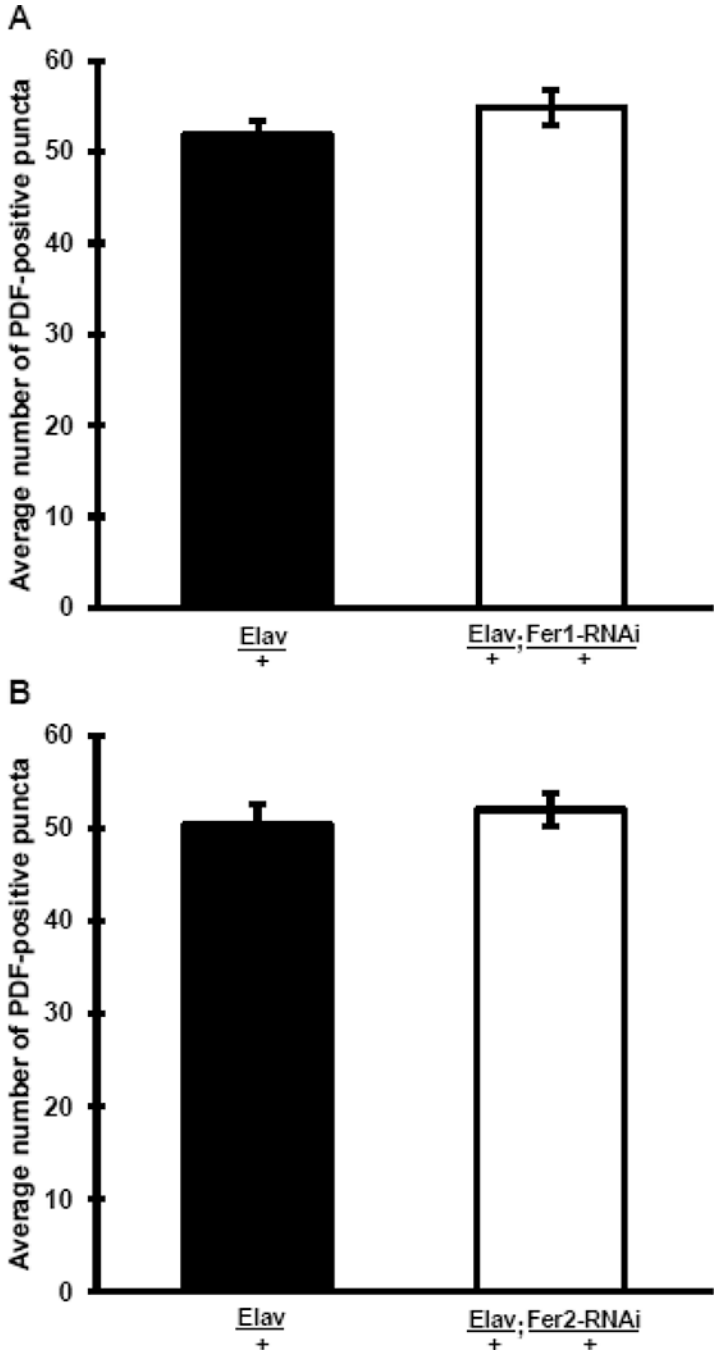

Fig 5. Neuronal knockdown of ferritin subunits does not affect PDF-positive synaptic development.

PDF-positive sLN_v synapses were quantified in flies neuronally expressing RNAi constructs to ferritin 1 (A) or ferritin 2 (B) and compared to Elav-gal4 heterozygous controls. Values are ± SEM