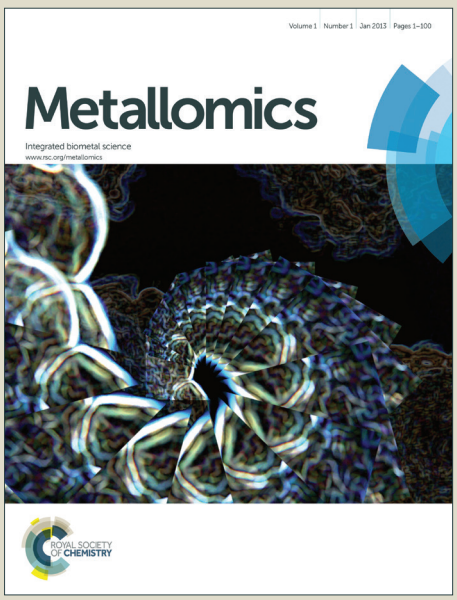
Metallomics

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/metallomics

Title:

Copper overload and deficiency both adversely affect the central nervous system of *Drosophila*

Authors:

Joab EC Hwang, Marinus de Bruyne, Coral G Warr and Richard Burke

Affiliations:

School of Biological Sciences, Monash University

ABSTRACT

The human copper homeostasis disorders Menkes and Wilson disease both have severe neurological symptoms. Menkes is a copper deficiency disorder whereas Wilson disease patients suffer from copper toxicity, indicating that tight control of neuronal copper levels is essential for proper nervous system development and function. Here we examine the consequences of neuronal copper deficiency and excess in the *Drosophila melanogaster* nervous system, using targeted manipulation of the copper uptake genes *Ctr1A* and *Ctr1B* and efflux gene *ATP7* in combination with altered dietary copper levels. We find that pan-neuronal over expression of *Ctr1B* and *ATP7* both result in a reduction in viability. The effects of *Ctr1B* over expression are exacerbated by dietary copper supplementation and rescued by copper limitation indicating a copper toxicity phenotype. Dietary manipulation has the opposite effect on *ATP7* over expression, indicating that this causes neuronal copper deficiency due to excessive copper efflux. Copper deficiency also causes a highly penetrant developmental defect in surviving adult flies which can be replicated by both copper excess and copper deficiency targeted specifically to a small subset of neuropeptidergic cells. We conclude that both copper overload and excess have detrimental effects on *Drosophila* neuronal function, reducing overall fly viability as well as impacting on a specific neuropeptide pathway.

Keywords

Drosophila, copper homeostasis, Ctr1, ATP7, peptidergic neurons

INTRODUCTION

Inherited human diseases caused by defective copper homeostasis commonly display symptoms of impaired neuronal development or neurodegeneration. Infants with Menkes disease, for instance, suffer an ultimately fatal systemic copper deficiency caused by loss-of-function mutations in the gene encoding the ATP7A copper-efflux pump [1, 2]. ATP7A is normally required for copper absorption through the intestinal enterocytes and delivery of copper to the brain via the blood-brain-barrier and Menkes patients exhibit seizures, mental retardation and severe developmental delay [2]. Recently, single amino acid substitutions in the C-terminus of the ATP7A protein have been shown to cause Distal Motor-Neuropathy (DMN), a late-onset degenerative disorder of the peripheral nervous system typified by muscle atrophy due to motor neuron loss [3].

Excess copper is also detrimental to neuronal health, as evidenced by the neuropathology seen in patients with Wilson disease, a copper toxicity disorder caused by mutations in a second copper efflux pump, ATP7B. A failure to properly excrete excess copper in Wilson disease patients results in accumulation of copper both in the liver and in the central nervous system (CNS) where it can cause neurological symptoms ranging from mild cognitive decline and behavioural changes to signs of parkinsonism [4].

Some of the roles of copper in nervous system development and function are clear. ATP7A has an important function in delivery of copper to the secretory pathway for incorporation into the active site of copper-dependent cupro-enzymes such as Dopamine-β-hydroxylase (DβH) [5], Peptidylglycine α-amidating monoxygenase (PAM) [6] and Cu,Zn-superoxide dismutase (SOD1) [7]. DβH is required to convert dopamine to norepinephrine while PAM is needed for the amidation and therefore activation of several neuropeptides. Mice heterozygous for PAM mutations display a wide array of behavioural abnormalities, including anxiety-like behaviours and seizures [8] and DβH deficiency in humans causes symptoms such as poor cardiovascular regulation and hypotonia [9].

Other neuronal functions of copper are less well defined. For instance, developmental regulation of ATP7A expression in neurons and its localization to the growing tip of

axons in the mouse olfactory bulb [10-12] suggest a requirement for copper in these tissues but evidence for function is currently lacking. Strikingly, translocation of ATP7A from the *trans* Golgi Network (TGN) to the outer plasma membrane has been shown to be induced by both copper and by stimulation of the NMDA receptor in cultured hippocampal neurons [13]. This translocation results in the release of copper into the synaptic cleft, where it plays a role in regulating the excitability of neurons and also helps to protect the neurons from excitotoxicity [13, 14]. However, there is currently no conclusive *in vivo* data indicating a critical requirement for copper transport in neurotransmission.

We have previously shown widespread expression of the sole *Drosophila melanogaster* copper efflux gene, *ATP7,* in neurons and neuropeptidergic cells [15], indicating a possible role in nervous system development and function in the fly. Furthermore RNA interference (RNAi)-induced *ATP7* knockdown has been shown to result in the loss of activity of Peptidylglycine alpha-hydroxylating monooxygenase (PHM), the fly orthologue of PAM, although no phenotypic consequences were reported [16]. Here, we further investigated the roles of copper in the *Drosophila* nervous system by genetically manipulating copper transport genes in all or specific subsets of neurons. We report both copper toxicity and copper deficiency phenotypes and highlight the particular importance of maintaining copper homeostasis in a small subset of neuropeptidergic neurons.

EXPERIMENTAL

Drosophila stocks

The following fly stocks were used: w1118 (BL (Bloomington *Drosophila* Stock Center, Bloomington, IN, USA)3605); UAS-GFP (BL32184, P{10XUAS-mCD8::GFP}attP2); Elav-Gal4 (BL458, P{GawB}elav^{C155}); double balancer (w; IF / CyO; MKRS / TM6b, gift from G. Hime, University of Melbourne, Australia); CCAP-Gal4 and CCAP-Gal80 [17, 18] (gift from B. White, NIH, USA); Overexpression lines UAS-ATP7, UAS-Ctr1A and UAS-Ctr1B have been described previously [19, 20]. RNAi lines obtained from the Vienna Drosophila RNAi Center included V8315 (*ATP7*), V46757 and V46758 (*Ctr1A*), V5804 and V5805 (*Ctr1B*), and V104028 (*PHM*).

Drosophila maintenance

All *Drosophila* strains and crosses were maintained on standard medium at 25°C unless stated otherwise. Standard medium was supplemented with either 300 μM bathocuproine disulfonate (BCS; Sigma-Aldrich, St. Louis, MO, USA) to make copper-deficient food medium, or 1 mM copper ($CuSO_4$.5H2O; Merck, Whitehouse Station, NJ, USA) to make copper-supplemented medium. *Drosophila* 1st instar larvae were transferred onto medium supplemented with aqueous $CuSO_4$ or BCS. Feeding experiments were density controlled with 50 larvae transferred per vial in triplicate.

Microscopy

Adult flies were anaesthetized using CO_2, then mounted directly onto Blu-Tack and monitored with a Leica MZ6 stereomicroscope. All images were recorded with a Leica DC300 digital camera using Leica Application Suite. Brains from wandering 3rd instar larvae were dissected in cold phosphate buffered saline and then fixed for 30 min in 4% paraformaldehyde at room temperature. For HRP staining, brains were permeabilized in 0.1% PBS-TritonX, then incubated overnight at 4°C in anti-HRP Cy3 (Jackson ImmunoResearch) at 1:1000. Finally, the brains were mounted onto glass slides in VectaShield (Vector Laboratories). Fluorescence was detected using a Nikon C1 upright confocal microscope.

RESULTS

Pan-neuronal copper deficiency leads to copper-sensitive lethality

Over expression of *ATP7* is predicted to result in copper deficiency due to increased cellular copper efflux, which occurs due to ectopic ATP7 localizing to the plasma membrane and exporting copper out of the cell [20]. Therefore to examine the requirement for copper in the nervous system, *ATP7* over expression was targeted to all *Drosophila* neurons using *Elav-Gal4*. This transgene combination gave a multifaceted phenotype. First, *Elav>ATP7^{OE}* flies showed reduced survival from 1st instar larval stage to adulthood compared to control flies (Fig. 1A). We found that this lethality was related to neuronal cell copper content. In feeding experiments performed by supplementation with the copper chelator bathocuproine disulfonate (BCS) or $CuSO_4$, an enhanced lethality was observed in flies raised on 300 μM BCS food compared to control flies, while rescue of the *Elav>ATP7^{OE}* lethality phenotype back to control levels was observed in flies raised on 1 mM $CuSO_4$ food (Fig. 1A). Lethality in the dying flies occurred largely at the pharate adult stage (Fig 1B). These results were consistent with the idea that *ATP7* over expression results in a copper deficiency in neuronal cells that is partially rescued by dietary copper supplementation and is exacerbated by copper limitation. We also examined whether *Elav>ATP7^{OE}* flies might show a delay in time to eclosion, however no developmental delays were observed between *Elav>ATP7^{OE}* and control flies on normal or BCS- / $CuSO_4$- supplemented foods (Fig. S1).

Neuronal copper deficiency causes additional wing expansion defects

An unexpanded wing phenotype of incomplete penetrance and varying severity was observed in surviving *Elav>ATP7^{OE}* flies (Fig. 2B-D). This was accompanied by a defect in tanning, as evidenced by a matte, non-reflective exoskeleton. To test whether the unexpanded wing phenotype may also be due to changes in neuronal copper levels, the wings of surviving *Elav>ATP7^{OE}* adult flies raised during larval development on basal, low and high copper food were assessed as being either fully expanded or unexpanded (Fig. 2E). While no unexpanded wings were observed in *Elav-Gal4* control flies on any of the food types, copper limitation caused a significant increase in the proportion of *Elav>ATP7^{OE}* flies showing unexpanded

wings, while copper supplementation was able to partially rescue the phenotype in a dosage dependent manner (Fig. 2E). Therefore *ATP7* over expression causes a functional cellular copper deficiency that also affects post-eclosion wing expansion behaviour.

Copper dyshomeostasis disrupts the Bursicon wing expansion pathway

Wing expansion behaviour in *Drosophila* is controlled by a cascade of neuropeptides including Eclosion hormone (EH), ecdysis triggering hormone (ETH), crustacean cardioactive peptide (CCAP) and Bursicon (which operates as a heterodimer with two subunits, Bursicon (Burs) and Partner of Bursicon (Pburs)). EH and ETH operate in a positive feedback loop upstream of CCAP and are involved in driving *CCAP* expression, which in turn may play a role in the regulation of Bursicon secretion [18, 21-23]. *EH* and *ETH* mutants show severe phenotypes and fail to moult properly [18, 22-24], while *Burs* and *Pburs* mutants exhibit a milder phenotype with wing expansion and tanning defects similar to those seen here in *Elav>ATP7*^{OE} flies [18, 21, 23]. When targeted ablation of CCAP-cells is carried out (CCAP-ablated flies), a similar phenotype to *Burs* and *Pburs* mutants is observed. In contrast, *CCAP* mutants show no phenotype, however *CCAP* and *Pburs* double mutants display a more severe phenotype compared to the *Pburs* mutants [18, 21] . In *Drosophila*, about 90% of all neuropeptides, including ETH and CCAP [23, 24], are amidated by PHM, the *Drosophila* homologue of the copper-dependent enzyme PAM [25].

Given the similarity in phenotypes between CCAP-ablated and *Elav>ATP7*^{OE} flies, we decided to test whether copper deficiency in CCAP-producing cells could recapitulate the unexpanded wing phenotype seen in *Elav>ATP7*^{OE} flies. Using *CCAP-Gal4* which drives expression specifically in CCAP-producing cells [18], severe copper deficiency (over expression of *ATP7* coupled with knockdown of the copper uptake gene *Ctr1A*) caused a highly penetrant unexpanded wing phenotype (Fig. 3A), which was not modified by either $CuSO_4$ or BCS supplementation (Fig. 3A).

The effects of copper deficiency might be due to a reduction in the ability of PHM to amidate neuropeptides in CCAP cells, including CCAP and myoinhibitory peptide [26] . If this were the case, knockdown of *Phm* itself would be expected to produce the same phenotype. However of knockdown of *Phm* in all neurons (*Elav-Gal4*)

produced no obvious phenotype alone nor in combination with *ATP7* knockdown (Fig. S2). Given the essential requirement for PHM at multiple developmental stages [25], the most likely explanation for this absence of phenotypes is insufficient knockdown by the *Phm* RNAi line.

To test if copper dyshomeostasis in CCAP cells was solely or partially responsible for the unexpanded wing phenotype originally observed in *Elav>ATP7*^{OE} flies, a *CCAP-Gal80* construct was used in tandem with *Elav>ATP7*^{OE}, allowing the overexpression of *ATP7* in all neuronal cells except the CCAP cells, where the Gal80 would inhibit Gal4 activation of *UAS-ATP7*. Reduction of *ATP7* over expression specifically in CCAP cells resulted in a significant rescue of the unexpanded wing phenotype only when flies were raised on $CuSO_4$ food, although a trend towards rescue was also observed on normal food (Fig. 3B). This result thus indicated that copper dyshomeostasis in CCAP cells was at least partially responsible for the unexpanded wing phenotype observed in *Elav>ATP7*^{OE} flies.

Copper overload also leads to lethality and an unexpanded wing defect

Over expression of the copper uptake gene *Ctr1B* under *Elav-Gal4* control also caused lethality at larval stages. Lethality on basal media was largely limited to males (Fig. 4A), which showed an average 5 +/- 2.5% of expected survival compared to sibling females (average 109 +/- 29% of expected survival). On a copper-supplemented diet, male *Elav>Ctr1B*^{OE} survival remained low while female survival was now effectively abolished. Copper-limitation had the opposite effect, rescuing the *Elav>Ctr1B*^{OE} male lethality back to 61 +/- 9.5 % of expected survival. Thus neuronal cell copper overload caused by *Ctr1B* over expression reduced survival rate to adulthood, had a stronger effect in males than females and was modified by manipulation of dietary copper levels.

While surviving *Elav>Ctr1B*^{OE} flies did not exhibit the unexpanded wing defect seen in *Elav>ATP7*^{OE} adult survivors (data not shown), *Ctr1B* over expression under *CCAP-Gal4* resulted in unexpanded wings in >30% of flies (Fig. 4B). Dietary copper depletion by BCS supplementation completely rescued this phenotype whereas increasing copper levels in the food increased the penetrance of the phenotype to almost 100% (Fig. 4B).

Copper toxicity results in CCAP cell death

Copper deficiency in CCAP-producing cells caused by *ATP7* over expression is predicted to reduce PHM activity since PHM requires copper ions for its catalytic activity. Why then would an increase in copper levels, caused by *Ctr1B* over expression, also cause an unexpanded wing phenotype? To determine the effect of copper deficiency and overload on neuronal cells, the morphology and number of CCAP cells was visualized by expression of mCD8-GFP (which localizes to the outer plasma membrane) under *CCAP-Gal4* control.

When raised at 29°C and on 2mM copper to generate the highest degree of copper overload, *CCAP>Ctr1B*^{OE} fly cells showed a greatly altered morphology compared to wild type, with many missing cell bodies, fewer connections between neurons and less axon branching observed (Fig. 5A and B). This corresponded with a much more severe pupal lethal phenotype (under conditions of 25°C and on normal food, *CCAP>Ctr1B*^{OE} flies survive to adulthood). Co-staining with anti-HRP was carried out and no obvious defects were observed outside of CCAP cells (Fig. S3). The reason for the wing expansion defect observed in *CCAP>Ctr1B*^{OE} flies is thus likely to be CCAP cell death due to excess copper. *CCAP>ATP7*^{OE}*; Ctr1A*^{RNAi} cells were also examined by co-expression of mCD8-GFP. In this case however, no obvious morphological change could be seen (Fig. 5C). This was consistent with the hypothesis that loss of PHM function due to insufficient cellular copper was causing failure of neuropeptide production pathway rather than general defects in these cells.

DISCUSSION

The range of neurological symptoms seen in patients with inherited copper homeostasis disorders indicates that maintenance of appropriate copper levels in neurons is critical for their correct development and / or function. To further investigate the exact role of copper ions in the nervous system, we examined whether the genetically tractable *Drosophila* system would serve as a suitable model. We found that, similar to the situation in Menkes and Wilson diseases, both copper deficiency and copper toxicity can be detrimental to neuronal activity.

When induced in all neurons, both excess and insufficient copper caused death of the fly during larval / pupal development. In the case of copper toxicity due to *Ctr1B* over expression, males were significantly more susceptible than females. A similar sex-specific difference in copper sensitivity has previously been described in *Malvolio* mutants [27] where mutant females showed reduced survival under conditions of copper limitation compared to males. Combined, these results imply that copper absorption is generally lower in females than in males. One possible explanation for this would be either reduced *ATP7* or *Ctr1A* expression in intestinal enterocytes. Both genes are X-linked so altered expression could be due to imperfect dosage compensation [28]. Which of these two possibilities is more likely would depend on which of these two genes provides the rate-limiting step in copper absorption.

The highly penetrant unexpanded wing phenotypes observed in surviving copper-deficient adult flies led us to examine its possible causes. Wing expansion is controlled by a cascade of neuropeptides which include EH, ETH, CCAP and Bursicon, of which CCAP and ETH are amidated by the copper-dependent enzyme PHM. Manipulation of copper transport genes in CCAP-producing cells resulted in an unexpanded wing phenotype in both copper deficiency and toxicity conditions. Copper toxicity was shown to induce death of CCAP cells which would explain the unexpanded wing phenotype; insufficient CCAP and Bursicon would be produced to promote expansion and tanning, similar to the phenotype observed in CCAP-ablated flies. In contrast copper-deficient CCAP cells appeared morphologically normal, indicating that PHM-activity is specifically impaired in these cells, most likely due to insufficient copper availability for this cupro-enzyme. This supports previous findings

that ATP7 is required for neuropeptide amidation in *Drosophila* [16] and shows for the first time the functional consequences of this deficiency.

If copper-delivery to the TGN is needed for PHM activation, *ATP7* knockdown would be predicted to reduce PHM activity and also cause the unexpanded wing phenotype. However *ATP7* knockdown, even at high temperatures and with multiple RNAi transgene copies gave no obvious defects in our hands. *Phm* knockdown also had no apparent effect. The most likely explanation is residual gene activity in both cases due to incomplete knockdown. While the *ATP7* RNAi line has previously been shown to be effective [19], neurons are notoriously resistant to RNAi [29]. Co-expression of dicer 2 could be used in future experiments to try and enhance *ATP7* knockdown, which has been shown to reduce neuropeptide amidation [16] but without the phenotypic defects reported here with *Ctr1B* or *ATP7* over expression. Indeed, *Ctr1A* knockdown was the only effective RNAi-mediated neuronal phenotype observed here and was only active in combination with *ATP7* over expression.

Knockdown of *ATP7* in the *Drosophila* midgut causes reduced systemic and brain copper levels and also reduced brain size [30]. This aberrant development of the nervous system is likely to be the reason for the overall reduced viability of *Elav>ATP7*^{OE} flies and their poor performance in the negative geotaxis assay. This result is also in line with the symptoms observed in Menkes patients, who display progressive neurodegeneration with time.

While our findings have highlighted the value of using *Drosophila* to examine the *in vivo* requirements of copper ions in neuronal function and development, the critical question of whether copper transport is important for neurotransmission has not yet been adequately addressed. The unexpanded wing phenotypes are best explained by CCAP cell death or reduction in copper-dependent PHM activity. The lethality caused by copper deficiency could be attributed to reduced neurotransmission but could equally be due to loss of neuropeptide activity. Direct physiological measurements of synaptic activity will be needed distinguish between these possibilities. The *Drosophila* larval neuromuscular junction would provide the ideal system for such investigations.

ACKNOWLEDGEMENTS

This work was supported by Project Grant funding from the National Health and Medical Research Council of Australia (RB, Grant ID # 606609). The Australian *Drosophila* Biomedical Research Support Facility assisted in the import and quarantine of fly strains used in this research. All transgenic *Drosophila* experiments carried out in this research were performed with the approval of the Monash University Institutional Biosafety Committee.

REFERENCES

1. Kodama, H. and Y. Murata, *Molecular genetics and pathophysiology of Menkes disease.* Pediatr Int, 1999. **41**(4): p. 430-5.
2. Kodama, H., Y. Murata, and M. Kobayashi, *Clinical manifestations and treatment of Menkes disease and its variants.* Pediatr Int, 1999. **41**(4): p. 423-9.
3. Kennerson, M.L., et al., *Missense mutations in the copper transporter gene ATP7A cause X-linked distal hereditary motor neuropathy.* Am J Hum Genet, 2010. **86**(3): p. 343-52.
4. Harada, M., *Wilson disease.* Med Electron Microsc, 2002. **35**(2): p. 61-6.
5. Kaler, S.G., C.S. Holmes, and D.S. Goldstein, *Dopamine beta-hydroxylase deficiency associated with mutations in a copper transporter gene.* Adv Pharmacol, 1998. **42**: p. 66-8.
6. Steveson, T.C., et al., *Menkes protein contributes to the function of peptidylglycine alpha-amidating monooxygenase.* Endocrinology, 2003. **144**(1): p. 188-200.
7. Kaler, S.G., *Menkes disease.* Adv Pediatr, 1994. **41**: p. 263-304.
8. Bousquet-Moore, D., et al., *Reversal of physiological deficits caused by diminished levels of peptidylglycine alpha-amidating monooxygenase by dietary copper.* Endocrinology, 2009. **150**(4): p. 1739-47.
9. Robertson, D. and E.M. Garland, *Dopamine Beta-Hydroxylase Deficiency*, in *GeneReviews(R)*, R.A. Pagon, et al., Editors. 1993, University of Washington, Seattle, University of Washington, Seattle. All rights reserved.: Seattle (WA).
10. El Meskini, R., et al., *The developmentally regulated expression of Menkes protein ATP7A suggests a role in axon extension and synaptogenesis.* Dev Neurosci, 2005. **27**(5): p. 333-48.
11. El Meskini, R., et al., *ATP7A (Menkes protein) functions in axonal targeting and synaptogenesis.* Mol Cell Neurosci, 2007. **34**(3): p. 409-21.
12. Niciu, M.J., et al., *Developmental changes in the expression of ATP7A during a critical period in postnatal neurodevelopment.* Neuroscience, 2006. **139**(3): p. 947-64.
13. Schlief, M.L., et al., *Role of the Menkes copper-transporting ATPase in NMDA receptor-mediated neuronal toxicity.* Proc Natl Acad Sci U S A, 2006. **103**(40): p. 14919-24.
14. Schlief, M.L. and J.D. Gitlin, *Copper homeostasis in the CNS: a novel link between the NMDA receptor and copper homeostasis in the hippocampus.* Mol Neurobiol, 2006. **33**(2): p. 81-90.
15. Burke, R., E. Commons, and J. Camakaris, *Expression and localisation of the essential copper transporter DmATP7 in Drosophila neuronal and intestinal tissues.* Int J Biochem Cell Biol, 2008. **40**(9): p. 1850-60.
16. Sellami, A., C. Wegener, and J.A. Veenstra, *Functional significance of the copper transporter ATP7 in peptidergic neurons and endocrine cells in Drosophila melanogaster.* FEBS Lett, 2012. **586**(20): p. 3633-8.
17. Luan, H., et al., *Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila.* J Neurosci, 2006. **26**(2): p. 573-84.

18. Park, J.H., et al., *Targeted ablation of CCAP neuropeptide-containing neurons of Drosophila causes specific defects in execution and circadian timing of ecdysis behavior.* Development, 2003. **130**(12): p. 2645-56.
19. Binks, T., et al., *Tissue-specific interplay between copper uptake and efflux in Drosophila.* J Biol Inorg Chem, 2010. **15**(4): p. 621-8.
20. Norgate, M., et al., *Essential roles in development and pigmentation for the Drosophila copper transporter DmATP7.* Mol Biol Cell, 2006. **17**(1): p. 475-84.
21. Lahr, E.C., D. Dean, and J. Ewer, *Genetic analysis of ecdysis behavior in Drosophila reveals partially overlapping functions of two unrelated neuropeptides.* J Neurosci, 2012. **32**(20): p. 6819-29.
22. McNabb, S.L., et al., *Disruption of a behavioral sequence by targeted death of peptidergic neurons in Drosophila.* Neuron, 1997. **19**(4): p. 813-23.
23. Peabody, N.C., et al., *Bursicon functions within the Drosophila CNS to modulate wing expansion behavior, hormone secretion, and cell death.* J Neurosci, 2008. **28**(53): p. 14379-91.
24. Park, Y., et al., *Molecular cloning and biological activity of ecdysis-triggering hormones in Drosophila melanogaster.* FEBS Lett, 1999. **463**(1-2): p. 133-8.
25. Jiang, N., et al., *PHM is required for normal developmental transitions and for biosynthesis of secretory peptides in Drosophila.* Dev Biol, 2000. **226**(1): p. 118-36.
26. Kim, Y.J., et al., *A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles.* Curr Biol, 2006. **16**(14): p. 1395-407.
27. Southon, A., et al., *Malvolio is a copper transporter in Drosophila melanogaster.* J Exp Biol, 2008. **211**(Pt 5): p. 709-16.
28. Gelbart, M.E. and M.I. Kuroda, *Drosophila dosage compensation: a complex voyage to the X chromosome.* Development, 2009. **136**(9): p. 1399-410.
29. Buckingham, S.D., et al., *RNA interference: from model organisms towards therapy for neural and neuromuscular disorders.* Hum Mol Genet, 2004. **13 Spec No 2**: p. R275-88.
30. Bahadorani, S., et al., *A Drosophila model of Menkes disease reveals a role for DmATP7 in copper absorption and neurodevelopment.* Dis Model Mech, 2010. **3**(1-2): p. 84-91.

FIGURE LEGENDS

Figure 1: Neuronal copper deficiency causes developmental lethality. A) Survival from 1st instar larval stages to adulthood was assessed for each genotype on normal, BCS- and $CuSO_4$-supplemented media. *Elav>ATP7*^{OE} flies showed significantly reduced survival on basal media compared to *Elav>+* control flies, which was worsened on copper-limited (BCS) media and significantly improved on 1 mM $CuSO_4$-supplemented media. N = 3 for each genotype. (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001). B) The number of dead parate adults (late pupal stages) was assessed for each of the genotype / diet combinations shown in A. Death at this stage appears to account for the majority of the missing adult flies.

Figure 2: Adult flies with neuronal copper deficiency display an unexpanded wing phenotype. Surviving *Elav>ATP7*^{OE} adult flies show a highly penetrant unexpanded wing phenotype that varies in severity, from mild (B), to moderate (C) and severe (D) compared to *Elav-Gal4* only control flies (A) that always display normal wing expansion behaviour. Increasingly defective tanning is also observed, as the exoskeleton becomes less glossy / reflective compared to control flies (arrows pointing to reflection which is clearly seen in control flies). E) Control and *Elav>ATP7*^{OE} flies raised in density-controlled conditions on basal, copper-limited and $CuSO_4$-supplemented conditions were assessed for the percentage of adult flies with normal wings >24 hours post-emergence. A significant decrease in the proportion of emerging *Elav>ATP7*^{OE} adults with fully expanded wings was seen under copper-limited conditions (BCS) compared to basal conditions, whereas $CuSO_4$ supplementation caused an increase in the proportion with fully expanded wings in a dosage dependent manner. (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001)

Figure 3: The unexpanded wing phenotype can be replicated by localized copper deficiency in CCAP-producing cells. A) Using *CCAP-Gal4* to drive expression of transgenes expected to reduce neuronal copper levels, only the combination of *Ctr1A* RNAi-knockdown and *ATP7* over expression led to a significant reduction in the proportion of individuals with normal wings on basal media. Dietary copper modulation had no appreciable effect on this phenotype. For each genotype / media combination, n = 5. (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001). B) *Elav-Gal4* was used to drive *ATP7* over expression in all neurons, alone and together with *CCAP-Gal80* which at 25ºC suppresses Gal4 activity only in CCAP-producing cells, allowing *ATP7* over expression in all neurons except the CCAP cells. Flies of each genotype were raised in density-controlled conditions on basal, copper-limited and copper-supplemented media. % of adults with normal wing morphology >24 hours post-emergence is shown for each genotype / media combination. Addition of *CCAP-Gal80* caused a significant reduction in the penetrance of the *Elav>ATP7*^{OE} unexpanded wing phenotype only on copper-supplemented media, although there was a trend towards rescue on basal media as well. (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001)

Figure 4: Copper overload also causes lethality and the unexpanded wing phenotype. A) Ectopic expression of *Ctr1B* under *Elav-Gal4* control resulted in a strong reduction in male survival on basal media while female survival was unaffected. Male survival was restored to control levels on BCS-supplemented media whereas female survival was reduced to zero on $CuSO_4$-supplemented media. (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001). B) Compared to *CCAP>+* flies that displayed fully-expanded wings on all media types, over expression of *Ctr1B* under *CCAP-Gal4* control caused a significant decrease in the proportion of individuals with normal wings on basal media which was exacerbated on copper-supplemented media and rescued to wild type levels on copper-limited media.

Figure 5: Severe copper overload causes a reduction in the number of CCAP-producing cells. CCAP-producing cells in the 3rd instar larval central nervous system are visualized in each panel with *CCAP>mCD8-GFP*. A) Control showing normal number (typically 25-30 cells) and morphology of CCAP-producing cells. B) Addition of $Ctr1B^{OE}$ construct results in a clear decrease in the number of visible cell bodies to <50% of control and a strong reduction in axonal GFP levels. C) Addition of the $Ctr1A^{RNAi}$; $ATP7^{OE}$ transgene combination has no appreciable effect on CCAP cell number / morphology. Brains of 3rd instar larvae were dissected and viewed at 20X using confocal microscopy. Representative images are shown for each genotype. N= 5 for each genotype

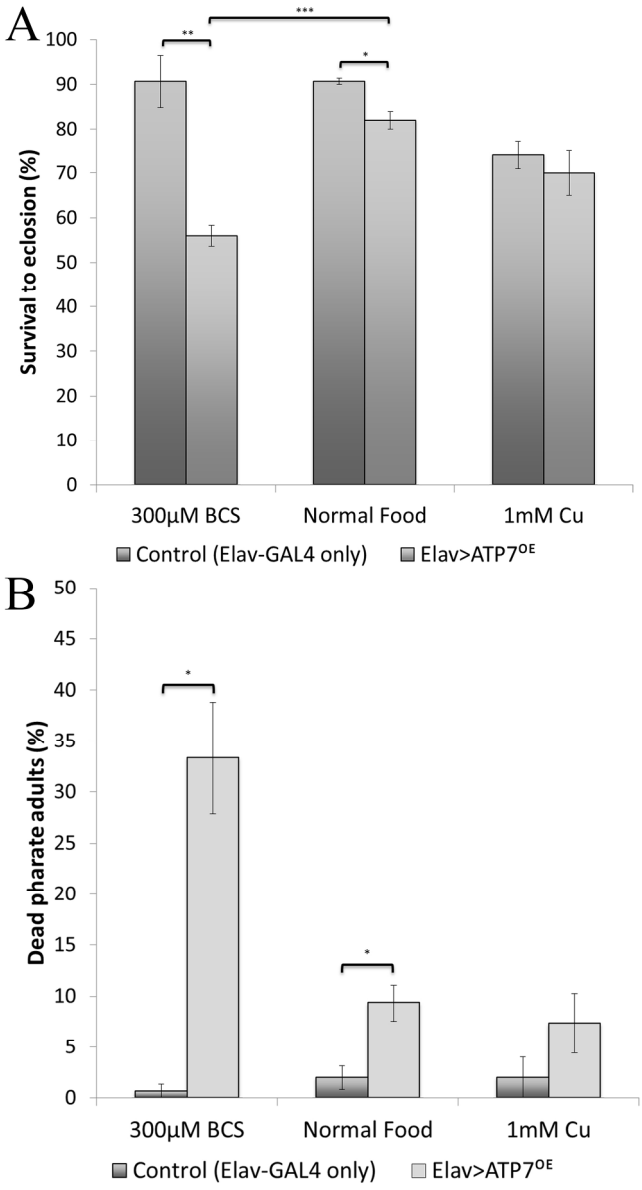


Figure 1
153x284mm (300 x 300 DPI)

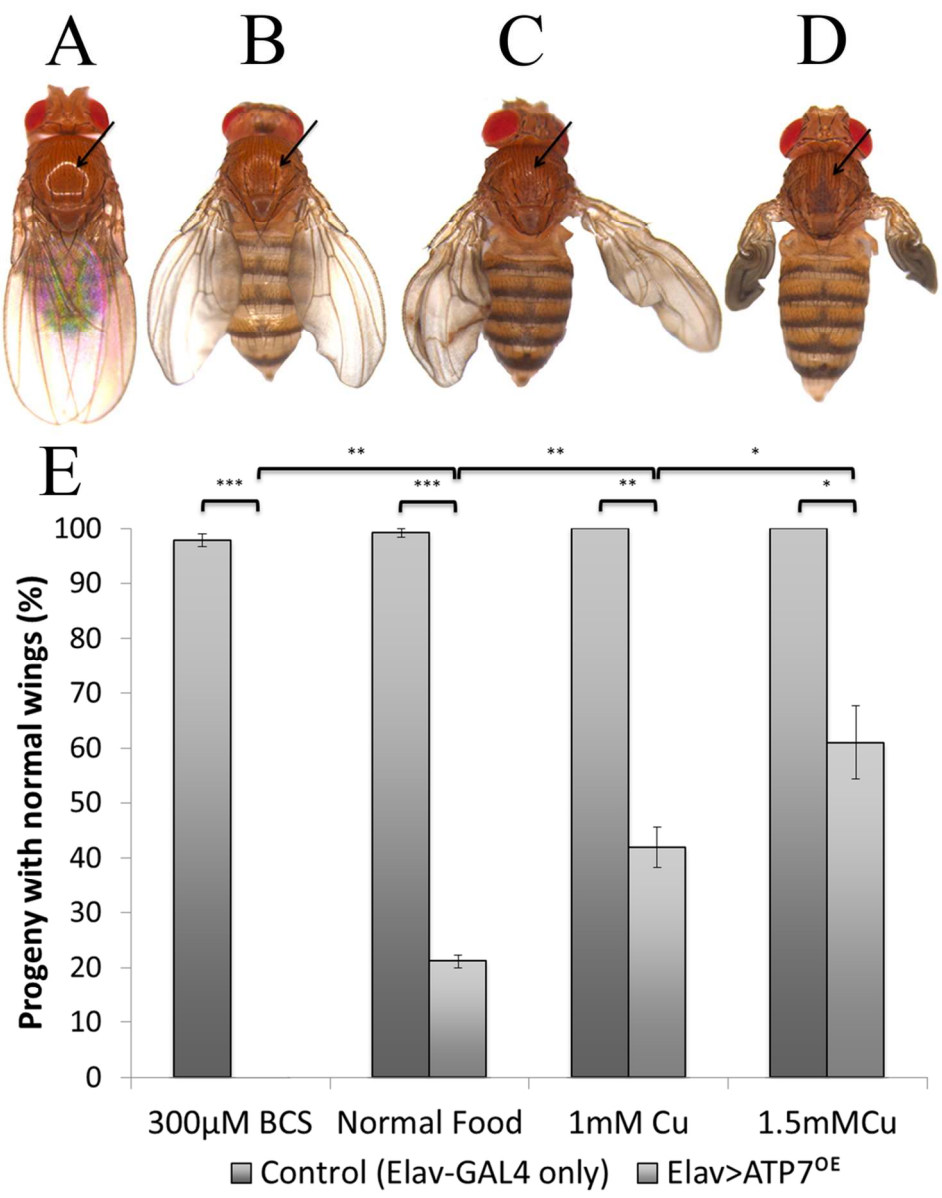


Figure 2
104x131mm (300 x 300 DPI)

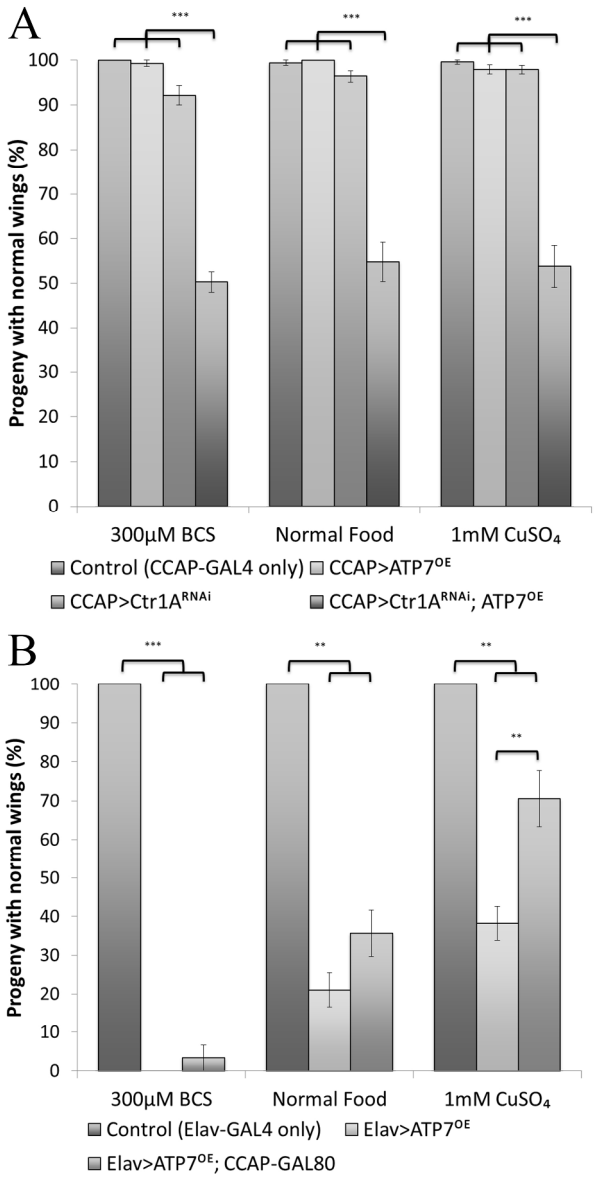


Figure 3
164x325mm (300 x 300 DPI)

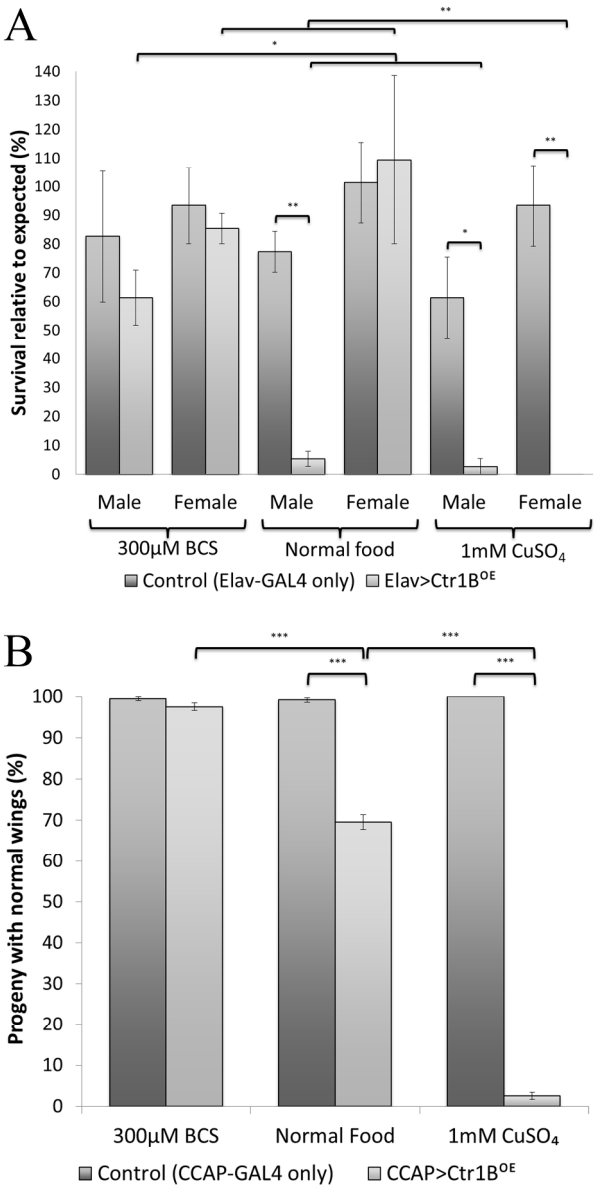


Figure 4
162x317mm (300 x 300 DPI)

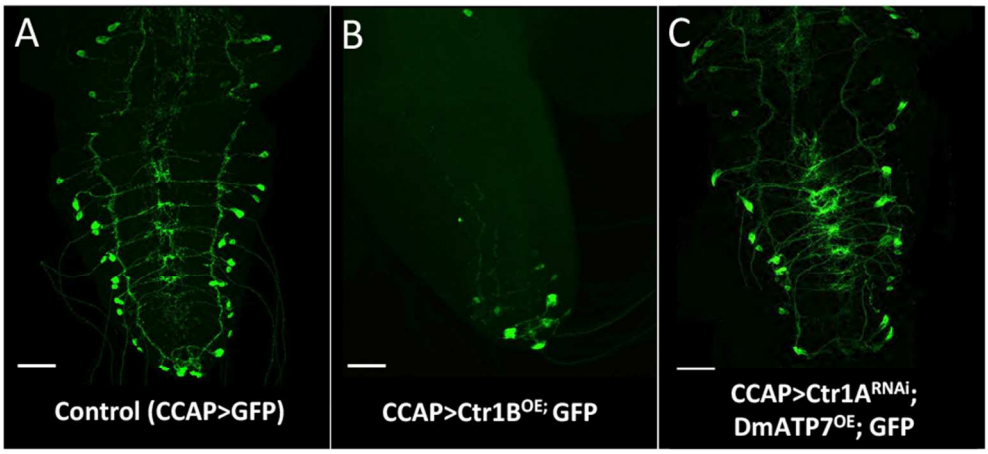


Figure 5
82x38mm (300 x 300 DPI)