A cAMP/PKA/Kinesin-1 Axis Promotes the Axonal Transport of Mitochondria in Aging Drosophila Neurons

Highlights

- cAMP/PKA pathway promotes mitochondrial transport in adult Drosophila wing neurons

- Pathway activation in aged flies suppresses age-related reduction in transport

- Levels of PKAc and kinesin-1 motor decline during aging

- Kinesin-1 upregulation is an important output of PKA activation in aged flies

Authors

Alessio Vagnoni, Simon L. Bullock

Correspondence

alessio.vagnoni@kcl.ac.uk (A.V.), sbulloch@mrc-lmb.cam.ac.uk (S.L.B.)

In Brief

Vagnoni and Bullock use the Drosophila wing to investigate the regulation of mitochondrial transport in aging neurons. They discover that acute activation of the cAMP/PKA pathway can suppress an age-related decline in mitochondrial motility. This effect is due in part to upregulation of the kinesin-1 motor, levels of which decline during aging.
A cAMP/PKA/Kinesin-1 Axis Promotes the Axonal Transport of Mitochondria in Aging Drosophila Neurons

Alessio Vagnoni1,2,3,* and Simon L. Bullock1,*

1Division of Cell Biology, MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, UK
2Division of Basic and Clinical Neuroscience, Maurice Wohl Clinical Neuroscience Institute, King’s College London, London SE5 9RX, UK
3Lead Contact
*Correspondence: alessio.vagnoni@kcl.ac.uk (A.V.), sbullock@mrc-lmb.cam.ac.uk (S.L.B.)
https://doi.org/10.1016/j.cub.2018.02.048

SUMMARY

Mitochondria play fundamental roles within cells, including energy provision, calcium homeostasis, and the regulation of apoptosis. The transport of mitochondria by microtubule-based motors is critical for neuronal structure and function. This process allows local requirements for mitochondrial functions to be met and also facilitates recycling of these organelles [1, 2]. An age-related reduction in mitochondrial transport has been observed in neurons of mammalian and non-mammalian organisms [3–6], and has been proposed to contribute to the broader decline in neuronal function that occurs during aging [3, 5–7]. However, the factors that influence mitochondrial transport in aging neurons are poorly understood. Here we provide evidence using the tractable Drosophila wing nerve system that the cyclic AMP/protein kinase A (cAMP/PKA) pathway promotes the axonal transport of mitochondria in adult neurons. The level of the catalytic subunit of PKA decreases during aging, and acute activation of the cAMP/PKA pathway in aged flies strongly stimulates mitochondrial motility. Thus, the age-related impairment of transport is reversible. The expression of many genes is increased by PKA activation in aged flies. However, our results indicate that elevated mitochondrial transport is due in part to upregulation of the heavy chain of the kinesin-1 motor, the level of which declines during aging. Our study identifies evolutionarily conserved factors that can strongly influence mitochondrial motility in aging neurons.

RESULTS AND DISCUSSION

To shed light on the regulation of mitochondrial transport in aging neurons, we exploited a tractable system for imaging of axonal transport in an adult animal: the sensory neurons of the translucent Drosophila wing [5, 8, 9] (Figure 1A). We previously showed that the number of actively transported mitochondria in wing neuron axons is ~5-fold lower at 30 days after eclosion from the pupal case (operationally defined as “aged” flies) than at 2 days after eclosion (“young” flies) [5] (Figure S1A). The decline in mitochondrial transport begins in the first week of adult life [5] and reflects reduced anterograde and retrograde movements (Figure S1B), equating to transport toward microtubule plus ends and minus ends, respectively [5]. The number of mitochondria in axonal tracts does not change during aging, demonstrating that a larger fraction of mitochondria becomes stationary over time [5].

Signaling pathways that increase lifespan are attractive candidates for exploring the regulation of axonal transport during aging [6, 10]. Cyclic AMP (cAMP) is an important second messenger in intracellular signaling, and elevation of its concentration can extend lifespan in Drosophila and mice [11, 12]. We therefore investigated whether cAMP influences axonal transport of mitochondria in aged flies.

We first targeted the cAMP phosphodiesterase Dunce (Dnc) in wing neurons using an RNA interference (RNAi) construct under the control of the Ga4–UAS system. Downregulation of Dnc increases the concentration of cAMP by inhibiting conversion to AMP [13]. To visualize mitochondria, we co-expressed GFP targeted to the mitochondrial matrix (mito::GFP). In aged flies, the number of transported mitochondria in axons of dnc RNAi neurons was ~4-fold higher than in neurons expressing a control RNAi construct (Figures 1B and 1C; Movie S1). RNAi of dnc increased transport in both the anterograde and retrograde directions (Figure S1C) without altering the number of mitochondria in the axonal tract (Figure 1C). Thus, prolonged inhibition of cAMP turnover in wing neurons of aged flies increases mitochondrial transport by mobilizing a fraction of stationary mitochondria.

Aging is characterized by a functional decline at the cellular and organismal level. A key question in aging research is to what extent prolonged reduction of cellular functions can be rescued by an acute intervention in later life [14]. We therefore asked whether an acute increase in cAMP can boost mitochondrial transport in aged flies. 28-day-old flies were fed with 100 µM 8-Br-cAMP (a hydrolysis-resistant cAMP analog) for 4 days prior to visualization of mitochondrial motility in wing neuron axons (Figure 1D). The number of motile mitochondria was significantly higher in flies fed with 8-Br-cAMP than in vehicle-fed controls (Figures 1E and 1F; Movie S2). Again, the increase in motility was evident in both the anterograde and retrograde directions (Figure S1D), with no change in the number of...
Figure 1. cAMP Boosts Mitochondrial Transport in Wing Neurons of Aged Flies

(A) Cartoon of the Drosophila wing. Green: sensory neurons used in this study. Magenta box: region imaged for transport studies, which contains bundled axons from several neurons.

(B) Top: stills from movies of GFP-labeled mitochondria in wing neuron axons of 30-day-old flies ("aged" flies) expressing UAS-luciferase (luc)RNAi (control) or UAS-dncRNAi. Bottom: traces of transported mitochondria in corresponding movies.

(C) Number of transported and total mitochondria per 50 μm of axonal tract in wings of aged flies. In these and other experiments, each wing was filmed for 3 min.

(D) Overview of 8-Br-cAMP feeding experiments. Green square: period of feeding. Microscope icon: visualization of mitochondrial transport.

(E) Top: stills from movies of GFP-labeled mitochondria in wing neuron axons of flies 32 days after eclosion following 4 days of feeding with vehicle or 8-Br-cAMP. Bottom: traces of transported mitochondria in corresponding movies.

(F and G) Number of transported and total mitochondria per 50 μm of axonal tract of wing neurons in aged (F) and young (G) flies after 4 days of feeding with vehicle or 8-Br-cAMP.

(H) Percentage of axonal segments of wing neurons in 5-week-old flies that contained focal accumulation of GFP after feeding throughout adulthood with vehicle or 8-Br-cAMP.

dpr-Gal4 is expressed in the chemosensory neurons of the wing. Statistical significance was evaluated with a Mann-Whitney U test (C, F, and G) or two-tailed Student’s t test (H). *p < 0.05. Magenta circles are values for individual wings, except in (H), where they are values from individual Z projections from 13 wings per genotype; error bars are SEM. Scale bars, 5 μm. See also Figures S1 and S2, Movies S1 and S2, and Table S1.
mitochondria in the axonal tract (Figures 1F). The number of transported mitochondria in wing neurons of aged flies fed with 8-Br-cAMP was not significantly different from that observed in flies fed with the vehicle control for the first 4 days of adulthood (Figures 1F and 1G). Interestingly, feeding flies with 8-Br-cAMP for the first 4 days of adult life (Figure 1D) did not alter the number of motile mitochondria compared to the age-matched controls fed with vehicle (Figure 1G). These results demonstrate that acute supply of cAMP can increase mitochondrial motility in aged but not young flies.

We next investigated whether elevating cAMP levels has broader effects on wing neurons of aged flies. Sustained feeding of aged but not young flies with 8-Br-cAMP reduced the appearance of focal accumulations of GFP (Figure 1H), indicative of improved protein homeostasis [5]. 8-Br-cAMP treatment also reduced the signal from an oxidative stress sensor (Figures S2A and S2B). Although the cAMP analog is likely to affect many processes in wing neurons of aged flies, these results are consistent with previous evidence that mitochondrial transport has a protective role in adult neurons of Drosophila [5, 8, 15], C. elegans [6, 16], and mice [17].

Several studies have reported that cAMP concentration modulates mitochondrial transport in cultured mammalian cells [18–20], although the underlying mechanisms have not been resolved. A key cellular role of cAMP is activation of the catalytic subunit of protein kinase A (PKAc) by triggering dissociation of the regulatory subunit. We investigated whether cAMP-mediated stimulation of mitochondrial transport in wing neurons involves PKA by expressing a constitutively active mouse PKAc (PKA*) from a heat-shock (hs)-mediated induction of PKA*. Green square: period of heat shock. Microscope icon: visualization of mitochondrial transport.

(B) Top: stills from movies of GFP-labeled mitochondria in wing neuron axons of aged control and hs-PKA* flies that have been subjected to heat shock. Bottom: traces of transported mitochondria in corresponding movies. (C and D) Number of transported and total mitochondria per 50 μm of axonal tract of wing neurons in aged (C) and young (D) control or hs-PKA* flies subjected to heat shock.

(E) Number of transported and total mitochondria per 50 μm of axonal tract of wing neurons of 2-day-old flies with expression of UAS-luciferaseRNAi1 or two independent UAS-Pka-C1RNAi constructs. appl-Gal4 marks both the mechanosensory and chemosensory neurons in the wing nerve. Statistical significance was evaluated with a Mann-Whitney U test (C and D) or a one-way ANOVA with Dunnett’s multiple comparison (E). *p < 0.05, **p < 0.01. Magenta circles are values for individual wings; error bars are SEM. Scale bar, 5 μm. See also Figures S1 and S2, Movie S3, and Table S1.
young flies (Figure 2A) did not alter the number of transported mitochondria (Figure 2D). The outcomes of PKA* expression in young and aged flies were very similar to those observed after feeding with 8-Br-cAMP, indicating that cAMP-mediated stimulation of mitochondrial transport in aged flies involves PKA.

We assessed the role of endogenous PKA during mitochondrial transport in wing neurons by targeting Pka-C1 mRNA, which encodes the major catalytic PKA subunit in Drosophila (PKAc). Two different Pka-C1 RNAi constructs reduced the number of transported mitochondria in young flies without altering mitochondrial number in the axonal tract (Figure 2E; Figure S1F). These experiments demonstrate that endogenous PKA activity promotes axonal transport of mitochondria. However, levels of PKA activation must not be limiting in young flies, because mitochondrial transport was insensitive to experimental elevation of cAMP or PKA*.

We next asked whether cAMP/PKA pathway activity could become limiting for transport in aged flies because of declining levels of cAMP or PKAc. Adult wings were used as a source of cell extracts for these experiments, because wing neurons constitute a large fraction of the living material in this sclerotized tissue [5, 21]. Whereas the concentration of cAMP in the wings of aged flies did not decline compared to those of young flies (Figure 3A), the level of PKAc did (Figure 3B). The reduction in PKAc level was not associated with a general decrease in protein abundance in aged flies (Figure 3B; Figure S2D). The level of Pka-C1 mRNA in wings was not lower in aged flies compared to young flies (Figure S2E), indicating that the decline in PKAc abundance is due to altered synthesis or turnover of the protein. Although we cannot rule out other mechanisms, a reduction in PKAc protein levels could explain why cAMP/PKA pathway activity is limiting for mitochondrial transport in aged flies. Presumably, increasing cAMP levels compensate for the reduction in PKAc concentration in aged neurons by activating the protein that is available.

We then explored how experimental elevation of cAMP/PKA upregulates mitochondrial transport in aging wing neurons. To test whether increased movement of mitochondria reflects a general increase in cargo transport, we monitored the transport of fluorescently labeled dense-core vesicles (DCVs) after heat-shock-mediated induction of PKA*. In contrast to what was observed for mitochondria, expression of PKA* in aged flies did not increase the frequency of DCV transport compared to heat-shocked controls (Figure 3C; Figures S2F and S2G). Although effects on motility of other cargoes remain to be tested, this experiment demonstrates that not all microtubule-based transport is elevated by PKA* expression. The selective effect of PKA* overexpression on transport of mitochondria and DCVs is consistent with these cargoes being differentially affected during aging, with DCVs only exhibiting a small reduction in motility during the first 30 days of adult life [3] (Figure S2H).

Although mitochondria and DCVs both use dynein for retrograde axonal transport in Drosophila neurons [5, 24, 25], they differ in the identity of the motors primarily used for anterograde motion. Anterograde movement of mitochondria is driven by kinesin-1 [5, 24], whereas DCVs use kinesin-3/Unc104 [25]. We therefore asked whether the sensitivity of mitochondria to normal aging and the experimental elevation of PKA activity could be related to their reliance on kinesin-1.

We observed a reduction in the level of the Kinesin-1 motor subunit (Kinesin-1 heavy chain, Khc) in wings of aged flies compared to wings of young flies (Figure 3D). This reduction must be due to altered synthesis or turnover of the protein, as Khc mRNA abundance did not decline with age (Figure S2E). These findings raised the possibility that reduced Khc abundance contributes to the decline in mitochondrial transport in aged flies. Limiting levels of Khc could in principle account for the age-related reduction in retrograde and anterograde motion because the activity of dynein in this system depends on kinesin-1 [5], as is the case elsewhere [26].

Next, we asked whether the level of Khc is altered by experimental activation of the cAMP/PKA pathway in aged flies. We observed a strong increase in Khc protein abundance in wing extracts of aged animals following heat-shock-mediated induction of PKA* (Figure 3E). Gel-based analysis of protein extracts revealed that several abundant proteins were also upregulated by PKA* expression, although the levels of other abundant proteins did not increase (Figure 3F). Thus, Khc is one of a number of proteins upregulated by cAMP/PKA pathway activation in aged flies.

cAMP/PKA pathway activation increases levels of Khc mRNA in Aplysia neurons [27]. We therefore monitored Khc mRNA abundance in wings of aged flies with heat-shock-mediated induction of PKA* or with heat-shock treatment alone. There was an ~6.5-fold increase in the amount of Khc transcript in response to PKA* expression, whereas the level of the mRNA encoding a “housekeeping” protein (ribosomal protein 49, Rp49) did not change (Figure 3G). Khc mRNA, but not Rp49 mRNA, was also upregulated by acute supply of 8-Br-cAMP to aged flies (Figure S2I). Thus, unlike the decline in Khc protein levels during aging, the increase in Khc protein levels upon experimental activation of cAMP/PKA is associated with changes in mRNA abundance. The best-characterized mechanism for PKA-mediated gene expression involves the cAMP response element-binding protein (CREB) transcription factor. CREB activity is stimulated by phosphorylation, which can be mediated by multiple kinases, including PKA [28]. The abundance of Khc mRNA in aged flies was decreased by heat-shock-mediated induction of a dominant-negative CREB transgene (hs-dCREB2-b; Figure 3H). By providing evidence that the Khc gene is responsive to CREB, these data suggest a mechanism for upregulation of Khc mRNA upon experimental activation of PKA in aged flies.

Khc is linked to mitochondria by the concerted action of two proteins, Milton and Miro [1]. The level of Milton mRNA in aged wings was not affected by PKA*, whereas the level of Miro RNA was increased by 1.7-fold (Figure 3G). Thus, the mRNAs encoding components of the anterograde mitochondrial transport complex, the one encoding Khc is most responsive to cAMP/PKA pathway activation. The level of Pka-C1 mRNA was also upregulated by PKA* (Figure 3G), consistent with the ability of PKAc to stimulate its own expression in mammalian cells [29]. Several other mRNAs tested also increased in abundance in response to PKA*, although the response was weaker than observed for Khc mRNA (Figure 3G). These results are consistent with the widespread transcriptional changes induced by PKA in mammalian cells [30, 31].

Our finding that many mRNAs are upregulated by PKA activation in aging wings raised the question of whether the increased abundance of Khc plays a significant role in boosting
mitochondrial transport. We therefore asked whether Khc overexpression is sufficient to boost mitochondrial motility in wing neurons of aged flies. For these experiments, we used a Drosophila Khc genomic rescue construct (P[Khc+]) to increase Khc protein levels (Figure 4A).

P[Khc+] increased the number of mitochondrial movements in aged flies by 2.1-fold without altering the number of mitochondria in the axonal tract (Figures 4B and 4C; Movie S4). The boost in transport was associated with an increase in both retrograde and anterograde movements (Figure S1G), again consistent

**Figure 3. Overexpression of Activated PKAc in Aged Flies Upregulates Khc Protein and mRNA**

(A) cAMP levels in wings of young (day 2) and aged (day 30) wild-type flies assessed by ELISA. Magenta circles are values per milliliter of extract (technical replicates from two independent experiments).

(B) Representative immunoblot for PKAc and mitochondrial complex-Vα (Vα) using wing extracts of young (day 2) and aged (day 30) wild-type flies. Vα signal indicates no global decline in protein levels during aging. Charts show quantification of normalized PKAc signal from three independent experiments.

(C) Percentage of DCVs that are transported in the axonal tract of wing neurons in heat-shocked control or hs-PKA* flies. Magenta circles are values for individual wings. DCVs were marked with rat prepro-atrial natriuretic factor peptide fused to the fluorescent protein Emerald (ANF::EMD).

(D) Representative immunoblot for Khc and Vα using wing extracts of young (day 2) and aged (day 30) wild-type flies. Chart shows quantification of normalized Khc signal from four independent experiments.

(E) Representative immunoblots for Khc using wing extracts of 32-day-old control or hs-PKA* flies following 4-day heat shock. Chart shows quantification of normalized Khc signal from three independent experiments.

(F) SYPRO Ruby-stained gel of wing extracts of 32-day-old heat-shocked control or hs-PKA* flies used in (E). Circles and arrowheads indicate, respectively, abundant proteins that are or are not responsive to PKA*.

(G) Reverse-transcription digital droplet-PCR (RT-ddPCR) analysis of the relative abundance of mRNAs in wings of control and hs-PKA* 31-day-old flies subjected to heat shock for the preceding 24 hr. Magenta circles are values from individual technical replicates from two independent reverse transcription reactions per genotype. Rp49 has been used as a “housekeeping” gene in RT-PCR experiments with wings [22]. Rap2L, 14-3-3, and eIF-1A mRNAs were previously detected in RT-PCR experiments in Drosophila heads [23]. Sgg/Gsk3β upregulates anterograde mitochondrial motility in mammalian neurons [20].

(H) RT-ddPCR analysis of the relative abundance of Khc mRNA in wings of 32-day-old control and hs-dCREB2-b flies following 4 days of heat shock. Magenta circles are values from individual technical replicates.

Statistical significance was evaluated with the Mann-Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars are SEM. See also Figure S2 and Table S2.
with the tightly coupled activities of dynein and kinesin-1 in this system. Khc overexpression did not strongly affect the length and velocity of mitochondrial movements, as was also the case following manipulation of the cAMP/PKA pathway (Table S1). Thus, both types of intervention predominantly affect the initiation of transport.

P[Khc+] also reduced the signal from an oxidative stress sensor in wing neurons of aged flies (Figure S3). This finding is consistent with protective effects of mitochondrial transport in this system [5], although elevated transport of other kinesin-1 cargoes could also contribute to this phenotype. It will be important in the future to understand the specific contribution of upregulated mitochondrial motility to energy metabolism in the wing nerve.

The frequency of mitochondrial transport in wing neurons of young flies was not increased by P[Khc+] (Figure 4D). The finding that kinesin-1 activity was not limiting at this stage is consistent with the endogenous level of Khc protein being higher than in aged flies (Figure 3D). Although we cannot rule out additional influences of activated PKA, such as direct phosphorylation of proteins that affect mitochondrial dynamics [32–37], our data indicate that Khc upregulation is a key functional output of experimental activation of PKA in the context of mitochondrial transport in aged neurons.

Perspective

Reduced axonal transport of mitochondria has been implicated in the pathogenesis of several age-related neurodegenerative diseases [38, 39]. It has therefore been proposed that stimulating mitochondrial transport could be of therapeutic benefit, potentially in combination with agents that target other cellular processes [38–41]. However, it was not clear to what extent a chronic decline in mitochondrial motility can be reversed. We find that feeding aged Drosophila with a small-molecule agonist of PKA is sufficient to ameliorate a decline in mitochondrial transport that begins in the first few days of adult life.

We provide evidence that the stimulation of transport upon cAMP/PKA pathway activation is associated with upregulation of the kinesin-1 motor subunit, which counteracts the reduction in levels of this protein that occurs during aging. The observation that cytosolic levels of the Khc ortholog decline in brains of aging monkeys [42] suggests that the regulatory processes we have uncovered in Drosophila could be of relevance in mammalian systems. Recent studies in mammalian neurons have shown that a progressive decrease in mitochondrial motility promotes developmental maturation of neurons [43–45]. It is tempting to speculate that whereas this attenuation of mitochondrial transport plays a positive role in early life, it becomes detrimental during aging when the functional demands of neurons change or stresses increase.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Drosophila strains and husbandry
- METHOD DETAILS
  - Axonal transport assays
  - 8-Br-cAMP feeding
SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, and four movies (ddPCR), members of the Drosophila community for sharing reagents, the Wohl Cellular Imaging Centre at King’s College London for help with light microscopy, MRC-LMB Visual Aids for artwork, and Giampietro Schiavo for support and critically reading the manuscript. This work was funded by the MRC (file reference number MC_U105178790; to S.L.B.), an NC3Rs David Sainsbury Fellowship (NC/N001753/1), and a Van Geest Fellowship in Dementia and Neurodegeneration (both to A.V.).

AUTHOR CONTRIBUTIONS

A.V. performed experiments and analyzed data; A.V. and S.L.B. conceived the study, designed experiments, and wrote the paper; and S.L.B. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Schwarz, T.L. (2013). Mitochondrial trafficking in neurons. Cold Spring Harb. Perspect. Biol. 5, a011304.
2. Sheng, Z.-H. (2017). The interplay of axonal energy homeostasis and mitochondrial trafficking and anchoring. Trends Cell Biol. 27, 403–416.
3. Takihara, Y., Inatani, M., Eto, K., Inoue, T., Kreymerman, A., Miyake, S., Ueno, S., Nagaya, M., Nakashishi, A., Iwao, K., et al. (2015). In vivo imaging of axonal transport of mitochondria in the diseased and aged mammalian CNS. Proc. Natl. Acad. Sci. USA 112, 10515–10520.
4. Milde, S., Adalbert, R., Elaman, M.H., and Coleman, M.P. (2015). Axonal transport declines with age in two distinct phases separated by a period of relative stability. Neurobiol. Aging. 36, 971–981.
5. Vagnoni, A., Hoffmann, P.C., and Bullock, S.L. (2016). Reducing Lissencephaly1 levels augments mitochondrial transport and has a protective effect in adult Drosophila neurons. J. Cell Sci. 129, 178–180.
6. Morsci, N.S., Hall, D.H., Driscoll, M., and Sheng, Z.-H. (2016). Age-related phasic patterns of mitochondrial maintenance in adult Caenorhabditis elegans neurons. J. Neurosci. 36, 1373–1385.
7. Adalbert, R., and Coleman, M.P. (2013). Review: axon pathology in age-related neurodegenerative disorders. Neuropathol. Appl. Neuropathol. 39, 90–108.
8. Fang, Y., Soares, L., Teng, X., Geary, M., and Bonini, N.M. (2012). A novel Drosophila model of nerve injury reveals an essential role of Nmnat in maintaining axonal integrity. Curr. Biol. 22, 590–595.
9. Vagnoni, A., and Bullock, S.L. (2016). A simple method for imaging axonal transport in aging neurons using the adult Drosophila wing. Nat. Protoc. 11, 1711–1723.
10. Li, L.B., Lei, H., Arey, R.N., Li, P., Liu, J., Murphy, C.T., Xu, X.Z., and Shen, K. (2016). The neuronal kinesin UNC-104/KIF1A is a key regulator of synaptic aging and insulin signaling-regulated memory. Curr. Biol. 26, 605–615.
11. Tong, J.J., Schriner, S.E., McCleary, D., Day, B.J., and Wallace, D.C. (2007). Life extension through neurofibromin mitochondrial regulation and antioxidant therapy for neurofibromatosis-1 in Drosophila melanogaster. Nat. Genet. 39, 476–485.
12. Wang, Z., Zhang, L., Liang, Y., Zhang, C., Xu, Z., Zhang, L., Fuji, R., Mu, W., Li, L., Jiang, J., et al. (2015). Cyclic AMP mimics the anti-aging effects of calorie restriction by up-regulating Sir2uin. Sci. Rep. 5, 12012.
13. Byers, D., Davis, R.L., and Kiger, J.A., Jr. (1981). Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in Drosophila melanogaster. Nature 289, 79–81.
14. Riera, C.E., and Dillin, A. (2015). Can aging be ‘drugged’? Nat. Med. 21, 1400–1405.
15. Avery, M.A., Rooney, T.M., Pandya, J.D., Wishart, T.M., Gillingwater, T.H., Geddes, J.W., Sullivan, P.G., and Freeman, M.R. (2012). WldS prevents axon degeneration through increased mitochondrial flux and enhanced mitochondrial Ca2+ buffering. Curr. Biol. 22, 596–600.
16. Han, S.M., Baig, H.S., and Hammarlund, M. (2016). Mitochondria localize to injured axons to support regeneration. Neuron 92, 1308–1323.
17. Cartoni, R., Norsworthy, M.W., Wei, F., Wang, C., Li, S., Zhang, Y., Gabel, C.V., Schwarz, T.L., and He, Z. (2016). The mammalian-specific protein Armck1 regulates mitochondrial transport during axon regeneration. Neuron 92, 1294–1307.
18. Hiruma, H., Saifo, A., Kusakabe, T., Takenaka, T., and Kawakami, T. (2002). Neuropeptide Y inhibits axonal transport of particles in neurites of cultured adult mouse dorsal root ganglion cells. J. Physiol. 543, 85–97.
19. Hwang, R.-D., Wiemerslage, L., LaBreck, C.J., Khan, M., Kannan, K., Wang, X., Zhu, X., Lee, D., and Fridell, Y.W. (2014). The neuroprotective effect of human uncoupling protein 2 (hUCP2) requires cAMP-dependent protein kinase in a toxin model of Parkinson’s disease. Neurobiol. Dis. 69, 180–191.
20. Ogawa, F., Murphy, L.C., Malavasi, E.L., O’Sullivan, S.T., Torrance, H.S., Porteous, D.J., and Millar, J.K. (2016). NDE1 and GSK3β associate with TRAK1 and regulate axonal mitochondrial motility: identification of cyclic AMP as a novel modulator of axonal mitochondrial trafficking. ACS Chem. Neurosci. 7, 553–564.
21. Johnson, S.A., and Milner, M.J. (1987). The final stages of wing development in Drosophila melanogaster. Tissue Cell 19, 505–513.
22. Raad, H., Ferveur, J.-F., Ledger, N., Capovilla, M., and Robichon, A. (2016). Functional gustatory role of chemoreceptors in Drosophila wings. Cell Rep. 15, 1442–1454.
23. Ling, D., and Salvaterra, P.M. (2011). Robust RT-qPCR data normalization: validation and selection of internal reference genes during post-experimental data analysis. PLoS ONE 6, e17782.
24. Pilling, A.D., Horuichi, D., Lively, C.M., and Saxton, W.M. (2006). Kinesin-1 and dynein are the primary motors for fast transport of mitochondria in Drosophila motor axons. Mol. Biol. Cell 17, 2057–2068.
25. Barkus, R.V., Klyachko, O., Horuichi, D., Dickson, B.J., and Saxton, W.M. (2008). Identification of an axonal kinesin-3 motor for fast anterograde vesicle transport that facilitates retrograde transport of neuropeptides. Mol. Biol. Cell 19, 274–283.
26. Jolly, A.L., and Gelfand, V.I. (2011). Bidirectional intracellular transport: utility and mechanism. Biochem. Soc. Trans. 39, 1126–1130.
27. Puthanveettil, S.V., Monje, F.J., Miniaci, M.C., Choi, Y.B., Karl, K.A., Kandros, E., Gawinowicz, M.A., Sheetz, M.P., and Kandel, E.R. (2008). A new component in synaptic plasticity: upregulation of kinesin in the neurons of the gill-withdrawal reflex. Cell 135, 960–973.
28. Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phospho-
rylation-dependent factor CREB. Nat. Rev. Mol. Cell Biol. 2, 599–609.
29. Skalhegg, B.S., and Tasken, K. (2000). Specificity in the cAMP/PKA
signaling pathway. Differential expression, regulation, and subcellular
localization of subunits of PKA. Front. Biosci. 5, D678–D693.
30. Impay, S., McCorkle, S.R., Cha-Molstad, H., Dwyer, J.M., Yochum, G.S.,
Boss, J.M., McWeeney, S., Dunn, J.J., Mandel, G., and Goodman, R.H.
(2004). Defining the CREB regulon: a genome-wide analysis of transcrip-
tion factor regulatory regions. Cell 79, 1041–1054.
31. Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J.,
Chen, H., Jenner, R., Herbsolheimer, E., Jacobsen, E., et al. (2005). Genome-wide analysis of cAMP-response element binding protein occu-
pancy, phosphorylation, and target gene activation in human tissues.
Proc. Natl. Acad. Sci. USA 102, 4459–4464.
32. Okada, Y., Sato-Yoshitake, R., and Hirokawa, N. (1995). The activation of
protein kinase A pathway selectively inhibits anterograde axonal transport
of vesicles but not mitochondria transport or retrograde transport in vivo.
J. Neurosci. 15, 3053–3064.
33. Chang, C.R., and Blackstone, C. (2007). Cyclic AMP-dependent protein kinase
phosphorylation of Drp1 regulates its GTPase activity and mito-
ochondrial morphology. J. Biol. Chem. 282, 21583–21587.
34. Cribbs, J.T., and Strack, S. (2007). Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mito-
ochondrial fission and cell death. EMBO Rep. 8, 939–944.
35. Dagda, R.K., Guodon, A.M., Pien, I., Strack, S., Green, S., Li, C., Van
Houten, B., Cherra, S.J., and Chu, C.T. (2011). Mitochondrially localized
PKA reverses mitochondrial pathology and dysfunction in a cellular model
of Parkinson’s disease. Cell Death Differ. 18, 1914–1923.
36. Merrill, R.A., Dagda, R.K., Dickey, A.S., Cribbs, J.T., Green, S.H.,
Usachev, Y.M., and Strack, S. (2011). Mechanism of neuroprotective mito-
ochondrial remodeling by PKA/AKAP1. PLoS Biol. 9, e1000612.
37. Scherer, J., Yi, J., and Vallee, R.B. (2014). PKA-dependent dynein switch-
ing from lysosomes to adenosine: a novel form of host-virus competition.
J. Cell Bioi. 205, 163–177.
38. Hinckelmann, M.V., Zala, D., and Saudou, F. (2013). Releasing the brake:
restoring fast axonal transport in neurodegenerative disorders. Trends Cell
Bioi. 23, 634–643.
39. De Vos, K.J., and Hafiezparast, M. (2017). Neurobiology of axonal trans-
port defects in motor neuron diseases: opportunities for translational research?
Neurobiol. Dis. 105, 283–299.
40. Bil iso, L.G., Sahai, E., Kelly, G., Golding, M., Greensmith, L., and Schiavo, G. (2010). Deficits in axonal transport precede ALS symptoms
in vivo. Proc. Natl. Acad. Sci. USA 107, 20523–20528.
41. d’Ydewalle, C., Krishnan, J., Chibe, D.M., Van Damme, P., Iobi, J.,
Kozikowski, A.P., Vanden Berghe, P., Timmerman, V., Robberecht, W.,
and Van Den Bosch, L. (2011). HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease.
Nat. Med. 17, 968–974.
42. Kimura, N., Imamura, O., Ono, F., and Terao, K. (2007). Aging attenuates
dynein-dynein interaction: down-regulation of dynein causes accumula-
tion of endogenous tau and amyloid precursor protein in human neuro-
bioma cells. J. Neurosci. Res. 85, 2909–2916.
43. Faits, M.C., Zhang, C., Soto, F., and Kerschensteiner, D. (2016). Dendritic
mitochondria reach stable positions during circuit development. eLife 5,
e11583.
44. Lewis, T.L., Jr., Turi, G.F., Kwon, S.-K., Losonczy, A., and Polleux, F.
(2016). Progressive decrease of mitochondrial motility during maturation of cortical axons in vitro and in vivo. Curr. Biol. 26, 2602–2608.
45. Smit-Rigter, L., Rajendran, R., Silva, C.A.P., Spierenburg, L., Groeneweg,
F., Ruimschotel, E.M., van Versendaal, D., van der Toogt, C., Eyssel, U.T.,
Heimel, J.J.A., et al. (2016). Mitochondrial dynamics in visual cortex are
limited in vivo and not affected by axonal structural plasticity. Curr. Biol.
26, 2609–2616.
46. Lane, M.E., and Kalderon, D. (1993). Genetic investigation of cAMP-
dependent protein kinase function in Drosophila development. Genes Dev.
7, 1229–1243.
47. Walker, J.A., Gozli, J.Y., Long, J.B., Huang, S., Maher, R.C., Xia, H.,
Khall, K., Ray, A., Van Vactor, D., Bernards, R., and Bernards, A. (2013).
Genetic and functional studies implicate synaptic overgrowth and ring
gland cAMP/PKA signaling defects in the Drosophila melanogaster
neurofibrromatosis-1 growth deficiency. PLoS Genet. 9, e1003958.
48. Martin, M., Iyadurai, S., Gassman, A., Gindhart, J.G., Jr., Hays, T.S., and
Saxton, W.M. (1999). Cytoplasmic dynein, the dynactin complex, and
kinesin are interdependent and essential for fast axonal transport. Mol.
Biol. Cell 10, 3717–3728.
49. Sykioisis, G.P., and Bohmann, D. (2008). Keap1/Nrf2 signaling regulates
oxidative stress tolerance and lifespan in Drosophila. Dev. Cell 14, 76–85.
50. Yin, J.C., Wallach, J.S., Del Vecchio, M., Wilder, E.L., Zhou, H., Quinn,
W.G., and Tully, T. (1994). Induction of a dominant negative CREB trans-
gene specifically blocks long-term memory in Drosophila. Cell 79, 49–58.
51. Nakamura, M., Baldwin, D., Hannaford, S., Palka, J., and Monteil, C.
(2002). Defective proboscis extension response (DPR), a member of the Ig
superfamily required for the gustatory response to salt. J. Neurosci.
22, 3463–3472.
52. Torroja, L., Chu, H., Kotsovsky, I., and White, K. (1999). Neuronal overex-
pression of APP, the Drosophila homologue of the amyloid precursor pro-
ctein (APP), disrupts axonal transport. Curr. Biol. 9, 489–492.
53. Rao, S., Lang, C., Levitan, E.S., and Deitcher, D.L. (2001). Visualization of
neuropeptide expression, transport, and exocytosis in Drosophila
melanogaster. J. Neurobiol. 49, 159–172.
54. Hao, Y., Frey, E., Yoon, C., Wong, H., Nestorovsky, D., Holzman, L.B.,
Giger, R.J., DiAntonio, A., and Collins, C. (2016). An evolutionarily
conserved mechanism for cAMP elicited axonal regeneration involves
direct activation of the dual leucine zipper kinase DLK. eLife 5, e14048.
55. Godena, V.K., Brookes-Hocking, N., Moller, A., Shaw, G., Oswald, M.,
Sancho, R.M., Miller, C.C., Whitworth, A.J., and De Vos, K.J. (2014).
Increasing microtubule acetylation rescues axonal transport and locomo-
tor deficits caused by LRRK2 Roc-COR domain mutations. Nat. Commun.
5, 5245.
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| rabbit anti-Kinesin-1 heavy chain | Cytoskeleton Inc. | Cat# AKIN01-B; RRID:AB_10707921 |
| mouse anti-Complex-Vα | Thermo Fisher Scientific | Cat# 43-9800; RRID: AB_2533548 |
| mouse anti-PKAc | Santa Cruz Biotechnology | Cat# sc-28315; RRID:AB_628136 |
| rabbit anti-Drosophila PKAc (DC0) | Columbia University [46] | Produced and characterized by Daniel Kalderon |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| 8-Br-cAMP | Santa Cruz Biotechnology | Cat# sc-201564 |
| PhosSTOP Phosphatase Inhibitor | Sigma-Aldrich | Cat# 4906837001 |
| cOmplete Protease Inhibitor | Sigma-Aldrich | Cat# 11836170001 |
| DTT | Sigma-Aldrich | Cat# 10197777001 |
| LDS sample buffer | ThermoFisher | Cat# NP0008 |
| SYPRO Ruby | Lonza | Cat# 50562 |
| iScript Select cDNA synthesis kit | Bio-Rad | Cat# 1708996 |
| EvaGreen Supermix | Bio-Rad | Cat# 1864034 |
| Droplet Generator Oil | Bio-Rad | Cat# 1864005 |
| RNase-free DNase Set | QIAGEN | Cat# 79254 |
| **Critical Commercial Assays** |        |            |
| cAMP Parameter Assay Kit | R&D Systems | Cat# KGE002B |
| RNeasy MINI kit | QIAGEN | Cat# 74104 |
| Amersham ECL Prime Western Blotting Detection Reagent | GE-Healthcare | Cat# RPN2232 |
| Amersham ECL Western Blotting Detection Kit | GE-Healthcare | Cat# RPN2108 |
| **Experimental Models: Organisms/Strains** |        |            |
| D. melanogaster: Oregon-R | Bloomington Drosophila Stock Center | Cat# 5; RRID:BDSC_5 |
| D. melanogaster: w1118 P[w+mC = UAS-mito-HA-GFP.AP]3, e1 | Bloomington Drosophila Stock Center | Cat# 31599; RRID:BDSC_31599 |
| D. melanogaster: hs-PKA* | Harvard Medical School [47] | Produced and characterized by James Walker |
| D. melanogaster: hs-dCREB2-b | University of Wisconsin-Madison [50] | Produced and characterized by Jerry Yin |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by Alessio Vagnoni (alessio.vagnoni@kcl.ac.uk)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila strains and husbandry

The wild-type strain was Oregon-R. Strains containing the following transgenes were obtained from the Bloomington Drosophila Stock Center (Indiana University, USA): dpr-Gal4 ([8, 51]; BL#25083), UAS-mito::GFP ([24]; BL#8442 or BL#8443); appl-Gal4 ([52]; BL#32040); UAS-preproANF::EMD ([53]; BL#7001); UAS-luciferase-RNAi (BL#31603); UAS-dunce-RNAi ([54]; BL#27250); UAS-Pka-C1-RNAi (BL#31277 - RNAi1; BL#31599 - RNAi2). The RNAi lines were generated by the Transgenic RNAi Project (TRiP) at Harvard Medical School, USA. hs-PKA* flies [47] were provided by James Walker (Harvard University, USA). P[Khc+] flies (‘PK9a’ stock [48]) were provided by Isabel Palacios (University of Cambridge, UK). The gstD-GFP reporter line [49] was provided by Sean Sweeney (University of York, UK). The hs-dCREB2-b flies [50] were provided by Mani Ramaswami (Trinity College Dublin, Ireland). Each transgene was present in one copy in the experimental genotypes. Within an experimental series, cohorts of flies were cultured using the same fly food. Flies were transferred to new food twice a week. Flies were cultured at 25°C unless stated otherwise using a 12-h-light–12-h-dark cycle. For experiments involving heat-shock, fly cultures were reared at 18°C throughout development and shifted to 30°C typically within 12 h of eclosion (for imaging young flies) or after 28 or 30 d (for 4-d or 24-h heat shock of aged flies, respectively).

METHOD DETAILS

Axonal transport assays

We recently published an in-depth protocol for the visualization and analysis of cargo transport in the wing nerve [9]. Briefly, flies that had been anaesthetised with CO2 were immobilised, with wings outstretched, on a cover glass with a fine layer of Halocarbon oil (VWR). A second coverglass was then added on top of the fly to stabilize the sample. Wing nerves were imaged by spinning disk microscopy. Two spinning disk systems were used during the study, with the same system used for an entire experimental series: (1) PerkinElmer Ultraview ERS with a CSU21 scanning head (Yokogawa) and an inverted microscope stand (IX71 (Olympus)) equipped with a 60x PlanApo oil-immersion objective (1.4 NA) and a CCD camera (Ora ER (Hamamatsu)); (2) Nikon spinning disk system with a CSU-X1 scanning head (Yokogawa) and an inverted microscope stand (Eclipse Ti-E (Nikon)) equipped with a 60 x CFI Apo oil-immersion objective (1.4 NA) and an EM-CCD camera (Du 897 iXon Ultra (Andor)). Frame rates were 0.5/s and 1/s for mitochondria and DCVs, respectively. Image series were captured for 3 min. Tracking of mitochondria was performed manually (MTrackJ) on the raw movies by marking the start and end of each run. Tracking was performed blind to the experimental condition or genotype using the Randomizer macro (Tiago Ferreira, McGill University) in ImageJ. In cases where the sample shifted during filming, correction with...
were starved by removal from food for \( C/2^4 \) h at 25 °C and placed on Whatman glass microfiber filter papers (GF/C grade, GE-Healthcare) soaked with 400 μL of a solution containing 10% sucrose alone (vehicle) or 10% sucrose and 100 μM 8-Br-cAMP (Santa Cruz Biotechnology). Filters were changed daily for 4 d before recording axonal transport in wing neurons. At the time of imaging, the age of the flies was 4 d after eclosion (young flies) and 32 d after eclosion (aged flies). For prolonged drug feeding, 8-Br-cAMP was added to standard fly food to a final concentration of 100 μM. Standard fly food was used in the vehicle control. Flies were transferred into fresh food (±8-Br-cAMP) twice a week until imaging.

**Immunoblotting and protein staining**

Protein extracts from wings and heads were prepared following a protocol adapted from Vagnoni et al., 2016 [5]. Wings and heads were collected by cutting at the wing root close to the thorax of the animal or at the base of the head, respectively, with fine spring scissors. Typically, 100 wings or 20 heads were collected in a 1.5 mL Eppendorf tube kept on dry ice. Tissues were collected by brief centrifugation at 16,100 × g and ground on ice with a plastic hand-held pestle before adding 300 μL (for heads) or 200 μL (for wings) of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1x PhosSTOP Phosphatase Inhibitor (Roche) and 1x cComplete Protease Inhibitor (Roche)). After addition of Triton X-100 to a final concentration of 1%, the samples were further homogenized by multiple passages through a 23G syringe needle. Lysates were spun at 16,100 × g for 30 min at 4 °C. 40 mM DTT was added to the collected supernatant, mixed with LDS sample buffer (Novex-Life Technologies) and boiled for 10 min at 90 °C before electrophoresis. After gel electrophoresis and protein transfer, membranes were incubated with the following primary antibodies: rabbit anti-Kinesin-1 heavy chain (AKIN01, Cytoskeleton Inc., diluted 1:1000), mouse anti-Complex-V \( \alpha \) (MitoSciences-Life Technologies, clone 15H4C4, diluted 1:5000 for wings and 1:10,000 for heads), mouse anti-PKAc (Santa Cruz Biotechnology, clone A2, diluted 1:1000) or rabbit anti-Drosophila PKAc (DC0) ([46]; diluted 1:1000 (a gift from Daniel Kalderon (Columbia University, USA)). After washing with PBS containing 0.1% Tween, HRP-conjugated secondary antibodies and chemiluminescent substrate (ECL or ECL Prime detection systems (GE-Healthcare)) were used to reveal the protein-derived signal on X-ray films (GE-Healthcare). Typically, between 2% and 10% of total lysate sample was loaded per gel lane. Background corrected band intensities were quantified using the Gel Analyzer tool in ImageJ. Signals from the target protein were normalized to the mean signal for the control sample or young fly sample on the same film. For the data in Figures 3B, 3D, and 4A, signals from the target protein were additionally normalized for the Complex \( \alpha \) signal for the same sample. For staining with SYPRO Ruby (Lonza), gels were fixed in 50% methanol/7% acetic acid for 1 h following electrophoresis. Overnight incubation in SYPRO Ruby gel stain was followed by washing in 10% methanol/7% acetic acid for 40 min before imaging with a ChemiDoc MP imaging system (Bio-Rad).

**Assessment of cAMP levels**

cAMP abundance in *Drosophila* wings was assayed with the cAMP Parameter Assay Kit (R&D Systems). 100 wings were homogenized in 600 μL of Cell Lysis Buffer from the kit and the lysate centrifuged twice at 600 × g for 5 min at 4 °C to remove cellular debris. After collecting the supernatant, the assay was carried out immediately according to the manufacturer’s instructions. The absorbance was measured at 450 nm with a PHERAsstar FS microplate reader (BMG Labtech) with correction wavelength set at 570 nm. The final cAMP concentration per mL of extract was calculated by interpolating the absorbance values on a cAMP standard curve.

**RT-ddPCR**

Wings were collected as described above. RNA was isolated with a RNeasy MINI kit (QIAGEN) from 140 wings per condition, and DNase treatment performed on-column with RNase-free DNase Set (QIAGEN) according to the manufacturer’s instruction. RT reactions were carried out at 42 °C for 80 min with Oligo(dT)\(_{20}\) and the iScript Select cDNA synthesis kit (Bio-Rad). 10 ng purified RNA was added to each reaction. Prior to ddPCR, end-point PCR was used to check the quality of the RT reactions and verify the presence of the mRNA of interest. ddPCR was performed with the QX200 Droplet Digital PCR System (Bio-Rad), following the manufacturer’s instructions. 1 μL of RT reaction was mixed with the EvaGreen Supermix (Bio-Rad) and forward and reverse primers (Table S2) in a final volume of 20 μL. The reaction mix was loaded into a D8 cartridge (Bio-Rad) and 70 μL of Droplet Generator Oil (Bio-Rad) was added before droplet production in the QX200 Droplet Generator (Bio-Rad). 40 μL of droplets was then used for each ddPCR replicate with the cycling conditions recommended by the manufacturer, except the annealing/extension temperature was set at 99 °C. The ddPCR output was read in a QX200 Droplet Reader (Bio-Rad) and the results visualized with QuantaSoft software (Bio-Rad). For each reaction, data from > 15,000 droplets were analyzed, and partitioned into two clearly distinguishable populations of positive droplets (where target amplification took place) and negative droplets (no target amplification).
Oxidative stress and focal protein accumulation assays
Flies containing the gstD-GFP transgene were mounted in an imaging chamber for spinning disk confocal microscopy, as described above. Z stacks of neuronal areas were acquired in multiple positions along the wing nerve. The different genotypes within a single experiment were imaged with the same laser power and camera gain on the same day. Reporter fluorescent intensity was measured from cell bodies using Z-projections of 5 focal planes. The average pixel fluorescence intensity of each region of interest was then calculated in ImageJ after subtraction of the background fluorescence signals from each image. The ‘Smooth’ filter of ImageJ was applied to images for presentation purposes. Flies overexpressing the UAS-GFP transgene present in the dpr-GAL4 stock were used to quantify the amount of wing nerve affected by focal protein accumulation in the fifth week after eclosion, as described previously [5]. Briefly, the wing nerve was divided into 8 equal segments, with a segment scored as positive if at least one focal accumulation of GFP was present.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis and data plotting was performed using Excel (Microsoft) and Prism (GraphPad). Details of the quantification methods for the different experiments are provided under each heading in the Method Details section. Details of statistical evaluations are provided in the figure legends, and the numbers of samples indicated in the figures. Non-parametric tests were used when the sample size was $\leq 10$. Multiple comparison corrections were applied when more than two groups were compared.