Axonal Mitochondria Modulate Neuropeptide Secretion Through the Hypoxic Stress Response in *Caenorhabditis elegans*

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ABSTRACT Neurons are highly dependent on mitochondrial function, and mitochondrial damage has been implicated in many neurological and neurodegenerative diseases. Here we show that axonal mitochondria are necessary for neuropeptide secretion in *Caenorhabditis elegans* and that oxidative phosphorylation, but not mitochondrial calcium uptake, is required for secretion. Oxidative phosphorylation produces cellular ATP, reactive oxygen species, and consumes oxygen. Disrupting any of these functions could inhibit neuropeptide secretion. We show that blocking mitochondria transport into axons or decreasing mitochondrial function inhibits neuropeptide secretion through activation of the hypoxia inducible factor HIF-1. Our results suggest that axonal mitochondria modulate neuropeptide secretion by regulating transcriptional responses induced by metabolic stress.

KEYWORDS mitochondria; neuropeptide secretion; hypoxia; reactive oxygen species; *Caenorhabditis elegans*

MUTATIONS that perturb mitochondrial function are linked to several neurodevelopmental and neurodegenerative disorders (Johri and Beal 2012; Pease and Segal 2014; Pickrell and Youle 2015; Kerr *et al.* 2017; Krench and Littleton 2017). For these reasons, there is significant interest in defining the cell biological mechanisms through which mitochondria regulate neuronal function and development. Mutations (or drug treatments) that impair mitochondrial function cause widespread cellular and developmental defects. Given these pleiotropic effects, identifying the precise molecular pathways through which mitochondria regulate neural development and behavior has proved challenging. One strategy to circumvent this problem has been to analyze mutants in which mitochondria transport or localization in axons is blocked (Guo *et al.* 2005; Verstreken *et al.* 2005; Kang *et al.* 2008; Ma *et al.* 2009; Russo *et al.* 2009). In these transport mutants, mitochondrial defects are restricted to

axons, whereas other cellular functions of mitochondria remain intact.

Analysis of mitochondria transport mutants suggests mitochondria can act through multiple pathways to influence neuronal function and development. Several aspects of neuronal function are especially dependent upon cellular energy stores (e.g., maintenance of membrane potential, intracellular transport to axons and dendrites, and membrane-fusion and endocytosis reactions). In addition to producing ATP, mitochondria also act as an intracellular calcium store; consequently, axonal mitochondria alter the duration of presynaptic calcium transients, thereby regulating neurotransmitter release at synapses (Werth and Thayer 1994; Tang and Zucker 1997; Billups and Forsythe 2002; Medler and Gleason 2002). Finally, mitochondria promote axon regeneration following injury (Rawson *et al.* 2014; Han *et al.* 2016). Given the widespread effects of mitochondria on cellular physiology, it is likely that additional mechanisms will be discovered that link mitochondria to neuronal function and development.

To further investigate how they affect neuronal function, we asked if axonal mitochondria regulate secretion of neuropeptides. Neuropeptides are a chemically diverse set of neuromodulators that are recognized by G protein-coupled receptors (GPCRs). GPCRs typically bind their cognate ligands with extremely high affinity and stimulate production of

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second messengers through G-protein activation of enzymatic targets. Consequently, neuropeptides (and other metabolic agonists) often produce relatively long-lasting signals over very large spatial domains. Thus, we hypothesized that axonal mitochondria could alter global behavioral states by regulating neuropeptide secretion. Here we show that axonal mitochondria promote neuropeptide secretion and that they do so by regulating a cell-autonomous stress response to hypoxia.

Materials and Methods

Caenorhabditis elegans strains and drug assays

Strains were maintained at 20° as described (Brenner 1974). The wild-type reference strain was N2 Bristol. Full descriptions of all alleles can be found at <http://www.wormbase.org>. Strains used in this study are as follows:

KP8711 *ric-7(nu447)* V.
 KP8730 *ric-7(nu447)* V; *nuEx1853[psnb-1::UNC-116-GFP-TOMM-7]*.
 KP8714 *nuIs183[punc129::NLP-21-YFP]* III KP8717 *nuIs183 III*; *nuEx1851[punc-129::UNC-116-tagRFP-TOMM-7]*.
 KP8724 *nuIs183 III*; *ric-7(nu447)* V KP8725 *nuIs183 III*; *ric-7(nu447)* V; *nuEx1851[punc-129::UNC-116-tagRFP-TOMM-7]*.
 KP9044 *nuSi187[punc-129::NLP-21-mNeonGreen]* IV.
 KP9073 *nuSi187 IV*; *ric-7(nu447)* V.
 KP9082 *nuSi187 IV*; *nuEx1851[punc-129::UNC-116-tagRFP-TOMM-7]*.
 KP9083 *nuSi187 IV*; *ric-7(nu447)* V; *nuEx1851[punc-129::UNC-116-tagRFP-TOMM-7]*.
 KP8755 *nuIs183 III*; *mcu-1(tm6026)* IV.
 KP8756 *nuIs183 III*; *mcu-1(ju1154)* IV.
 KP8749 *nuIs183 III*; *mev-1(kn1)* III.
 KP8748 *nuIs183 III*; *isp-1(qm150)* IV.
 KP8759 *nuo-6(qm200)* I; *nuIs183 III*.
 KP8777 *nuo-6(qm200)* I; *nuIs183 III*; *nuEx1863[punc-129::NUO-6]*.
 KP8804 *sod-2(ok1030)* I; *nuIs183 III*.
 KP8874 *sod-2(ok1030)* I; *nuIs183 III*; *nuEx1887[punc-129::SOD-2]*.
 KP9099 *sod-2(ok1030)* I; *nuSi187 IV*.
 KP9100 *sod-2(ok1030)* I; *nuSi187 IV*; *ric-7(nu447)* V.
 KP8753 *vhl-1(ok161)* X; *nuIs183 III*.
 KP8778 *vhl-1(ok161)* X; *nuIs183 III*; *nuEx1864[punc-129::VHL-1]*.
 KP9109 *vhl-1(ok161)* X; *nuSi187 IV*.
 KP9110 *vhl-1(ok161)* X; *nuSi187 IV*; *ric-7(nu447)* V.
 KP9111 *vhl-1(ok161)* X; *nuSi187 IV*; *hif-1(ia4)* V.
 KP9097 *nuSi187 IV*; *hif-1(ia4)* V.
 KP9098 *nuSi187 IV*; *ric-7(nu447)* V; *hif-1(ia4)* V.
 KP9677 *sod-2(ok1030)* I; *nuSi187 IV*; *hif-1(ia4)* V.
 KP9067 *nuIs183 III*; *aldo-1(tm5782)* III.
 KP9069 *pfkb-1.1(ok2733)* I; *nuIs183 III*.
 KP9071 *gpd-3(ok2870)* X; *nuIs183 III*.

KP8803 *sod-1(tm776)* II; *nuIs183 III*.
 KP8839 *sod-3(tm760)* X; *nuIs183 III*.
 KP8831 *nuIs183 III*; *sod-4(gk101)* III.
 KP8832 *sod-5(tm1146)* II; *nuIs183 III*.
 KP9106 *nuSi187 IV*; *unc-31(e928)* IV.
 KP9112 *vhl-1(ok161)* X; *nuSi187 IV*; *unc-31(e928)* IV.
 KP9095 *daf-16(mgDf47)* I; *nuSi187 IV*.
 KP9096 *daf-16(mgDf47)* I; *nuSi187 IV*; *ric-7(nu447)* V.

Constructs and transgenes

Transgenic strains were isolated by microinjection of various plasmids using either *Pmyo-2::NLS-GFP* or *Pmyo-2::NLS-mCherry* as co-injection markers. The *kin-Tom7* construct was adapted from the original version (Rawson *et al.* 2014) to contain the following elements (listed 5' to 3'): the *snb-1* promoter (for locomotion and aldicarb assays) or the *unc-129* promoter (for imaging), the complementary DNA (cDNA) for *unc-116*, linker sequence, GFP (for locomotion and aldicarb) or TagRFP (for imaging) with syntons, attachment recombination site + second linker, the genomic sequence of *tomm-7*, and the *let-858* 3'-UTR.

The TOMM-20-mCherry construct contains the *unc-129* promoter, the sequence encoding the first 53 amino acids of TOMM-20 carrying the mitochondrial localization signal, attachment recombination site, mCherry with syntons, and the *unc-54* 3'-UTR. The *Punc-129::SNB-1-GFP* construct was previously described (Sieburth *et al.* 2005).

For the *Punc-129::NLP-21-mNeonGreen* construct, the *unc-129* promoter, the *nlp-21*-minigene (coding region plus two syntons), mNeonGreen with syntons, and the *let-858* 3'-UTR were inserted into the miniMOS vector pCFJ910. Single copy insertion (*nuSi187*) of this transgene was obtained as described (Frokjaer-Jensen *et al.* 2014). The *Punc-129::NLP-21-YFP (nuIs183)* transgenic line was previously described (Sieburth *et al.* 2007).

The cell-specific rescue constructs contain the *unc-129* promoter, the open reading frame including introns (*nuo-6*, *vhl-1*) or the cDNA (*sod-2*) of the rescuing gene, and the *unc-54* 3'-UTR.

Drug, hypoxia, and RNA-interference assays

Acute aldicarb assays were performed blind in five replicates on young adult worms as described (Nurrish *et al.* 1999). Locomotion was measured by transferring adults to unseeded plates and recording their movement at 2 Hz for 30 sec on a Zeiss Discovery Stereomicroscope using AxioVision software. The centroid velocity of each animal was analyzed at each frame using object-tracking software in AxioVision. The speed of each animal was calculated by averaging the velocity value at each frame.

For treatment of worms with varying oxygen levels, worms were picked onto a fresh plate at the L4 stage and the plate was put into a Hypoxia Incubator Chamber (STEMCELL Technologies). Using a MINIOS 1 Oxygen Analyzer (MEDIQUIP), the

chamber was then filled with oxygen (for hyperoxia) or nitrogen until the oxygen test concentration was reached. Worms were left to grow at control and test oxygen levels for 24 hr and coelomocytes were imaged subsequently.

RNA interference (RNAi) was performed in the neuronal RNAi hypersensitive mutant background (*nre-1 lin-15b*) (Schmitz *et al.* 2007). One-day-old hermaphrodites were dropped into bleach on RNAi plates. When the progeny reached L4 stage, worms were transferred onto a fresh RNAi plate and scored for coelomocyte fluorescence 24 hr later.

Fluorescence microscopy and quantitative analysis

Worms were immobilized on 10% agarose pads with 0.1- μ m diameter polystyrene 501 microspheres (Polysciences 00876-15, 2.5% w/v suspension). For axonal fluorescence measurements, the dorsal nerve cord (midway between the vulva and the tail) was captured using a 60 $\times$ objective (NA 1.45) on an Olympus FV-1000 confocal microscope at 5 $\times$ digital zoom. Maximum intensity projections of z-series stacks were made using Metamorph 7.1 software (Molecular Devices, Sunnyvale, CA). Line scans of dorsal cord fluorescence were analyzed in Igor Pro (WaveMetrics, Lake Oswego, OR) using custom-written software (Burbea *et al.* 2002; Dittman and Kaplan 2006). The intensity of NLP-21 axonal puncta was measured as peak punctal fluorescence normalized to the baseline of diffuse axonal fluorescence [$\Delta F/F = (F_{\text{puncta}} - F_{\text{axon}})/F_{\text{axon}}$].

For coelomocyte fluorescence measurements, worms were imaged 24 hr after the L4 stage. Image stacks of the second-most-posterior coelomocyte were captured using a Zeiss Axioskop I, Olympus PlanAPO 100 $\times$ 1.4 NA objective, and a CoolSnap HQ CCD camera (Roper Scientific, Tuscon, AZ). Maximum intensity projections were obtained using Metamorph 7.1 software (Molecular Devices). For quantification, the five brightest vesicles were analyzed for each coelomocyte and the mean fluorescence for each vesicle was logged. For each worm, coelomocyte fluorescence was calculated as the mean of the vesicle values in that animal subtracted by the background fluorescence (measured at an empty region on the slide). All fluorescence values are normalized to wild-type controls to facilitate comparison.

Data availability

Strains and plasmids are available upon request. Supplemental Material, Figure S1 and Figure S2 contain measurements of NLP-21 secretion in additional genotypes. Supplemental material available at Figshare: <https://doi.org/10.25386/genetics.6849056>.

Results

Axonal mitochondria promote neuropeptide secretion

In a prior study, we showed that mutations inactivating the *C. elegans ric-7* gene significantly reduced neuropeptide secretion [which is mediated by exocytosis of dense core vesicles

(DCVs)] but had little effect on acetylcholine release [which is mediated by exocytosis of synaptic vesicles (SVs)] (Hao *et al.* 2012). These results imply that RIC-7 plays a specific role in promoting exocytosis of DCVs but not SVs. Decreased neuropeptide secretion in *ric-7* mutants was accompanied by decreased locomotion rate and increased resistance to the paralytic effects of aldicarb (an acetylcholinesterase inhibitor) (Hao *et al.* 2012). Homologs of *ric-7* are found in other nematodes but not in other metazoans. The *ric-7* gene encodes an unfamiliar protein that lacks any previously described structural domain. Thus, the sequence of the RIC-7 protein failed to provide any clues as to the cell biological mechanism underlying RIC-7's function in neuropeptide secretion.

A subsequent study showed that RIC-7 is required for kinesin-1-mediated transport of mitochondria into axons (Rawson *et al.* 2014). These results suggest that the behavioral and neuropeptide secretion defects observed in *ric-7* mutants could be caused by the absence of axonal mitochondria. To test this idea, we expressed a chimeric kinesin construct (kin-Tom7) in which UNC-116/KIF5 is fused to the outer mitochondrial membrane protein TOMM-7 (Figure 1A, Rawson *et al.* 2014). Expression of this construct restores the mitochondrial distribution in *ric-7* mutant axons (Rawson *et al.* 2014). Neuronal expression of kin-Tom7 significantly improved locomotion and decreased the aldicarb resistance of *ric-7* mutants (Figure 1, B and C), suggesting that *ric-7* behavioral defects are caused by the lack of axonal mitochondria.

To assay neuropeptide secretion, we expressed the pro-neuropeptide NLP-21 tagged with YFP in the cholinergic DA/DB motor neurons (using the *unc-129* promoter). We quantified NLP-21::YFP secretion by analyzing YFP fluorescence in the endolysosomal compartment of coelomocytes, which are scavenger cells that endocytose proteins secreted into the body cavity (Fares and Greenwald 2001). In parallel, we assayed the abundance of NLP-21-containing vesicles in DA/DB axons by quantifying the fluorescence intensity of YFP puncta in dorsal cord axons (Sieburth *et al.* 2007). As previously reported, NLP-21 coelomocyte fluorescence was significantly decreased in *ric-7* mutants while axonal puncta fluorescence was significantly increased (Figure 1, D–F; Hao *et al.* 2012), both indicating decreased NLP-21 secretion in *ric-7* mutants. Both the coelomocyte and axonal NLP-21 fluorescence defects exhibited by *ric-7* mutants were rescued by expressing kin-Tom7 in the DA/DB neurons (Figure 1, D–F). Thus, the *ric-7* behavioral and neuropeptide secretion defects are both caused by the absence of axonal mitochondria.

Neuropeptide secretion requires oxidative phosphorylation but not mitochondrial calcium uptake

Loss of axonal mitochondria is predicted to result in multiple defects. For example, mitochondria regulate cytoplasmic calcium levels by taking up calcium through the mitochondrial calcium uniporter (MCU) (Kirichok *et al.* 2004; Baughman *et al.* 2011; De Stefani *et al.* 2011), which could contribute to

Figure 1 Axonal mitochondria are required for locomotion and neuropeptide release. (A) Schematic of the kin-Tom7 construct in which UNC-116/KIF5 was fused to TagRFP and the outer mitochondrial protein TOMM-7. This illustration was modified from that shown in the original article describing kin-Tom7 (Rawson *et al.* 2014). (B and C) Locomotion rate (B) and aldicarb-induced paralysis (C) of wild-type, *ric-7* mutant, and *ric-7* mutant worms that express kin-Tom7 in neurons. (D and E) Representative images (D) and quantification (E) of NLP-21 coelomocyte fluorescence in wild-type and *ric-7* mutant worms with and without the DA/DB-specific expression of kin-Tom7. (F) Dorsal cord axonal NLP-21 fluorescence in cholinergic motor neurons of wild-type and *ric-7* mutant worms with and without the DA/DB-specific expression of kin-Tom7. Number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated. **** $P < 0.0001$, ANOVA with Tukey's multiple comparisons test. Scale bar, 5 μm . WT, wild type.

the *ric-7* neuropeptide secretion defect. To test this idea, we analyzed two independent *mcu-1* null mutants, finding no significant changes in NLP-21 coelomocyte and axonal puncta fluorescence (Figure 2, A and B). These results suggest that changes in mitochondrial calcium uptake are unlikely to account for the *ric-7* mutant neuropeptide secretion defects.

Next, we asked if neuropeptide secretion requires mitochondrial respiration. Mutations in the *nuo-6*, *mev-1*, and *isp-1* genes (which encode components of complex I, II, and III, respectively) reduce respiration rate (Ishii *et al.* 1998; Yang and Hekimi 2010b; Pfeiffer *et al.* 2011; Yee *et al.* 2014). These oxidative phosphorylation mutants also had decreased

NLP-21 coelomocyte fluorescence and increased axonal fluorescence, indicating decreased neuropeptide secretion (Figure 2, C–F). The *nuo-6* NLP-21 secretion defect was rescued by a transgene restoring NUO-6 expression specifically in the DA/DB neurons (Figure 2, E and F). These results support the idea that mitochondrial respiration acts in a cell-autonomous manner to promote neuropeptide secretion from DA/DB neurons.

Increased mitochondrial reactive oxygen species inhibits neuropeptide secretion

Disrupting oxidative phosphorylation can lead to reduced ATP levels and altered production of reactive oxygen species (ROS) (Senoo-Matsuda *et al.* 2001; Yang and Hekimi 2010a; Yee *et al.* 2014), both of which could affect neuropeptide secretion. Several steps in neurotransmission, such as establishment and maintenance of membrane potential and membrane fusion, are known to require ATP. Accordingly, we found that mutants of glycolytic enzymes also had decreased neuropeptide secretion (Figure S1A).

Conflicting results have been reported for the effects of *mev-1*, *isp-1*, and *nuo-6* mutations on ATP levels (Senoo-Matsuda *et al.* 2001; Yang and Hekimi 2010a,b; Yee *et al.* 2014). In addition, several findings suggest that glycolysis plays a more important role than oxidative phosphorylation in supplying the metabolic requirements for neurotransmission (Rangaraju *et al.* 2014; Jang *et al.* 2016; Ashrafi and Ryan 2017; Ashrafi *et al.* 2017). Prompted by these results, we investigated other potential mechanisms for neuropeptide secretion defects in the oxidative phosphorylation deficient mutants. Since *mev-1*, *isp-1*, and *nuo-6* mutants have all been shown to increase ROS levels (Ishii *et al.* 1998; Senoo-Matsuda *et al.* 2001; Lee *et al.* 2010; Yang and Hekimi 2010a,b; Yee *et al.* 2014), we hypothesized that altered ROS production might contribute to decreased secretion in these mutants.

To test this idea, we measured NLP-21 coelomocyte fluorescence in mutants lacking ROS detoxification enzymes. Superoxide dismutases (SODs) convert superoxide to hydrogen peroxide (H_2O_2), which can then be broken down to water by catalase (Figure 3A). In *C. elegans*, the genes *sod-1* and *sod-5* encode cytoplasmic Cu/ZnSODs (Larsen 1993; Jensen and Culotta 2005), *sod-2* and *sod-3* encode mitochondrial MnSODs (Giglio *et al.* 1994; Suzuki *et al.* 1996; Hunter *et al.* 1997), and *sod-4* encodes an extracellular Cu/ZnSOD (Fujii *et al.* 1998). We analyzed NLP-21 secretion in all five SOD mutants (Figure S1, B and C). NLP-21 secretion was significantly reduced in *sod-2* mutants (which lack a mitochondrial MnSOD), as indicated by decreased NLP-21 coelomocyte fluorescence and increased NLP-21 axonal puncta fluorescence, both of which were rescued by a transgene restoring *sod-2* expression in DA/DB neurons (Figure 3, B and C). Thus, *ric-7* and *sod-2* mutants exhibited very similar defects in NLP-21 secretion, suggesting that SOD-2 and RIC-7 may function together to promote NLP-21 secretion. Consistent with this idea, we found that the NLP-21 coelomocyte

Figure 2 Neuropeptide release requires oxidative phosphorylation but not mitochondrial calcium uptake. (A and B) Mutation of the mitochondrial calcium uniporter *mcu-1* did not alter NLP-21 (A) coelomocyte or (B) axonal fluorescence. (C–F) Oxidative phosphorylation is required for neuropeptide secretion cell autonomously. (C) Mutation of the complex-II component *mev-1* or the complex-III component *isp-1* reduced NLP-21 coelomocyte fluorescence. (D) Mutation of *isp-1* increased NLP-21 axonal fluorescence. Mutation of the complex-I component *nuo-6* (E) decreased NLP-21 coelomocyte and (F) increased NLP-21 axonal fluorescence, both of which were rescued by expression of a wild-type *nuo-6* transgene in cholinergic DA/DB motor neurons only. Number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated. **** $P < 0.0001$, Kruskal–Wallis test with Dunn test for multiple comparisons. n.s., not significant; resc, rescue; WT, wild type.

fluorescence defect observed in *sod-2; ric-7* double mutants was not significantly different from that observed in *ric-7* single mutants (Figure 3D). Unlike *sod-2* mutants, NLP-21 coelomocyte fluorescence was not significantly reduced in *sod-3* mutants, which lack a second mitochondrial MnSOD (Figure S1B), perhaps because *sod-3* is primarily expressed in dauer larvae (Doonan *et al.* 2008; Van Raamsdonk and Hekimi 2012). Collectively, these results suggest that perturbing mitochondrial ROS detoxification in axons inhibits NLP-21 secretion.

To determine if increased cytoplasmic ROS levels regulate NLP-21 secretion, we analyzed *sod-1* and *sod-5* mutants, which lack the cytoplasmic SODs. NLP-21 secretion was significantly increased in *sod-1* mutants whereas decreased se-

Figure 3 Mitochondrial superoxide detoxification is required for neuropeptide secretion. (A) Oxidative phosphorylation generates superoxide as by-products, which is converted to H_2O_2 by superoxide dismutases SOD-1 and SOD-5 in the cytoplasm and SOD-2 and SOD-3 in mitochondria. H_2O_2 is membrane diffusible and is subsequently detoxified by catalases. (B and C) Mutation of *sod-2* decreased NLP-21 coelomocyte fluorescence (B) and increased NLP-21 axonal fluorescence (C), both of which were rescued by expressing a wild-type *sod-2* construct in cholinergic DA/DB motor neurons only. (D) The *sod-2* and *ric-7* mutations did not have additive effects on NLP-21 secretion. NLP-21 coelomocyte fluorescence was compared in wild type, *sod-2* and *ric-7* single mutants, and *sod-2; ric-7* double mutants. Number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Kruskal–Wallis test with Dunn test for multiple comparisons. n.s., not significant; oxphos, oxidative phosphorylation; resc, rescue; WT, wild type.

cretion was observed in *sod-5* mutants (Figure S1, B and C). These results suggest that increased cytoplasmic ROS does indeed regulate NLP-21 secretion but opposite effects are observed depending on which cytoplasmic SOD is impaired. Taken together, these results support the idea that neuropeptide secretion is strongly inhibited by a variety of mitochondrial defects, including mutations disrupting axonal transport of mitochondria, mutations disrupting mitochondrial respiration, and mutations increasing mitochondrial ROS levels. Furthermore, in each case, mitochondria act cell autonomously to promote neuropeptide secretion.

Hypoxia inhibits neuropeptide secretion

The neuropeptide secretion defects observed in mitochondrial mutants could result from decreased levels of ATP or other metabolites. Alternatively, decreased neuropeptide secretion could result from activation of stress response pathways. Several stress response pathways are induced in mitochondrial mutants, including those mediated by the hypoxia inducible factor (HIF-1), SKN-1/Nrf2, the mitochondrial unfolded protein response, and the AMP-activated protein kinase (Sena and Chandel 2012; Hwang *et al.* 2014; Blackwell

et al. 2015; Chang et al. 2017; Dues et al. 2017; Shpilka and Haynes 2018).

We focused on *HIF-1* because *ric-7* mutants display hypoxic phenotypes (Jang et al. 2016) and mutations decreasing mitochondrial respiration stabilize *HIF-1* (Lee et al. 2010; Hwang et al. 2014). Prompted by these results, we hypothesized that *ric-7* mutants might undergo a hypoxic response that inhibits neuropeptide release. We did several experiments to test this idea. First, we asked if growth in reduced oxygen tension inhibits neuropeptide secretion. Consistent with this idea, growing wild-type animals in a hypoxic chamber (5 or 10% oxygen) for 24 hr significantly reduced *NLP-21* coelomocyte fluorescence (Figure 4A). By contrast, growth in hyperoxic conditions (100% oxygen) for the same duration had no effect (Figure S2A). Thus, hypoxia was sufficient to inhibit *NLP-21* secretion.

Next, we asked if activation of *HIF-1* was sufficient to inhibit *NLP-21* secretion. To test this idea, we analyzed *vhl-1* mutants. *VHL-1* is the *C. elegans* ortholog of the von Hippel-Lindau tumor suppressor protein, which promotes *HIF-1* ubiquitination under normoxic conditions. In *vhl-1* mutants, *HIF-1* is stabilized and constitutively activates the hypoxic transcriptional response (Kaelin 2007). As expected, *NLP-21* secretion was significantly decreased in *vhl-1* mutants and this defect was rescued by a transgene that restores *VHL-1* expression specifically in the DA/DB neurons (Figure 4, B and C). The *NLP-21* secretion defect in *vhl-1* mutants was abolished in *hif-1*; *vhl-1* double mutants, confirming that the *vhl-1* secretion defect was a consequence of stabilized *HIF-1* (Figure 4D). Taken together, these results suggest that activation of the *HIF-1* hypoxia response in DA/DB neurons is sufficient to inhibit *NLP-21* secretion.

Does activation of *HIF-1* account for the *ric-7* neuropeptide secretion defect? We did two experiments to test this idea. First, if *ric-7* mutations inhibit neuropeptide secretion by stabilizing *HIF-1*, then *ric-7* and *vhl-1* mutations should not have additive effects on *NLP-21* secretion in double mutants. Consistent with this idea, *NLP-21* coelomocyte fluorescence in *vhl-1*; *ric-7* double mutants was not significantly different from that in either single mutant (Figure 4D). By contrast, *vhl-1* and *unc-31*/CAPS mutations did have additive effects on *NLP-21* secretion in double mutants (Figure S2B). Second, if *ric-7* mutations inhibit neuropeptide secretion by activating the hypoxic response, then *hif-1* mutations should restore secretion in these mutants. As predicted, *ric-7* *hif-1* double mutants had significantly higher *NLP-21* coelomocyte fluorescence than *ric-7* single mutants (Figure 4E). Similarly, the *NLP-21* secretion defect of *sod-2* mutants was fully suppressed in *sod-2*; *hif-1* double mutants (Figure 4F). *HIF-1*'s effect was specific because inactivating two other stress-activated transcription factors (*DAF-16*/FOXO and *SKN-1*/Nrf) did not increase *NLP-21* coelomocyte fluorescence in *ric-7* mutants (Figure S2, C and D). Collectively, these results support the idea that disrupting mitochondrial transport into axons (in *ric-7* mutants) or disrupting mitochondrial function (in *sod-2* mutants) elicits a cell-autonomous hypoxic re-

Figure 4 *ric-7* and *sod-2* mutations inhibit neuropeptide secretion through activation of the hypoxic response. (A) *NLP-21* coelomocyte fluorescence in worms that were either grown at atmospheric oxygen levels, at 5% oxygen, or at 10% oxygen for 24 hr. Worms grown under hypoxic conditions exhibited reduced *NLP-21* coelomocyte fluorescence. (B and C) Mutation of *vhl-1* decreased *NLP-21* coelomocyte fluorescence (B) and increased *NLP-21* axonal fluorescence (C), both of which were rescued by expressing wild-type *vhl-1* in cholinergic DA/DB motor neurons. (D–F) Comparison of *NLP-21* coelomocyte fluorescence for the indicated genotypes. (D) Mutation of *hif-1* suppressed the decreased *NLP-21* coelomocyte fluorescence exhibited by *vhl-1* mutants. *vhl-1* and *ric-7* mutations did not have additive effects on *NLP-21* coelomocyte fluorescence. (E) Mutation of *hif-1* partially restored *NLP-21* coelomocyte fluorescence in *ric-7* mutants. (F) Mutation of *hif-1* fully restored *NLP-21* coelomocyte fluorescence in *sod-2* mutants. Number of animals analyzed is indicated for each genotype. Error bars indicate SEM. Values that differ significantly are indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Kruskal–Wallis test with Dunn test for multiple comparisons. n.s., not significant; resc, rescue; WT, wild type.

sponse that stabilizes *HIF-1* and inhibits neuropeptide secretion.

Discussion

Here we show that axonal transport of mitochondria is critical for neuropeptide release and locomotion in *C. elegans*. Impaired mitochondrial transport, reduced oxidative phosphorylation rates, and increased mitochondrial ROS levels all inhibited neuropeptide secretion cell autonomously; whereas interfering with mitochondrial calcium uptake had no effect. The effects of axonal mitochondria on neuropeptide release

are mediated (at least in part) by changes in HIF-1 activity. Activating HIF-1 inhibited neuropeptide secretion while *hif-1* null mutations restored secretion in mutants with impaired mitochondrial transport or function. These results demonstrate that modifications of mitochondrial transport or function lead to cell-autonomous changes in neuropeptide secretion, thus providing a mechanism whereby the metabolic or redox state of a neuron could alter the global behavioral state of the animal.

Mitochondrial transport mutants as a model to identify mitochondrial function in neurotransmission

Blocking mitochondrial transport into axons can result in a variety of synaptic defects by a number of different mechanisms. For example, mouse syntabulin knockouts are defective for mitochondrial transport in cultured superior cervical ganglion neurons, which is accompanied by accelerated synaptic depression, slowed recovery after SV depletion, and impaired short-term plasticity (Ma *et al.* 2009). In contrast, decreased axonal mitochondria in rat syntaphilin mutant hippocampal neurons slow presynaptic calcium clearance and enhance short-term facilitation (Kang *et al.* 2008). Here we find that the absence of axonal mitochondria induces a hypoxic response that inhibits neuropeptide secretion, thus identifying a new mechanism by which mitochondrial transport defects alter neurotransmission. Although *ric-7* is not conserved beyond nematodes and has no recognizable structural domains, it is conceivable that RIC-7 functions analogously to syntabulin (which is a KIF5B cargo adaptor for mitochondrial transport) (Ma *et al.* 2009). While it is likely that RIC-7 promotes transport of other unidentified cargoes, restoring axonal mitochondria in *ric-7* mutants (by expressing kin-Tom7) rescued the NLP-21 secretion defects. Thus, mitochondrial transport defects alone account for the impaired neuropeptide secretion of *ric-7* mutants.

Neuropeptide secretion does not require the mitochondrial calcium uniporter MCU-1

Mitochondrial calcium uptake regulates synaptic strength in a variety of systems (Werth and Thayer 1994; Tang and Zucker 1997; Billups and Forsythe 2002; Medler and Gleason 2002; David and Barrett 2003; Talbot *et al.* 2003; Kim *et al.* 2005; Chouhan *et al.* 2010; Shutov *et al.* 2013; Kwon *et al.* 2016). However, other studies reported limited or undetectable effects of mitochondrial calcium uptake on neurotransmitter release (Guo *et al.* 2005; Verstreken *et al.* 2005; Ma *et al.* 2009), indicating that mitochondrial effects on local calcium may vary between cell types and systems. Using two independent *mcu-1* null alleles, we show that neuropeptide secretion from *C. elegans* motor neurons is independent of the mitochondrial uniporter. This suggests that other calcium clearance mechanisms (such as the endoplasmic reticulum, Na⁺/Ca²⁺ pumps, or membrane Ca²⁺ ATPases) may have the dominating effect in DA/DB neurons (Rizzuto *et al.* 1998; Emptage *et al.* 2001; Liang *et al.* 2002; Bouchard *et al.* 2003; de Juan-Sanz *et al.* 2017).

Three matrix dehydrogenases are activated by calcium ions, thus coupling mitochondrial calcium levels to bioenergetics and metabolic flux (Denton and McCormack 1980). Accordingly, knockout of MCU attenuates metabolic rate in a variety of cell types (Kamer and Mootha 2015). One might therefore expect that *mcu-1* mutant animals should inhibit neuropeptide secretion indirectly due to changes in mitochondrial metabolism. However, previous studies reported that *mcu* knockouts in *C. elegans* are viable and fertile, while *mcu* knockout mice have reduced exercise capacity but have unaltered basal metabolic rates (Pan *et al.* 2013; Xu and Chisholm 2014). Mitochondrial calcium levels are strongly reduced (25% of wild-type levels) but not eliminated in *mcu* knockouts, implying that alternate mechanisms for calcium entry into mitochondria must exist (Feng *et al.* 2013; Murphy *et al.* 2014). The residual mitochondrial calcium levels may be sufficient to allow *mcu-1* mutant worms to sustain normal levels of neuropeptide secretion.

Increased mitochondrial ROS inhibits neuropeptide secretion

Two findings in this study suggest that increased mitochondrial ROS inhibits neuropeptide secretion cell autonomously. First, neuropeptide secretion is reduced in mutants lacking the major mitochondrial superoxide dismutase, *sod-2*. Inactivating the second mitochondrial SOD (SOD-3) had no effect on secretion, most likely because *sod-3* is predominantly expressed in dauer larvae and not in adult worms (Doonan *et al.* 2008; Van Raamsdonk and Hekimi 2012; Suthamarak *et al.* 2013). Second, mutations in the genes *nuo-6*, *mev-1*, and *isp-1* all lead to elevated mitochondrial ROS levels (Senoo-Matsuda *et al.* 2001; Yang and Hekimi 2010a,b; Yee *et al.* 2014) and decreased neuropeptide secretion. Because of their chemical reactivity, ROS can damage lipids, proteins, and DNA (Cross *et al.* 1987), and oxidative stress has been associated with many neurodegenerative diseases. ROS can also function as a second messenger in signal transduction pathways by oxidizing critical thiols within proteins, thereby regulating numerous biological processes, including metabolic adaptation, differentiation, and proliferation (Schieber and Chandel 2014; Reczek and Chandel 2015).

Intracellular ROS can activate stress response pathways in distant tissues. For example, ROS-induced activation of the mitochondrial unfolded protein response in multiple classes of *C. elegans* neurons elicits a stress response in the intestine (Berendzen *et al.* 2016; Shao *et al.* 2016). In this case, the nonautonomous effect of ROS is mediated by increased neuronal secretion of serotonin and FLP-2. By contrast, we observed decreased NLP-21 secretion in motor neurons when mitochondrial function was impaired. Taken together, these results suggest that mitochondrial stress can either promote (FLP-2) or inhibit (NLP-21) neuropeptide secretion, depending on the activating stimulus and cell type.

One surprising observation was that mutations in *sod-1* and *sod-5* (the two cytoplasmic SODs) had opposite effects on neuropeptide secretion. Different phenotypes have

previously been reported for these mutations: while *sod-1* mutant worms are hypersensitive to drug-induced oxidative stress, *sod-5* mutant worms are unaffected, presumably because *sod-5* is predominantly expressed in the dauer larva (Doonan *et al.* 2008; Van Raamsdonk and Hekimi 2009, 2012). Notably, *sod-1* mutants have increased ROS in both cytoplasm and mitochondria (Yanase *et al.* 2009). Recent findings suggest that the location of ROS production can influence downstream events (Van Raamsdonk 2015). For example, in a ROS-sensitized *clk-1* mutant background of *C. elegans*, further increase of cytoplasmic ROS by mutation of *sod-1* shortens life span, whereas increase of mitochondrial ROS by *sod-2* mutation increases life span (Schaar *et al.* 2015). How different subcellular localizations of ROS produce distinct phenotypes is poorly understood. The opposite effects of *sod-1* and *sod-5* mutations could indicate that accumulation of cytoplasmic ROS in different tissues or at different times during development can either promote or inhibit NLP-21 secretion. Further experiments will be required to determine how SOD-1 and SOD-5 regulate neuropeptide secretion.

Activation of the hypoxic response

Acute neural activity relies on an increase in oxidative phosphorylation for ATP supply, which results in decreased oxygen levels (Hall *et al.* 2012; Rangaraju *et al.* 2014). Therefore, oxygen depletion and HIF-1 activation provide a way to sense and adapt to metabolic stress. At normal oxygen levels, HIF-1 is continually hydroxylated by oxygen-dependent prolyl hydroxylases. Hydroxylated HIF-1 is ubiquitinated by an E3 ligase containing VHL-1, which results in HIF-1 degradation. During hypoxia, the prolyl hydroxylases are inactive and HIF-1 becomes stabilized, initiating transcription of a variety of genes to adapt to the hypoxic condition. Prior studies suggest that mutations altering mitochondrial respiration can also stabilize HIF-1 (Lee *et al.* 2010; Hwang *et al.* 2014). Mitochondria have been proposed to regulate HIF-1 stability through a variety of mechanisms. Mitochondrially produced ROS are proposed to directly activate HIF-1 (Chandel *et al.* 2000; Brunelle *et al.* 2005; Guzy *et al.* 2005; Mansfield *et al.* 2005). Other studies suggest that altered abundance of mitochondrial metabolites stabilize HIF-1 (Lu *et al.* 2005; Hewitson *et al.* 2007; Koivunen *et al.* 2007; Mishur *et al.* 2016). Therefore, HIF-1 could become activated in *ric-7* and *sod-2* mutants due to elevated ROS production or altered abundance of mitochondrial metabolites. NLP-21 secretion was inhibited when HIF-1 was activated by *vhl-1* mutations, by hypoxic growth conditions, or by mutations altering mitochondrial respiration and ROS levels. Thus, our results are compatible with either mechanism.

Activating HIF-1 initiates a comprehensive transcriptional program that, among others, reduces oxidative phosphorylation and promotes glycolysis (Wang *et al.* 1995; Rankin and Giaccia 2016). Activated HIF-1 also promotes axon regeneration following injury (Nix *et al.* 2014; Cho *et al.* 2015; Alam *et al.* 2016), suggesting a neuroprotective role in certain con-

ditions. This study identifies a new role for HIF-1 in inhibiting neuropeptide secretion, indicating that in addition to cell-autonomous metabolic rewiring, activation of HIF-1 might (directly or indirectly) alter intercellular signaling, thus facilitating the communication of cell-specific stresses to different cells and tissues to induce broad physiological changes.

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