Glycolytic Enzymes Localize to Synapses under Energy Stress to Support Synaptic Function

Highlights
- A metabolic compartment forms in vivo near synapses to meet local energy demands
- Under energy stress, glycolytic proteins redistribute to form clusters at synapses
- The glycolytic metabolon is needed for the synaptic vesicle cycle
- Disruption of glycolytic metabolon impairs synaptic recovery and affects locomotion

Authors
SoRi Jang, Jessica C. Nelson, Eric G. Bend, ..., Katherine Underwood, Erik M. Jorgensen, Daniel A. Colón-Ramos

Correspondence
daniel.colon-ramos@yale.edu

In Brief
Changes in synaptic activity cause local changes in energy demands. Jang and Nelson et al. discover glycolytic microcompartments, or “glycolytic metabolons,” that form dynamically near presynaptic sites to meet local energy demands and support synaptic function.
Glycolytic Enzymes Localize to Synapses under Energy Stress to Support Synaptic Function

SoRi Jang,1,6 Jessica C. Nelson,1,6 Eric G. Bend,2 Lucelenie Rodríguez-Laureano,1 Felipe G. Tueros,3 Luis Cartagenova,1 Katherine Underwood,1 Erik M. Jorgensen,2 and Daniel A. Colón-Ramos1,4,*

1Program in Cellular Neuroscience, Neurodegeneration, and Repair, Department of Cell Biology and Department of Neuroscience, Yale University School of Medicine, P.O. Box 9812, New Haven, CT 06536-0812, USA
2Department of Biology, Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT 84112-0840, USA
3Laboratorio de Microbiologia, Facultad de Ciencias Biológicas, Universidad Ricardo Palma, P.O. Box 1801, Lima 33, Perú
4Instituto de Neurobiología, Recinto de Ciencias Médicas, Universidad de Puerto Rico, 201 Boulevard del Valle, San Juan 00901, Puerto Rico
5Co-first author
6Correspondence: daniel.colon-ramos@yale.edu
http://dx.doi.org/10.1016/j.neuron.2016.03.011

SUMMARY

Changes in neuronal activity create local and transient changes in energy demands at synapses. Here we discover a metabolic compartment that forms in vivo near synapses to meet local energy demands and support synaptic function in Caenorhabditis elegans neurons. Under conditions of energy stress, glycolytic enzymes redistribute from a diffuse localization in the cytoplasm to a punctate localization adjacent to synapses. Glycolytic enzymes colocalize, suggesting the ad hoc formation of a glycolysis compartment, or a “glycolytic metabolon,” that can maintain local levels of ATP. Local formation of the glycolytic metabolon is dependent on presynaptic scaffolding proteins, and disruption of the glycolytic metabolon blocks the synaptic vesicle cycle, impairs synaptic recovery, and affects locomotion. Our studies indicate that under energy stress conditions, energy demands in C. elegans synapses are met locally through the assembly of a glycolytic metabolon to sustain synaptic function and behavior.

INTRODUCTION

The brain consumes more energy than any other organ in the body, accounting for ~20% of total body energy consumption (Bélander et al., 2011; Mink et al., 1981). Within the brain, synapses are primary sites of ATP consumption—the synaptic vesicle cycle being one of the main sources of activity-driven metabolic demands (Harris et al., 2012; Rangaraju et al., 2014). Conditions that alter the metabolic state of the brain, such as hypoxia, starvation, and hypoglycemia, have profound effects on synaptic transmission and cognitive function (Cherubini et al., 1989; Gold et al., 1995). Even brief interruptions of activity-stimulated ATP synthesis can result in severe impairment of synaptic function (Rangaraju et al., 2014).

ATP is predominantly produced by either glycolysis or oxidative phosphorylation. But not all ATP is created equal, and these two sources contribute differentially to various metabolic processes (Pfeiffer et al., 2001). Oxidative phosphorylation, which is mediated by the mitochondria, is an efficient process that produces high yields of ATP molecules, but at low rates of production. Glycolysis, on the other hand, can act independently from the mitochondria to produce lower yields of ATP, but at faster rates (Pfeiffer et al., 2001). Tissues that consume ATP at higher rates, including the brain (but also muscles, developmental tissues, and cancer cells), heavily rely on the glycolytic machinery to meet their energy demands (Vander Heiden et al., 2009; Wojtas et al., 1997). While glycolysis is inhibited by oxygen in most cells, in these tissues glycolysis is active under aerobic conditions. The preferential use of glycolysis over oxidative phosphorylation even in aerobic conditions is referred to as the Warburg effect, or aerobic glycolysis (Warburg et al., 1927). Aerobic glycolysis plays an important role in brain metabolism and function (Gjedde and Marrett, 2001; Magistretti and Allaman, 2013) and increases locally upon conditions of increased neuronal activity (Vaishnavi et al., 2010).

Neuronal activity can change the energy demands at synapses (Harris et al., 2012; Rangaraju et al., 2014). Because the diffusion rate of intracellular ATP is limited (Hubley et al., 1996), synapses must rely on local production of ATP to meet these transient changes in energy demands and sustain synaptic function. Mitochondria, which mediate oxidative phosphorylation, are actively transported to neuronal synapses, and defects in localization have been linked to neurodegenerative disorders such as Parkinson’s disease (Burté et al., 2015; Lin and Sheng, 2015; Schwarz, 2013). Unlike the mitochondrion, which is a membrane-bound organelle, glycolytic enzymes are soluble proteins in the cytosol. Glycolytic enzymes, however, are not uniformly distributed throughout the cytosol (Masters, 1991; Me nanard et al., 2014), and in neurons, biochemical studies demonstrated that glycolytic proteins are enriched in synaptic fractions (Knoll, 1978, 1980; Knoll and Fillmore, 1985). The physiological importance of the localization of glycolytic enzymes, and their role in meeting local energy demands at synapses, remains poorly understood.
In this study, we identify, from forward genetic screens in *C. elegans*, a role for the glycolytic machinery in powering the synaptic vesicle cycle. We demonstrate that under conditions of energy stress, glycolytic proteins dynamically colocalize near presynaptic sites into a metabolic compartment. We also demonstrate that presynaptic scaffolding proteins are necessary for the ad hoc localization of glycolytic proteins to presynaptic sites, and that the local assembly of this metabolic compartment is necessary for synaptic function and locomotion under energy stress. Our studies indicate that energy demands in *C. elegans* neurons are met locally through the assembly of a glycolytic metabolon to sustain synaptic function and behavior.

**RESULTS**

**Glycolytic Proteins Are Required to Maintain Synaptic Vesicle Protein Clusters during Energy Stress**

We conducted unbiased forward genetic screens to identify molecules required for the localization of synaptic vesicle proteins in the serotonergic NSM neurons in the nematode *C. elegans* (Figures 1A–1D and 1H; genetic screen described in Figure S1 and Supplemental Experimental Procedures, available online). From this screen we identified allele *ola72*, which displayed diffuse distribution of the synaptic vesicle proteins VMAT/CAT-1 and synaptobrevin under hypoxic conditions (Figures 1E–1G, 1I, and 1N). Positional cloning of the *ola72* allele revealed a missense mutation (C562Y) in *ola72* allele revealed a missense mutation (C562Y) in *pfk-a1* gene in *C. elegans* (Figure S2A)–one of two *C. elegans* genes that encode phosphofructokinase-1. In mutant animals carrying the *ola72* allele, synaptic vesicle proteins cluster normally under normoxic conditions (Figures 1J, 1M, 1O, S1C, and S1L). However, under hypoxic conditions (induced by mounting animals under a glass coverslip [Pitts and Toombs, 2004] or by incubation in a hypoxia chamber), these synaptic vesicle proteins become diffusely distributed throughout the neurite in *ola72* animals (Figures 1J, 1N, 1P, and S1F; Movie S1). This phenotype was not observed in wild-type animals, which maintained punctate localization of synaptic vesicle proteins under the same hypoxic conditions (Figures 1J–1L and S1E). Three independent alleles, *pfk-a1* (gk549413), *pfk-a1* (gk758818), and *pfk-a1* (gk922689), phenocopy and fail to complement the *ola72* allele (Figures 2C, 2I, and S2C). Expression of a wild-type copy of the *pfk-a1* gene in *ola72* mutant animals rescues punctate localization of synaptic vesicle proteins under hypoxic conditions (Figure S2B). These findings indicate that phosphofructokinase-1 is required to maintain the localization of synaptic vesicle proteins at synapses under hypoxic conditions.

The *pfk-a1* phenotype is not due to a disruption of the synapses themselves, since the localization of the presynaptic active-zone protein ELKS is not altered in *pfk-a1* mutants (Figures S3A–S3E). In addition, the requirement for *pfk-a1* is not limited to the NSM neuron, as it is also required for the maintenance of vesicle protein clustering in all neurons examined (Figures 1K–1P and S3A–S3J). Consistent with the pan-neuronal phenotype, a GFP reporter under the *pfk-a1* promoter is expressed in all examined neurons, including the NSM neuron (Figures S2D–S2G). Moreover, NSM neuron-specific expression of the *pfk-a1* cDNA in *pfk-a1* (ola72) mutants led to rescue of the synaptic vesicle phenotype in the NSM neuron (Figures 2B and 2I). Our findings indicate that the phosphofructokinase enzyme is required cell autonomously in neurons to cluster synaptic vesicle proteins under hypoxic conditions.

Phosphofructokinase-1 is a rate-limiting enzyme that catalyzes the first committed step during glycolysis. To determine if the glycolytic pathway is required for the maintenance of synaptic vesicle protein clusters in neurons under hypoxic conditions, we examined mutants of other glycolytic enzymes (Figure S2H). Mutants for phosphofructokinase-2/fructose-2,6-bisphosphatase/*pfkb-a1* (ok2733), glyceraldehyde 3-phosphate dehydrogenase/*gpd-a3* (ok2870), aldolase/*aldo-a1* (tm5782), and phosphoglycerate kinase/*pgk-a1* (tm5613) phenocopy the hypoxia-dependent synaptic vesicle protein phenotype of *pfk-a1* mutant animals (Figures 2C–2I).

Hypoxia inhibits oxidative phosphorylation (Cohen, 1972). To determine if disruption of oxidative phosphorylation phenocopied the effects of hypoxia, we pharmacologically blocked cytochrome oxidase with sodium azide (NaN₃) and the ATP synthase with oligomycin (Bogucka and Wojtczak, 1966; Chappell and Grieve, 1961). We observed that *pfk-a1* mutants exhibit hypersensitivity to sodium azide and oligomycin, similar to their acute response to lowered oxygen, but do not exhibit hypersensitivity to glycolysis inhibitor 2-deoxy-D-glucose (2-DG) (Woodward and Hudson, 1954) (Figures 1J and S3K). Together, our findings indicate that in vivo, under conditions of energy stress in which the activity of the oxidative phosphorylation pathway is decreased (either pharmacologically or by hypoxia), the glycolytic pathway is required in *C. elegans* neurons for the maintenance of synaptic vesicle protein clusters.

**Endocytosis Is Disrupted in pfk-a1 Mutants during Hypoxia**

Synaptic vesicle endocytosis is vulnerable to ATP levels, and pharmacological or genetic inhibition of glycolysis dramatically and specifically reduces endocytosis at presynaptic sites (Rangaraju et al., 2014; Wang et al., 2004). To examine if *pfk-a1* mutants disrupt synaptic vesicle endocytosis, we first visualized vesicle protein clusters in endocytic mutant unc-57/ endophilin and *pfk-a1*; unc-57/endophilin double mutants. Mutants lacking *unc-57*/endophilin display a diffuse distribution of synaptic vesicle proteins throughout the neurite even under normoxic conditions (Schuske et al., 2003) (Figures 3A–3F). This phenotype resembles that seen for glycolytic mutants under conditions of energetic stress (Figures 2C–2I). Consistent with endophilin/*unc-57* and *pfk-a1* acting in the same genetic pathway, we observed that mutations in endophilin did not enhance the hypoxia-induced *pfk-a1* phenotype (Figure S3J). These observations suggest that the synaptic vesicle defect observed in *pfk-a1* mutant animals could result from inhibition of endocytosis due to decreased rates of glycolysis.

If *pfk-a1* mutants disrupt synaptic vesicle endocytosis during energy stress, vesicle proteins would remain trapped on the plasma membrane, as has been observed for other endocytosis mutants (Bai et al., 2010; Kraszewski et al., 1996). To examine this hypothesis, we analyzed the mobility of vesicle protein synaptobrevin using fluorescence recovery after photobleaching (FRAP). Proteins associated with the plasma membrane exhibit...
greater mobility than proteins associated with synaptic vesicles (Bai et al., 2010; Kraszewski et al., 1996). We observed that synaptobrevin exhibited greater mobility in endocytic mutants than in wild-type animals, as expected (Figures 3K and 3L). Consistent with the hypothesis that pfk-1.1 mutants affect endocytosis during energy stress, we observed that synaptobrevin also exhibited greater mobility in pfk-1.1 mutants under hypoxic conditions, and that the mobility phenocopied that seen for endocytosis mutants (Figures 3K and 3L). If pfk-1.1 mutants affect endocytosis during energy stress, we would expect that blocking
exocytosis would suppress the \textit{pfk-1.1} mutant phenotype. Indeed, we observed that mutants for the synaptic vesicle docking protein UNC-13 partially suppressed the hypoxia-induced \textit{pfk-1.1} phenotype (Figures 3G–3J). The observed partial suppression may be due to the hypomorphic nature of the \textit{unc-13} allele, or to potential contributions to the phenotype from other, \textit{unc-13}-independent pathways. The phenotype in \textit{pfk-1.1; unc-13} double mutants, however, indicates that a defect in the synaptic vesicle cycle significantly contributes to the \textit{pfk-1.1} mutant phenotype. Our results are consistent with previous studies (Rangaraju et al., 2014; Wang et al., 2004) and suggest that under conditions of energy depletion, the glycolytic pathway becomes critical in maintaining the energy supplies necessary for sustaining endocytosis and the synaptic vesicle cycle.

\textbf{Glycolytic Enzymes Localize to Presynaptic Sites during Energy Stress}

Where is PFK-1.1 localized to meet local energy demands at synapses? We examined the subcellular localization of PFK-1.1 in neurons. We observed that under normoxic conditions, PFK-1.1 is localized in a punctate pattern at some of the cell somas (Figures S4A and S4A') and largely diffuse throughout the neurites (Figures 4A and 4C). However, upon exposure to hypoxia, PFK-1.1 became clustered in neurites (Figures 4A–4E; Movie S3), preferentially localizing to synaptic-rich regions (Figure 4F) and within 0.2 \textmu m from vesicle release sites (Figures 4G and 4H).

Glycolytic proteins have been hypothesized to interact and form functional supercomplexes, termed glycolytic metabolons (or glycolons), which sustain the accelerated rates of glycolysis (Clarke and Masters, 1975; Kurganov et al., 1985; Ureta, 1985). The concept of the glycolytic metabolon derives from evidence generated primarily from work in fixed tissues or biochemical assays, and less is known about the existence in vivo of this complex or its physiological importance (Bronstein and Knul, 1981; Campanella et al., 2008; Knul et al., 1980; Kurganov et al., 1985; Masters, 1984; Sullivan et al., 2003; Zhou et al., 2005).

To examine if other glycolytic enzymes colocalize with PFK-1.1 in vivo, we visualized the subcellular localization of ALDO-1 and GPD-3. ALDO-1 and GPD-3, like PFK-1.1, were diffusely distributed throughout the cytosol under normoxic conditions (data not shown). Upon exposure to hypoxia, both enzymes clustered near synapses (Figures 4J and 4N). The GPD-3 and ALDO-1 clusters colocalized with the PFK-1.1 clusters (Figures 4I–4P), suggesting that these enzymes dynamically form a complex near presynaptic sites in response to demands for ATP.

Glycolysis normally occurs in the brain and increases locally upon conditions of increased neuronal activity (Vaishnavi et al., 2010). To test if the glycolytic metabolon is necessary at synapses in response to high levels of neuronal activity, we stimulated neurons using a pharmacological approach. \textit{C. elegans} GABA neurons can be stimulated through the bath application of levamisole, an agonist of acetylcholine receptors (such as \textit{lev-8}) present in the GABA neurons (Towers et al., 2005). By applying levamisole under normoxic conditions, we examined whether \textit{pfk-1.1} was required to support vesicle cycling during periods of strong neuronal stimulation. We observed that \textit{pfk-1.1} mutant animals were sensitized to levamisole treatment compared to wild-type animals (Figures 5A–5C). This sensitization is specific to the GABA neurons that express levamisole-sensitive acetylcholine receptors (data not shown). These
findings suggest that even in aerobic conditions, glycolytic proteins are required to maintain the synaptic vesicle cycle during periods of high neuronal activity, which are known to result in increased energy demands at synapses (Rangaraju et al., 2014). Together, our findings suggest that glycolysis is critical for the maintenance of the synaptic vesicle cycle under conditions that either reduce energy supplies (such as hypoxia) or increase energy demands (such as neuronal stimulation).

Does PFK-1.1 cluster at synapses upon increased neuronal stimulation? Optogenetic stimulation of GABA neurons expressing channelrhodopsin caused clustering of PFK-1.1 in normoxic conditions (Figures S5D–S5F). Therefore, PFK-1.1 can dynamically localize near presynaptic sites upon neuronal stimulation. To test whether this relocalization is dependent on metabolic needs at the synapse, we examined, in hypoxic conditions, PFK-1.1 localization in unc-13 mutants, in which exocytosis and synaptic activity are greatly reduced (Richmond et al., 1999). We observed that the subcellular localization of PFK-1.1 to presynaptic sites is reduced in the neurites (but not the somas) of unc-13(e51) mutants (Figures 5G, 5H, and S4A). In normoxic conditions, optogenetic stimulation of GABA neurons did not promote PFK-1.1 clustering in unc-13 mutants, indicating that synaptic PFK-1.1 clustering does not simply depend on depolarization (Figure S4B). These results suggest that PFK-1.1 clustering to presynaptic sites relies on energy demands at synaptic sites.

**Presynaptic Localization of PFK-1.1 Depends on Synaptic Scaffolding Proteins**

How do glycolytic proteins localize to presynaptic sites? Glycolytic proteins copurify with synaptic vesicles and are required for their transport (Burré and Volkman, 2007; Ikemoto et al., 2003; Ishida et al., 2009; Zala et al., 2013). Glycolytic proteins are also known to associate with the mitochondria, which are actively transported to synaptic sites (Giege et al., 2003). Therefore, we...
next examined if the localization of glycolytic proteins to presynaptic sites depended on synaptic vesicle transport or on mitochondrial transport to synapses. We observed that in unc-104(/kinesin 3/kif1A) mutant animals, synaptic vesicles fail to cluster at presynaptic sites, as previously reported (Hall and Hedgecock, 1991; Nelson and Colón-Ramos, 2013). However, PFK-1.1 clustering under hypoxic conditions was not affected in unc-104(e1265) mutant animals (Figures 6A–6D and 6O). Our data indicate that the clustering of PFK-1.1 is not dependent on vesicle transport to synapses.

To determine if the localization of PFK-1.1 to presynaptic sites depends on the mitochondria, we simultaneously visualized the subcellular localization of PFK-1.1 and mitochondrial outer-membrane protein TOM-20 in mutant backgrounds that affect
mitochondria localization in neurites. We observed that in the mutant backgrounds of kinesin klp-6(sy511), ric-7(nu447), and mitochondrial fission drp-1(tm408), there was a reduction (or altered distribution) of mitochondria in the neurites, as previously reported (Labrousse et al., 1999; Rawson et al., 2014; Tanaka et al., 2011; data not shown). While the mitochondria localization was affected in these animals, we did not detect a reduction in the number of animals displaying PFK-1.1 clusters in the neurites (Figures 6E–6J and 6O). Instead, we observed that klp-6(sy511), ric-7(nu447), and drp-1(tm408) mutants displayed PFK-1.1 clustering in neurites even before animals were exposed to hypoxic conditions (Figure 6O). Our findings indicate that glycolytic protein localization to presynaptic sites is not dependent on the mitochondria or unc-104/kinesin 3/kif1A synaptic vesicle transport. Our findings also uncover a relationship between PFK-1.1 cluster formation and mitochondrial dysfunction.

SYD-2 is a synaptic scaffolding protein required for synaptic release site formation and maintenance (Zhen and Jin, 1999). To examine if the integrity of the presynaptic sites was necessary for the localization of glycolytic clusters in the neurite, we visualized PFK-1.1 in syd-2(ok217) null allele (Wagner et al., 2009) and the syd-2(ju37) loss-of-function allele (Zhen and Jin, 1999), PFK-1.1 clustering at the neurites was significantly suppressed (Figures 6K, 6L, 6O, and 6P). Cell-specific expression of the wild-type SYD-2 in GABA neurons rescued the PFK-1.1 clustering in GABA neurons of syd-2(ju37) mutants (Figures 6M–6O), demonstrating the cell-autonomous role of this synaptic scaffolding protein in PFK-1, and underscore a functional link
Figure 6. Presynaptic Localization of PFK-1.1 Depends on Synaptic Scaffolding Proteins

(A–N) Localization of PFK-1.1 in GABA ventral neurites (pseudocolored magenta) and its respective pixel fluorescence values along the neurite after 10 min of hypoxia in wild-type (A and B); synaptic vesicle kinesin mutant unc-104(e1265) (C and D); mitochondria localization and distribution mutants klp-6(sy511) (E and F), ric-7(nu447) (G and H), and dpr-1(tm408) (I and J); synaptic scaffolding mutant syd-2(ju37) (K and L); and syd-2(ju37) mutant animals expressing a wild-type rescuing array of syd-2 cell specifically in GABA neurons (M and N).

(O) Percentage of animals displaying PFK-1.1 clusters in GABA neurites in varying genotypes after 0 or 10 min of exposure to hypoxic conditions, as indicated. For syd-2(ju37), PFK-1.1 clustering was examined in the absence (−) or in the presence (+) of a wild-type rescuing array of syd-2 cell specifically in GABA neurons. Number of animals tested is indicated at the bottom of each column.

(P) Quantification of the synaptic enrichment of PFK-1.1 (ΔF/F) in the NSM neurons for wild-type (black circles) and syd-2(ok217) (white circles) under hypoxic conditions. The circles in the graph represent individual animals.

Scale bar, 5 μm. Error bars: SEM. *p < 0.05, **p < 0.01, ***p < 0.001 between indicated groups.
between the presynaptic release sites and the clustering of glycolytic proteins.

Localization of PFK-1.1 to Synaptic Sites Is Important for Maintaining the Synaptic Vesicle Cycle during Energy Stress

To test the importance of the subcellular localization of PFK-1.1 in sustaining energy levels at synapses, we identified a dominant-negative version of PFK-1.1 (PFK-1.1G532E). PFK-1.1G532E contains a lesion in the regulatory domain of PFK-1.1, fails to form clusters (Figures S4C–S4E), and fails to rescue the pfk-1.1 mutant phenotype (data not shown). We observed that expression of PFK-1.1G532E in wild-type animals altered wild-type PFK-1.1 localization to presynaptic sites, suggesting that PFK-1.1G532E acts as a dominant negative, affecting the subcellular localization of wild-type PFK-1.1 (Figures 7A–7D). In addition, these wild-type animals expressing PFK-1.1G532E display a dominant-negative synaptic vesicle protein clustering phenotype similar to that observed for pfk-1.1 loss-of-function mutant animals (Figures 7E–7I). Because expression of PFK-1.1G532E affects wild-type PFK-1.1 localization, we hypothesize that the resulting phenotype occurs because of a disruption in the localization of the wild-type PFK-1.1 to presynaptic sites and, therefore, its capacity to sustain energy levels at the synapse. Consistent with these findings, we also observed that when we alter the localization of a wild-type version of PFK-1.1 with a nuclear localization sequence, the wild-type PFK-1.1 is incapable of rescuing the synaptic vesicle phenotype of pfk-1.1 mutants (Figures S4F–S4H). Our findings are consistent with studies in Drosophila flight muscles, which demonstrated that even when the full complement of glycolytic enzymes is present in the muscle, disruption of glycolytic protein colocalization to sarcomeres results in inability to fly (Wojtas et al., 1997). Together, our findings suggest that the presence of functional glycolytic enzymes in the neurons is not sufficient to power synaptic function. Instead, subcellular localization of these glycolytic enzymes near synapses is necessary to power synaptic function.

PFK-1.1 Is Required to Sustain Synaptic Activity and Locomotion during Energy Stress

Is PFK-1.1 required to sustain synaptic activity? We examined the requirement of pfk-1.1 in synaptic recovery following fatigue. Animals expressing the channelrhodopsin variant ChIEF were dissected to expose neuromuscular synapses of the body muscles (Richmond et al., 1998; Richmond and Jorgensen, 1999). Postsynaptic muscles were voltage clamped, and high-frequency
pulses of blue light were delivered to stimulate synapses for 30 s at 10 Hz. This stimulation caused rapid fatigue of acetylcholine neuromuscular junctions (Liu et al., 2009), presumably by the depletion of vesicle pools. We applied test pulses at increasing time increments after fatigue and assayed the recovery of evoked currents (Figures 8A and 8B). We observed that the absolute value of the first evoked response in the pfk-1.1 mutant is similar to the wild-type, demonstrating that the synapses of both wild-type and pfk-1.1 mutants are healthy and normal in response to an evoked stimulus (Figure 8C). Moreover, under normoxic conditions, both wild-type and pfk-1.1 mutant synapses reached full recovery (plateau amplitude ± SEM was as follows: WT = 1.1 ± 0.037; pfk-1.1 = 1.0 ± 0.046) with time constants of 8.9 and 8.6 s, respectively (Figure 8D). However, with two and three repetitions of the 30 s stimulation protocol, pfk-1.1 mutants exhibited reduced recovery (72% [plateau amplitude ± SEM was as follows: WT = 0.88 ± 0.071; pfk-1.1 = 0.63 ± 0.086] and 40% [WT = 0.55 ± 0.071; pfk-1.1 = 0.22 ± 0.043] for wild-type levels, respectively) (Figures 8E and 8F). Next, we included oligomycin to block oxidative phosphorylation. The rate of recovery at wild-type synapses was slightly decreased, but not significantly different from control conditions (time constant was as follows: oligo = 11.9 s versus control = 8.9 s). Furthermore, the drug did not affect the maximum recovery (plateau amplitude was as follows: WT = 0.88 ± 0.071; pfk-1.1 = 0.63 ± 0.086) and 40% (WT = 0.55 ± 0.071; pfk-1.1 = 0.22 ± 0.043) for wild-type levels, respectively (Figures 8E and 8F). Therefore oxidative phosphorylation or glycolysis alone support ATP demands under moderate stimulation. However, glycolysis is required for synaptic function under persistent stimulus conditions, or upon inhibition of oxidative phosphorylation.

To determine if glycolysis is required for normal behavior, we tested whether pfk-1.1 mutant animals exhibited fatigue during swimming. Worms thrash rapidly in liquid, so we scored the number of body bends in solution per minute (Figure 5SA). Consistent with behavioral impairments in pfk-1.1 mutants, we observed a significant reduction in the number of body bends in pfk-1.1 mutants compared to wild-type animals when oxidative phosphorylation was inhibited (Figures 5SB and 5SC). Together, these data suggest that disruption of the glycolytic metabolon impairs synaptic recovery and affects locomotion.

**DISCUSSION**

While many cellular processes rely on ATP, processes are differentially vulnerable to decreases in ATP production, depending on their $K_m$ values (Rangaraju et al., 2014). A recent study demonstrated that synaptic vesicle endocytosis is a particularly ATP-sensitive process and more vulnerable to metabolic perturbations than exocytosis (Rangaraju et al., 2014). Genetic studies in *Drosophila* also identified a role for the glycolytic enzyme phosphoglycerate kinase in regulating synaptic transmission and demonstrated that the physiological phenotypes of phosphoglycerate kinase mutants were related to problems in synaptic vesicle endocytosis due to reduced ATP levels (Wang et al., 2004). Our findings in *C. elegans* neurons now demonstrate that in vivo and under conditions of energy stress, the glycolytic machinery is required to sustain synaptic function. Disruption of glycolysis under energy stress affects the synaptic vesicle cycle, synaptic physiology, and animal behavior by inhibiting the energy-vulnerable process of synaptic vesicle endocytosis.

In most organisms, glycolytic proteins are soluble in the cytosol. An exception to this is the protozoan *Trypanosoma brucei*, which organizes its glycolytic enzymes in a membrane-bound organelle called the glycosome (Michels et al., 2006). This compartmentalization is essential for the regulation of the trypanosomatids’ metabolism and viability (Haanstra et al., 2015). Although membrane-bound glycosomes have not been observed in mammalian cells, it has been predicted for over 30 years that glycolytic proteins compartmentalize in the cytosol into a “glycolytic metabolon” to sustain the observed rates of glycolysis (Clarke and Masters, 1975; Kurganov et al., 1985; Ureta, 1985). In vivo evidence for the existence of this complex, and its physiological importance, has been lacking (Brooks and Storey, 1991; Menard et al., 2014).

We observe in *C. elegans* that glycolytic proteins dynamically cluster near presynaptic sites under conditions of energy stress, suggesting the ad hoc formation of a glycolytic metabolon. Disruption of presynaptic scaffolding proteins, or of the synaptic vesicle exocytosis protein, unc-13, suppresses the clustering of glycolytic proteins near presynaptic sites (but not their clustering at cell somas). Our findings are consistent with biochemical studies that demonstrated an enrichment of glycolytic enzymes and activity in synaptosomal fractions and lysed nerve endings (Knuld, 1978; Knuld and Fillmore, 1985; Wu et al., 1997), and extend findings demonstrating the presence of glycolytic proteins at postsynaptic sites and their role in synaptic transmission (Lasch et al., 2004). Interestingly, localization of glycolytic proteins to presynaptic sites does not depend on the synaptic vesicle transport protein UNC-104/Kinesin 3/ki1A. These findings indicate that clustering of glycolytic proteins to presynaptic sites is not dependent on active synaptic vesicle transport. Our findings also indicate that clustering of glycolytic proteins to presynaptic sites might also be powering other synaptic events besides the recycling of unc-104-dependent synaptic vesicles. Together, our study demonstrates a link between the dynamic localization of glycolytic proteins in vivo and their functional requirement at presynaptic sites.

The formation of the glycolytic metabolon meets local energy demands at presynaptic sites. Disrupting the formation of the glycolytic metabolon at synapses—by forcing the localization of PFK-1.1 to the nucleus or using a dominant-negative version of PFK-1.1—disrupts the capacity of the glycolytic pathway to sustain the synaptic vesicle cycle, synaptic physiology, and locomotory activity of animals under energy stress. Our observations in *C. elegans* neurons are consistent with studies in *Drosophila* muscles that demonstrated that colocalization of glycolytic proteins to sarcomeres was necessary for muscular function (Wojtas et al., 1997). Together, our findings underscore the in vivo importance of the local, ad hoc formation of glycolytic complexes near presynaptic sites in meeting energy demands and sustaining synaptic function and behavior.

Glycolysis is necessary to sustain the synaptic vesicle cycle when oxidative phosphorylation is inhibited. Mitochondria, which mediate oxidative phosphorylation, are actively transported to
Figure 8. PFK-1.1 Is Required for Synaptic Recovery Following Fatigue

(A) Representative electrophysiology experiment illustrating synaptic fatigue and recovery at C. elegans neuromuscular junctions as described in Supplemental Experimental Procedures. The blue line indicates blue-light illumination, and the black trace below is an example trace plotting postsynaptic current.

(B) Normalized plot of evoked amplitudes. Evoked currents (red Xs) from the representative trace in (A) are displayed with the curves fit to the cumulative fatigue (red) and recovery (dark blue) of wild-type synapses.

(C) Mean peak current for the first stimulus in the train for wild-type (black bar) and pfk-1.1(ola72) mutants (gray bar). Number of animals scored is indicated at the bottom of each column.

(D) Synaptic recovery of pfk-1.1(ola72) mutants is equivalent to wild-type recovery following one stimulus train. (N = 19 animals for the wild-type; N = 14 animals for pfk-1.1).

(E and F) Synaptic recovery is reduced in pfk-1.1(ola72) mutants following two (N = 12 animals for wild-type; N = 9 animals for pfk-1.1) (E) and three (N = 10 animals for wild-type; N = 8 animals for pfk-1.1) (F) 30 s stimulus trains.

(G) Synaptic recovery is significantly reduced in pfk-1.1(ola72) mutants in the presence of oligomycin following one stimulus train (p < 0.05; Mann-Whitney U test; for t = 10, 20, and 40 s; n = 3–7 animals per time point for the wild-type; n = 4–7 animals per time point for pfk-1.1; N = 12 animals for wild-type; N = 16 animals for pfk-1.1).

Error bars: SEM
neuronal synapses to meet energy demands. The physiological importance of mitochondria localization is perhaps best exemplified by the fact that defects in mitochondrial localization have been linked to neurodegenerative disorders (Burte et al., 2015; Lin and Sheng, 2015; Schwarz, 2013). In this study, we observe that inhibition of mitochondrial function, or of mitochondrial transport, enhances local clustering of glycolytic proteins to presynaptic sites. We hypothesize that this enhanced clustering of glycolytic proteins represents a response to energy demands at synapses in conditions of mitochondrial disruption. Our findings demonstrate that glycolysis can act redundantly with oxidative phosphorylation to sustain the synaptic vesicle cycle, particularly under conditions in which mitochondrial function at the synapse is compromised.

While many of our experiments were conducted under hypoxic conditions, we observed that pharmacological stimulation of neurons under normoxic conditions also requires glycolysis to sustain the synaptic vesicle cycle. We also observed that optogenetic stimulation of neurons under normoxic conditions results in clustering of glycolytic enzymes. This clustering is likely due to synaptic function and not mere depolarization, as it is suppressed in mutants with reduced exocytosis (unc-13 mutants). We therefore hypothesize that in physiological conditions, aerobic glycolysis would play an important role in sustaining synaptic function.

Synapses do not consume energy at a consistent rate, but rather have extended periods of low activity punctuated by periods of intense activity. How are changing energy demands at presynaptic sites dynamically met? Mitochondrial localization is an important mechanism for meeting local energy demands at the synapse, yet many presynaptic terminals, while rich in ATP, lack mitochondria (Chavan et al., 2015; Waters and Smith, 2003; Xu-Friedman et al., 2001). The capacity of the glycolytic machinery to produce ATP molecules at a faster rate than oxidative phosphorylation, and to dynamically assemble into metabolic compartments based on energy needs, might fill demands for changing levels of energy consumption at intensely active synapses, at synapses that lack mitochondria, or at synapses in which mitochondria has been damaged. Therefore, the observed dynamic localization of glycolytic proteins to synapses may be essential to sustain changes in the activity of synapses in physiology and disease.

**EXPERIMENTAL PROCEDURES**

Complete and detailed experimental procedures are available in the Supplemental Information.

**Inducing Hypoxia with Glass Coverslips and Slides**
Glass coverslips have been used to induce hypoxia in cell cultures (Pitts and Toombs, 2004). The reduced environment generated by mounting 10–15 live worms on glass slides was examined by using redox indicator resazurin (25 μg/mL) dissolved in water. Resazurin, when reduced to resorufin, gives off fluorescence under yellow-green light (O'Brien et al., 2000). Using resazurin, we confirmed that worms between a glass coverslip and slide experience reduced, or hypoxic, conditions; Leica DM500B compound fluorescence microscope was used to acquire the images (Figures S1M and S1N). Gas-permeable slides were made with Sylgard-184 (polydimethylsiloxane, or PDMS) (Dow Corning) according to manufacturer instructions.

**Inhibiting Oxidative Phosphorylation or Glycolysis Using a Hypoxia Chamber or Pharmacological Treatments**
Worms were mounted on a slide and exposed to nitrogen gas, carbon dioxide, 10 mM sodium azide (NaN3), 10 μM 2-DG, or 1 μM oligomycin for 10 min. Phenotype was scored immediately after the treatment.

**Neuronal Stimulation with Pharmacological Treatments or Optogenetics**
To pharmacologically stimulate GABA neurons, worms were mounted on a slide in 1 mM levamisole (Sigma) and imaged immediately in spinning-disc confocal microscope (PerkinElmer Life and Analytical Sciences). As a control, 50 mM muscimol (Abcam) was used. To optogenetically stimulate GABA neurons, a strain expressing channelrhodopsin 2 in GABA neurons (oxls352) (Liu et al., 2009) was stimulated with blue light (~0.6 mW/mm²) for 5 min.

**Quantification of Phenotypic Expressivity**
To quantify synaptically enriched of the indicated proteins (synaptic vesicle proteins or PFK-1.1), fluorescence values for individual neurons (ventral neurite for the NSM neuron, zone 3 for the AIY neuron, and dorsal and ventral neurite for GABA neurons) were obtained through segmented line scans using ImageJ. A sliding window of 2 μm was used to identify all the local fluorescence peak values and trough values for an individual neuron (the maximum and the minimum fluorescence values in a 2 μm interval, respectively). Synaptic enrichment was then calculated as ΔF/F as previously described (Bai et al., 2010; Dittman and Kaplan, 2006).

**Electrophysiology**
Worms expressing the channelrhodopsin I/II chimera ChIEF in acetylcholine neurons (oxs91[Punc-1::ChIEF]) and lacking GABA inputs to the NSM neuron, zone 3 for the AIY neuron, and dorsal and ventral neurite for GABA neurons) were dissected and patch-clamp physiology was conducted as previously described (Richmond et al., 1999; Richmond and Jorgensen, 1999). For oligomycin experiments, the dissected preps were exposed to 1 μM oligomycin for 5 min prior to patch-clamp recordings.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.03.011.

**AUTHOR CONTRIBUTIONS**

**ACKNOWLEDGMENTS**
We thank J. Bai, M. Zhen, J. Dittman, A. Esquibies, M. Hammarlund, M. Koelle, P. Lusk, R. Rawson, S. Margolis, S. Han, and members of the D.A.C.-R. lab for strains, reagents, and advice on the project. We thank J. Kim and A. Gitter for sharing unpublished data and advice. We thank N. Cook, J. Belina, and M. Omar for technical assistance. Some strains were provided by the CGC, which is funded by NIH (P40 OD010449) and by the Mitsuishi lab (Tokyo Women’s Medical University School of Medicine). Yui Kohara (National Institute of Genetics) provided the pks-r.1.1 cDNA clone. This work was funded by the following grants: to D.A.C.-R., R01 NS076558, a fellowship from the Klingenstein Foundation and the Alfred P. Sloan Foundation, and a March of Dimes Research Grant, and to E.M.J., NIH R01 NS034307 and NSF 0920069. S.J. was supported by the Cellular, Biochemical, and Molecular Sciences Predoctoral Training Program (T32 GM07223). E.M.J. is an Investigator of the Howard Hughes Medical Institute. J.C.N. was supported by a training grant 5 T32 NS 41228. L.R.-L. was supported by a diversity supplement to R01 NS076558.
F.G.T. was supported by a fellowship from Universidad Ricardo Palma, Perú and the Research Experience for Peruvian Undergraduates (REPU) program.

Received: July 14, 2015
Revised: January 12, 2016
Accepted: March 8, 2016
Published: April 7, 2016

REFERENCES


Supplemental Information

Glycolytic Enzymes Localize to Synapses under Energy Stress to Support Synaptic Function

SoRi Jang, Jessica C. Nelson, Eric G. Bend, Lucelenie Rodríguez-Laureano, Felipe G. Tueros, Luis Cartagenova, Katherine Underwood, Erik M. Jorgensen, and Daniel A. Colón-Ramos
Figure S1, related to Figure 1. *pfk-1.1(ola72)* fails to maintain synaptic vesicle protein clusters under hypoxic conditions

(A-L) Synaptic vesicle marker VMAT/CAT-1 in NSM of wild-type (B, E, H, and K) or *pfk-1.1(ola72)* mutants (C, F, I, and L) mounted as depicted in the cartoon diagrams (A, D, G, and J). Namely, on glass slides and immediately imaged (t = 0min), which is reflective of normoxic
state (A-C); after ten minutes (t = 10min) (D-F); immediately imaged after the glass coverslip was removed for ten seconds (and animals were briefly exposed to normoxic conditions) and replaced over the samples (G-I); imaged after the animals had been mounted for ten minutes using a polydimethylsiloxane (PDMS) slide instead of a glass slide (J-L). PDMS is a gas-permeable silicon-based material (Aoki, 1999). Note that the synaptic vesicle proteins become diffusely localized in pfk-1.1(ola72) after ten minutes under the coverslip and then fully recover after seconds of normoxic conditions. Also, pfk-1.1(ola72) animals do not express the phenotype when mounted on oxygen-permeable PDMS slides. Insets correspond to zoomed-in (3x) images of the indicated regions.

(M-N) Images of worms taken after 10 minutes of incubation with the redox indicator (resazurin) in the absence (M); or presence (N) of a glass coverslip. Resazurin, when reduced to resorufin, fluoresces under yellow-green light (O'Brien et al., 2000). In presence of a coverslip, there is an accumulation of fluorescence (N), indicating that the animals are experiencing a reduced (hypoxic) environment (note that exposure was increased in M to show the worms, otherwise the image would have been entirely dark, as there was no detectable fluorescence signal in this panel). These observations are consistent with former studies that demonstrated that mounting living cells between a glass slide and a glass cover slip results in hypoxic conditions (Pitts and Toombs, 2004).

As described in the Supplemental Experimental Procedures, the original screen that resulted in the identification of ola72 was designed to identify mutants in genes required for synaptogenesis in the serotonergic neurons, NSM. We screened through 2000 haploid genomes and identified 13 alleles with defects in synaptic patterning. These mutants fall into four categories: (1) Synaptic vesicle signal is diffusely distributed throughout neurites (6 mutants); (2) Synaptic vesicle signal
is very dim or completely absent (4 mutants); (3) Synaptic varicosities appear larger than average (2 mutant); (4) Synaptic vesicle signal appears wild-type upon first examination; after extended exposure to hypoxic conditions under a coverslip (Pitts and Toombs, 2004) the synaptic vesicles became diffusely distributed. This fourth category is the mutant class pfk-1.1(ola72) belongs to.
Figure S2, related to Figure 1 and Figure 2. Genetic lesions in *pfk-1.1* mutants and endogenous expression pattern
(A-B) Schematic of the genomic region of pfk-1.1 and locations of the genetic lesions of the pfk-1.1 alleles examined in this study (marked with arrows) (A); and percentage of animals displaying a diffuse distribution of synaptic vesicle proteins in rescuing backgrounds (B). Single Nucleotide Polymorphism (SNP) mapping was performed and the ola72 lesion was determined to be located between 2.77 Mb and 3.16 Mb on chromosome X (A). Fosmid WRM0616aB04, which contains the pfk-1.1 genomic region (as well as un-named gene, Y41G9A.5), was shown to rescue the diffuse distribution of synaptic vesicle protein phenotype in pfk-1.1(ola72) mutant animals exposed to 10 minutes of hypoxic conditions (induced by mounting on glass slide with coverslip (Pitts and Toombs, 2004)) (B). The pfk-1.1 promoter driving pfk-1.1 cDNA also rescues the synaptic vesicle clustering phenotype in pfk-1.1(ola72) mutant animals exposed to 10 minutes of hypoxic conditions (B). Number of animals scored is indicated at the bottom of each column. pfk-1.1(ola72) is the same as shown in Figure 1J for (B).

(C) Percentage of animals displaying a diffuse distribution of synaptic vesicle proteins after 10 minutes of hypoxic treatment (induced by mounting on glass slide with coverslip (Pitts and Toombs, 2004)). Note that all three independent pfk-1.1 alleles phenocopy pfk-1.1(ola72), and that pfk-1.1(gk549413) fails to complement pfk-1.1(ola72). Number of animals scored is indicated at the bottom of each column. Wild-type control and pfk-1.1 (ola72) is the same as shown in Figure 1J.

(D-G) Expression of the pfk-1.1 promoter is observed in neurons of the ventral and dorsal nerve cords (arrows) (D); and in head neurons, including the NSM neurons (E-G).

(H) Schematic of the glycolysis pathway and mutants examined in this study. Enzymes are shown in black, while substrates are in blue. Numbers inside parentheses indicate the number of identified C. elegans homologs for each given enzyme in the pathway. Solid red arrow denotes
enzymatic reactions in the glycolysis pathway. Dashed arrows denote regulatory steps: (+) for activation and (-) for inhibition. Asterisk marks genes examined in this study. We selected the specific enzymes for analyses based on availability of identified alleles from the Caenorhabditis Genomic Center (CGC). With the exception of 6-phosphofructokinase/pfk-1.1, which was identified in our forward genetic screen, all other enzymes were selected based on the availability of deletion alleles.

Scale bar represents 25µm for (D), and 5µm for (E). Error bars denote SEM. *, p < 0.05. **, p < 0.01. ***, p < 0.001 between indicated groups.
**Figure S3**, related to Figure1. Synaptic phenotypes in *pfk-1.1(ola72)* mutants under hypoxic conditions.

(A-E) Simultaneous visualization of synaptic vesicle clusters imaged with GFP::RAB-3 (A, C) and active zones imaged with ELKS-1::mCherry (B, D) in AIY neurons of wild-type (A, B); or *pfk-1.1(ola72)* mutant animals (C-E) mounted on glass slides and exposed to hypoxic conditions for 10 minutes (Pitts and Toombs, 2004). Note that in *pfk-1.1(ola72)* mutant animals, synaptic vesicle clusters become diffusely distributed (C); as compared to wild-type animals under similar conditions (A). Also note that active zone proteins remain punctate in both wild-type (B); and
pfk-1.1(ola72) mutant backgrounds (D, E). Insets correspond to zoomed-in (3x) images of the indicated regions.

(F-G) VENUS::RAB-3 in dorsal acetylcholine motor neurons of wild-type (F); and pfk-1.1(ola72) animals (G) mounted on glass slides and exposed to hypoxic conditions for 20 minutes (Pitts and Toombs, 2004). Insets correspond to zoomed-in (3x) images of the indicated regions.

(H-I) Pan-neuronal expression of GFP::RAB-3 in wild-type (H); and pfk-1.1(ola72) mutant animals (I) after being mounted on glass slides and exposed to hypoxic conditions for 20 minutes. Each inset (white and yellow outlined box) is of the ventral nerve cord and 3x magnified from corresponding regions indicated in the images.

(J) Percentage of animals displaying a diffuse distribution of synaptic vesicle proteins after 10 (or 20 minutes, as indicated) of exposure to hypoxic conditions (induced by mounting on glass slide with a glass coverslip (Pitts and Toombs, 2004); also refer to Figure S1). At 0 minutes, no differences are observed between the vesicle clusters of neurons in wild-type (black bars) and pfk-1.1(ola72) mutant animals (gray bars) for the four different types of neurons examined. By 10 (or 20) minutes, a significant percentage of pfk-1.1(ola72) mutant animals exhibit pronounced abnormal distribution of synaptic vesicle protein phenotypes in all neurons examined, but not the wild-type animals. NSM wild-type and pfk-1.1(ola72) are the same as shown in Figure 1J.

(K) Percentage of animals displaying a diffuse distribution of synaptic vesicle proteins in wild-type (black bars) or pfk-1.1(ola72) mutant animals (gray bars) under varying conditions. In the first eight columns, animals under normoxic conditions were treated for 10 minutes with pharmacological agents as indicated in the graph and described in the Supplemental Experimental Procedures. Note that in wild-type animals, inhibition of oxidative
phosphorylation (with 1uM oligomycin) and of glycolysis (with 10uM 2-deoxy-D-glucose, indicated as 2-DG) have only partial effects on the percentage of animals displaying diffuse synaptic vesicle (SV) clusters, while simultaneous inhibition of glycolysis and oxidative phosphorylation results in an enhancement of the phenotype. In pfk-1.1(ola72) animals, oligomycin, but not 2-DG, enhances the phenotype, suggesting that pfk-1.1(ola72) acts in the same pathway as 2-DG inhibition, namely, glycolysis. Note that in the last six columns of the graph, hypoxic conditions do not enhance the oligomycin effect for wild-type animals, but do enhance the 2-DG effect. Together, these analyses uncover a cooperative role between oxidative phosphorylation and glycolysis in maintaining the synaptic vesicle pattern in neurons, and indicate that under conditions of energy stress in which the activity of the oxidative phosphorylation pathway is decreased, the glycolytic pathway is required for the maintenance of synaptic vesicle clusters in vivo. Vehicle (or normoxic) and hypoxic are the same as shown in Figure 1J.

Scale bar represents 5µm; except for (H, I), in which scale bar represents 100µm. Error bars denote SEM. *, p < 0.05. **, p < 0.01. ***, p < 0.001 between indicated groups.
Figure S4, related to Figure 5 and Figure 7. Localization of PFK-1.1 to presynaptic sites is important for its function in sustaining the synaptic vesicle cycle.
(A–A’) Percentage of animals displaying PFK-1.1 clusters in the cell bodies of GABA neurons in wild-type (black bar), \textit{unc-13(e51)} (red bar), and \textit{syd-2(ok217)} (white bar) mutant animals immediately after mounting them under the coverslip (0 min) or after 10 minutes (10 min), causing hypoxic conditions (A). Even before hypoxic conditions ensue, PFK-1.1 (pseudocolored magenta) is observed as clustered (arrowheads) in the cell bodies (outlined by dotted lines) of wild-type animal, while the neurites (bracket) largely show a diffuse distribution of the PFK-1.1 (A’).

(B) Percentage of \textit{unc-13(e51)} mutant animals displaying PFK-1.1 clusters in the GABA neurons of \textit{oxIs352} animals (expressing channelrhodopsin cell-specifically in GABA neurons) and stimulated with blue light in the absence (-ATR) or presence (+ATR) of rhodopsin co-factor all-trans-retinal (ATR). PFK-1.1 clustering is suppressed even in the presence of ATR, or neuronal stimulation, in \textit{unc-13} mutants. Number of animals tested is indicated at the bottom of each column.

(C–E) PFK-1.1 (pseudocolored magenta) forms clusters under hypoxia (C). The dominant negative (DN) form, PFK-1.1(G532E), fails to form clusters under the same condition (D). Percentage of animals displaying PFK-1.1 (WT) or PFK-1.1(G532E) (DN) clusters under hypoxic conditions (E). Number of animals scored is indicated at the bottom of each column for (E). Wild-type control is the same as shown in Figure 5G for (E).

(F) PFK-1.1::eGFP (pseudocolored magenta) clusters to presynaptic sites when expressed in the NSM neurons of \textit{pfk-1.1(ola72)} animals and rescues the clustering of the synaptic vesicle protein RAB-3 under hypoxic conditions. Cell body is indicated by asterisk.

(G) PFK-1.1::eGFP::NLS (pseudocolored magenta) localizes to the nucleus, is not observed at synapses when expressed in the NSM neurons of \textit{pfk-1.1(ola72)} animals and fails to rescue the
clustering of RAB-3 under hypoxic conditions (quantified for three independent transgenic lines in (H)). Cell body is indicated by asterisk.

(H) Percentage of animals displaying abnormal distribution of synaptic vesicle proteins under hypoxic conditions in wild-type (black bar), pfk-1.1(ola72) (gray bar), pfk-1.1(ola72) expressing a rescuing construct PFK-1.1::eGFP cell-specifically in NSM (gray bar with black outline), and three independent transgenic lines of pfk-1.1(ola72) mutant animals expressing PFK-1.1::eGFP::NLS cell-specifically in NSM (white bars). Number of animals scored is indicated at the bottom of each column. Wild-type control and pfk-1.1(ola72) are the same as shown in Figure 1J.

Scale bar represents 5µm. Error bars denote SEM. *, p < 0.05. **, p < 0.01. ***, p < 0.001 between indicated groups.
Figure S5, related to Figure 8. *pfk-1.1* mutants have impaired locomotion compared to wild-types upon inhibition of oxidative phosphorylation.

(A) Cartoon illustration of one full body bend in *C. elegans* as indicated by the arrows.

(B-C) Number of body bends measured for 1 minute after a four-minute bath in M9 buffer alone (B) or in a 10mM sodium azide (NaN3) (C). M9 buffer alone produced no difference in the number of body bends between wild-type and *pfk-1.1*(ola72) mutant animals while *pfk-1.1*(ola72) (B) mutants showed significant decrease in the number of body bends compared to that of the wild-type under sodium azide treatment (C). Number of animals scored is indicated at the bottom of each column.

Error bars denote SEM. *, p < 0.05. **, p < 0.01. ***, p < 0.001 between indicated groups.
SUPPLEMENTAL MOVIES

Movie S1, related to Figure 1. Time lapse movie of synaptobrevin/SNB-1::GFP in GABA neuron of *pfk-1.1(ola72)* mutant animal under hypoxic conditions.

Images acquired at one minute interval from 5 to 20 minutes during hypoxic treatment.
Corresponds to Figures 1M and 1N.

Movie S2, related to Figure 4. Time lapse movie of PFK-1.1::eGFP (pseudo colored magenta) localization in NSM neuron in wild-type animal under hypoxic conditions.

Images acquired at one minute interval from approximately 10 minutes to 30 minutes during hypoxic treatment. Correspond to Figures 4A and 4A’.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Strains and genetics

Worms were raised on nematode growth media plates at 20°C using OP50 *Escherichia coli* as a food source (Brenner, 1974). For wild-type nematodes, *C. elegans* Bristol strain N2 was used.

The following mutant strains were obtained through the *Caenorhabditis* Genetics Center:

*juIs1* [Punc-25::snb-1::gfp; lin-15(+)], *jsIs682* [Prab-3::gfp::rab-3], pfk-1.1(gk549413), pfk-1.1(gk758818), pfk-1.1(gk922689), pfkb-1.1(ok2733), gpd-3(ok2870), unc-57(ok310), unc-11(e47), unc-26(s1710), unc-13(e450), unc-13(e51), unc-104(e1265), klp-6(sy511), ric-7(nu447), syd-2(ju37), syd-2(ok217), and unc-49(e407). *aldo-1(tm5782)* and *pgk-1(tm5613)* were received from Shohei Mitani (Tokyo Women’s Medical University, Tokyo, Japan).

*nuls168* [Pmyo-2::gfp; *Punc-129::Venus::rab-3*] was provided by Jihong Bai (Fred Hutchinson Cancer Research Center, Seattle, Washington). *lev-8(ok1519)* was provided by Michael Koelle (Yale University, New Haven, Connecticut). *drp-1(tm408)* was provided by Marc Hammarlund (Yale University, New Haven, Connecticut). *syd-2(ju37); juIs330* [Punc-25::syd-2] was provided by Mei Zhen (University of Toronto, Canada). Other strains used in the study are as follows: *olaIs1* [Ptph-1::mCherry; Ptph-1::cat-1::gfp], *WyEx505* [Pttx-3::mCherry::erc; Pttx-3::GFP::rab-3], *oxSi91* [Punc-17::ChIEF::mCherry], and *oxIs352* [Punc-47::ChR2::mCherry; lin-15(+)].

Molecular biology and transgenic lines

Expression clones were generated using the Gateway system (Invitrogen). Detailed cloning information will be provided upon request. Transgenic strains (0.5–30 ng/µl) were generated using standard techniques (Mello and Fire, 1995) and coinjected with markers *Punc-122::gfp* or *Punc-122::rfp*. The following strains were generated: *olaEx1556* [WRM016aB04; Pttx-


3::mCherry], olaEx2337 [Ppfk-1.1::pfk-1.1], olaEx1923 [Ppfk-1.1::pfk-1.1], olaEx1920 [Ptph-1::pfk-1.1], olaEx2085 [Ppfk-1.1::egfp], olaEx1641 [Ppfk-1.1::egfp; Ptph-1::mCherry], olaEx2014 [Ptph-1::pfk-1.1::egfp; Ptph-1::mCherry], olaEx2016 [Ptph-1::pfk-1.1::egfp; Ptph-1::mCherry::rab-3], olaEx2209 [Ptph-1::pfk-1.1::egfp; Ptph-1::mCherry::rab-3], olaEx2241 [Ptph-1::pfk-1.1::mCherry; Ptph-1::aldo-1::egfp], olaEx2249 [Ptph-1::pfk-1.1::mCherry; Ptph-1::gpd-3::egfp], olaEx2274 [Punc-47::pfk-1.1::egfp], olaEx2245 [Pttx-3::sl2::pfk-1.1::egfp; Pttx-3::mCh::rab-3], olaEx2544 [Ptph-1::pfk-1.1G532E::egfp; Ptph-1::mCherry::rab-3], olaEx2545 [Ptph-1::pfk-1.1G532E], olaEx2330 [Ptph-1::pfk-1.1::egfp::NLS; Ptph-1::mCherry::rab-3], olaEx2290 [Ptph-1::pfk-1.1::egfp::NLS; Ptph-1::mCherry::rab-3], olaEx2291 [Ptph-1::pfk-1.1::egfp::NLS; Ptph-1::mCherry::rab-3], and olaex2671 [Ptph-1::mCherry::rab-3].

Screen and positional cloning

Worms expressing CAT-1::GFP and cytosolic mCherry in NSM neuron (olaIs1) were mutagenized with ethyl methanesulfonate (EMS) as described previously (Brenner, 1974). The original screen was designed to identify mutants in genes required for synaptogenesis in the serotonergic neurons (NSM). The screen was performed as previously described (Colon-Ramos et al., 2007; Jin, 2005; Schaefer et al., 2000; Shao et al., 2013; Shen and Bargmann, 2003; Sieburth et al., 2005; Yeh et al., 2005). Briefly, F₁ progeny of mutagenized P₀ worms were cloned onto individual plates, and F₂ progeny were screened using a Leica DM500B compound fluorescent microscope to visualize the distribution of the synaptic vesicular monoamine transporter (VMAT), CAT-1::GFP. NSM neurons were first inspected for appropriate cellular morphology and position, as well as axon and dendrite extension and arborization (by using the cytoplasmic mCherry marker). Next, the position, number, intensity, and distribution of the synaptic vesicle signal was assessed using our CAT-1::GFP marker. We screened through 2000
haploid genomes and identified 13 alleles with defects in synaptic patterning. These mutants fall into four categories: (1) Synaptic vesicle signal is diffusely distributed throughout neurites (6 mutants); (2) Synaptic vesicle signal is very dim or completely absent (4 mutants); (3) Synaptic varicosities appear larger than average (2 mutant); (4) Synaptic vesicle signal appears wild-type upon first examination; after extended exposure to hypoxic conditions under a coverslip (Pitts and Toombs, 2004) the synaptic vesicles became diffusely distributed. This fourth category is the mutant class pfk-1.1(ola72) belongs to.

The ola72 allele was mapped to a 0.39Mbp region on chromosome X using single nucleotide polymorphisms and the CB4856 strain as described (Davis et al., 2005). Fourteen fosmids that cover this region were injected into ola72 mutants and examined for rescue of synaptic vesicle clustering defects. ola72/pfk-1.1 trans-heterozygotes were examined for complementation. Sanger sequencing was performed to identify the genetic lesion in the ola72 allele.

**Cell autonomy and rescue of ola72**

The ola72 mutant phenotype was rescued by both the full pfk-1.1 genomic sequence and pfk-1.1 cDNA under the regulation of its endogenous promoter (fragment 1.5kb upstream of pfk-1.1). Cell-specific rescue in NSM was achieved with a plasmid driving the expression of pfk-1.1 cDNA under the NSM-specific tph-1 promoter fragment as described (Nelson and Colón-Ramos, 2013). pfk-1.1 cDNA tagged with eGFP at its C-terminus under the NSM-specific promoter also rescued the ola72 mutant phenotype (Figure S4H).

**Inducing hypoxia with glass coverslips and slides**

Glass coverslips have been used to induce hypoxia in cell cultures (Pitts and Toombs, 2004). The reduced environment generated by mounting 10-15 live worms on glass slides was examined by
using redox indicator resazurin (25ug/mL) dissolved in water. Resazurin when reduced to resorufin gives off fluorescence under yellow-green light (O'Brien et al., 2000). Using resazurin, we confirmed that worms between a glass coverslip and slide experience reduced, or hypoxic, conditions; Leica DM500B compound fluorescent microscope was used to acquire the images (Figures S1M and S1N). Gas permeable slides were made with Sylgard-184 (polydimethylsiloxane, or PDMS) (Dow Corning) according to manufacturer instructions.

**Inhibiting oxidative phosphorylation or glycolysis using a hypoxia chamber or pharmacological treatments**

Hypoxia assays were performed using a chamber (similar to Billups-Rothernberg modular incubator chamber MIC-101) generously shared by Dr. Americo Esquibies (Yale University School of Medicine). Worms were mounted on a slide, placed in a hypoxia chamber and flushed for 4 minutes with nitrogen gas. The chamber was sealed and the worms were exposed to hypoxic conditions for 10 minutes. After the exposure, phenotypes were scored immediately under a Leica DM500B compound microscope. For carbon dioxide exposure, a smaller chamber was built using glass bottom culture dish (MatTek). Worms were placed in the culture dish and exposed to carbon dioxide for 10 minutes, and scored immediately. For pharmacological disruption of oxidative phosphorylation or glycolysis, worms were mounted on a slide with 10mM sodium azide (NaN₃), 10uM of 2-deoxy-D-glucose (2-DG), or 1uM of oligomycin dissolved in muscimol, and scored after 10 minutes.

**Neuronal stimulation with pharmacological treatments or optogenetics.**

To pharmacologically stimulate GABA neurons, worms were mounted on a slide in 1mM levamisole (Sigma) and imaged immediately in spinning-disc confocal microscope (PerkinElmer Life and Analytical Sciences). As a control, 50mM of muscimol (Abcam) was used. To
optogenetically stimulate GABA neurons, a strain expressing channelrhodopsin 2 in GABA neurons (oxIs352) (Liu et al., 2009) was used. All-trans-retinal and OP50-seeded plates were prepared as described previously (Liu et al., 2009). Briefly, 4uL of 100uM all-trans-retinal was added to 200uL of OP50 culture to seed each plate. Channelrhodopsin expressing worms were grown in all-trans-retinal seeded plate for minimum of 16 hours before stimulation. Worms were then mounted to a gas permeable PDMS slide set up, immobilized in muscimol, exposed to blue light for 5 minutes using a Leica DM500B compound microscope (approximately 0.6mW/mm²), and examined for formation of PFK-1.1 clusters. As a control, the same strains were grown without all-trans-retinal, and tested under the same conditions.

**Electrophysiology**

Worms expressing the channelrhodopsin I/II chimera ChIEF in acetylcholine neurons (oxSi91) were dissected and patch-clamp physiology was conducted as previously described (Richmond et al., 1999; Richmond and Jorgensen, 1999). For oligomycin experiments, the dissected preps were exposed to 1uM oligomycin for 5 minutes prior to patch clamp recordings. The stimulation protocol included a pre-pulse followed by a 5-second recovery period and 30-second stimulation train (10Hz). Test pulses were delivered at increasing time increments and normalized to the first spike in the train to assess recovery. Two test-pulses were delivered per animal at ≥15 seconds apart. By analyzing the overlap of the first and second pulses at intermediate time-points, we determined that a single test-pulse did not affect the amplitude of the second pulse. For repeated stimulus trains, the full protocol was repeated with 70 seconds between the end of the first train and the beginning of the following train. Peak current was measured for each evoked response (red Xs in Figure 8B) and normalized to the amplitude of the first pulse of the stimulus train. The evoked amplitudes were plotted vs. time.
Behavioral experiments

*C. elegans* nematodes move in a wave-like fashion by executing dorsal-ventral body bends, and this sinusoidal body motion can be observed both on solid substrates and when worms are placed in an aqueous environment (Ghosh and Emmons, 2008). To examine how the locomotion of these animals is affected under hypoxic conditions, an Anaerobe Pouch System (Becton Dickinson & Co) was used to induce hypoxia. Animals were placed in M9 solution on slides, which were then placed inside the gas-impermeable pouch along with a wet paper towel and anaerobe gas-generating sachet. To examine how the locomotion of animals is affected under conditions of energetic stress, animals were placed in 10mM sodium azide (NaN$_3$) solution dissolved in M9 on top of a slide. After 4 minutes of acclimation, the number of body bends was scored for 1 minute under a dissection scope. Bending of the head of the worm to one side and back to the initial side was counted as one body bend as illustrated in Figure 8H. M9 solution was used as a control and, as expected, no difference in the number of body bends between wild-type and *pfk-1.1* mutant animals were observed in this control (data not shown). *p values* were calculated using the Mann-Whitney *U* test.

Microscopy, FRAP, and imaging

Images of fluorescently tagged fusion proteins were captured in live *C. elegans* nematodes using a 60 CFI Plan Apo VC, numerical aperture 1.4, oil-immersion objective on an UltraView VoX spinning-disc confocal microscope (PerkinElmer Life and Analytical Sciences). Worms were immobilized using 50mM muscimol (Abcam). Image J and Photoshop were used to analyze images, which were oriented anterior to the left and dorsal up. Maximum projections were used for all the confocal images, unless otherwise stated. Ratiometric images were generated by using the Volocity software (Perkin Elmer). For the Fluorescence Recovery After Photobleaching
(FRAP) experiment, the spinning-disc confocal microscope and Volocity FRAP Plugin (Perkin Elmer) were used. Single plane images were acquired at 3-second intervals post-photobleaching. Acquired data was normalized using FRAP analysis in Volocity and fitted to a single exponential curve. For FRAP analysis, \( p \) values were calculated using the Mann-Whitney \( U \) test.

**Quantification of phenotypic expressivity**

To quantify synaptic enrichment of the indicated proteins (synaptic vesicle proteins or PFK-1.1), fluorescence values for individual neurites (ventral neurite for the NSM neuron, Zone3 for the AIY neuron, and dorsal and ventral neurite for GABA neurons) were obtained through segmented line scans using ImageJ. A sliding window of 2\( \mu \)m was used to identify all the local fluorescence peak values and trough values for an individual neuron (the maximum and the minimum fluorescence values in a 2\( \mu \)m interval, respectively). Synaptic enrichment was then calculated as \( \% \Delta F/F \) as previously described (Bai et al., 2010; Dittman and Kaplan, 2006). Briefly, all the identified local maximum and minimum fluorescence values in a given neurite (local \( F_{\text{peak}} \) and local \( F_{\text{trough}} \)) were averaged and used to calculate \( \% \Delta F/F \), with \( \% \Delta F/F \) being the percent difference between average peak-to-trough fluorescence (\( F \)) defined as \( 100 \times \frac{(F_{\text{peak}} - F_{\text{trough}})}{F_{\text{trough}}} \). All the images used in the quantification analyses were obtained using identical microscopy settings. For the quantification of the average number of PFK-1.1 puncta in AIY neurons, confocal images of AIY neurons were analyzed and scored for PFK-1.1 puncta in synaptic and asynaptic regions (Colon-Ramos et al., 2007; White et al., 1986). Statistical analyses were performed with Prism (GraphPad) and \( p \) values were calculated using the Mann-Whitney \( U \) test.

**Quantification of phenotypic penetrance**
Animals were scored as displaying either “punctate” or “diffuse” phenotypes for synaptic vesicles proteins or PFK-1.1 after specified manipulations. “Punctate” or “diffuse” phenotypes were first qualitatively defined for the purposes of the genetic screens. To provide a quantitative definition that described the phenotypes we saw, we present what was meant by “diffuse” based on DF/F values. Briefly, we scored the distribution of synaptic vesicle proteins in wild-type or mutant animals by performing line scans, and calculated the DF/F for wild-type (N=16) and pfk-1.1(ola72) mutants (N=19). The average value for wild-type animals is 5.17 DF/F (fold increase of peak-to-trough fluorescence), while the average value for pfk-1.1(ola72) mutants is 2.94 DF/F fold. We then defined the “diffuse” distribution of vesicle clusters based on this average value observed for pfk-1.1(ola72) mutants and established a threshold at 3 DF/F for identifying an animal as either “diffuse” or “punctate”. Calculations, using this value, of the number of animals with a “diffuse” distribution of vesicle clusters reveal that approximately 20% of wild-type animals vs. 60% of pfk-1.1(ola72) mutant animals display a diffuse clustering phenotype under hypoxic conditions. We used these parameters as guidelines for scoring the phenotypic penetrance of all conditions and genotypes described in this study. We validated the approach by qualitatively scoring blindly the percentage of animals that exhibit a diffuse distribution of synaptic vesicle proteins for wild-type and for pfk-1.1(ola72) animals. We determined that the number we obtained after qualitative assessments of the phenotype and blind scoring were similar to the ones which were generated by measuring synaptic distribution of vesicular proteins using line scans, calculating the DF/F signal and then scoring individuals animals based on the 3 DF/F threshold definition of “diffuse”. Statistical analyses were performed with Prism (GraphPad) and p values were calculated using Fisher’s exact test.

**Quantification of colocalization**
To quantify the relative distance between two fluorescently tagged proteins in the colocalization experiments, we used ImageJ and line scans of neurites to score the pixel fluorescence values in the green and red channels. The relative location corresponding to the maximum pixel fluorescence values for each channel was then used to calculate the distance between the two fluorescently tagged proteins.
SUPPLEMENTAL REFERENCES