

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

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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

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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 (Bélangier 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 (Hubble 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-nard et al., 2014), and in neurons, biochemical studies demonstrated that glycolytic proteins are enriched in synaptic fractions (Knull, 1978, 1980; Knull 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 *pfk-1.1* (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-1.1(gk549413)*, *pfk-1.1(gk758818)*, and *pfk-1.1(gk922689)*, phenocopy and fail to complement the *ola72* allele (Figures 2C, 2I, and S2C). Expression of a wild-type copy of the *pfk-1.1* 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-1.1* 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-1.1* mutants (Figures S3A–S3E). In addition, the requirement for *pfk-1.1* 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-1.1* promoter is expressed in all examined neurons, including the NSM neuron (Figures S2D–S2G). Moreover, NSM neuron-specific expression of the *pfk-1.1* cDNA in *pfk-1.1(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-1.1(ok2733)*, glyceraldehyde 3-phosphate dehydrogenase/*gpd-3(ok2870)*, aldolase/*aldo-1(tm5782)*, and phosphoglycerate kinase/*pgk-1(tm5613)* phenocopy the hypoxia-dependent synaptic vesicle protein phenotype of *pfk-1.1* 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 Greville, 1961). We observed that *pfk-1.1* 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-1.1* 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-1.1* mutants disrupt synaptic vesicle endocytosis, we first visualized vesicle protein clusters in endocytic mutant *unc-57*/endophilin and *pfk-1.1*;*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-1.1* acting in the same genetic pathway, we observed that mutations in endophilin did not enhance the hypoxia-induced *pfk-1.1* phenotype (Figure 3J). These observations suggest that the synaptic vesicle defect observed in *pfk-1.1* mutant animals could result from inhibition of endocytosis due to decreased rates of glycolysis.

If *pfk-1.1* 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

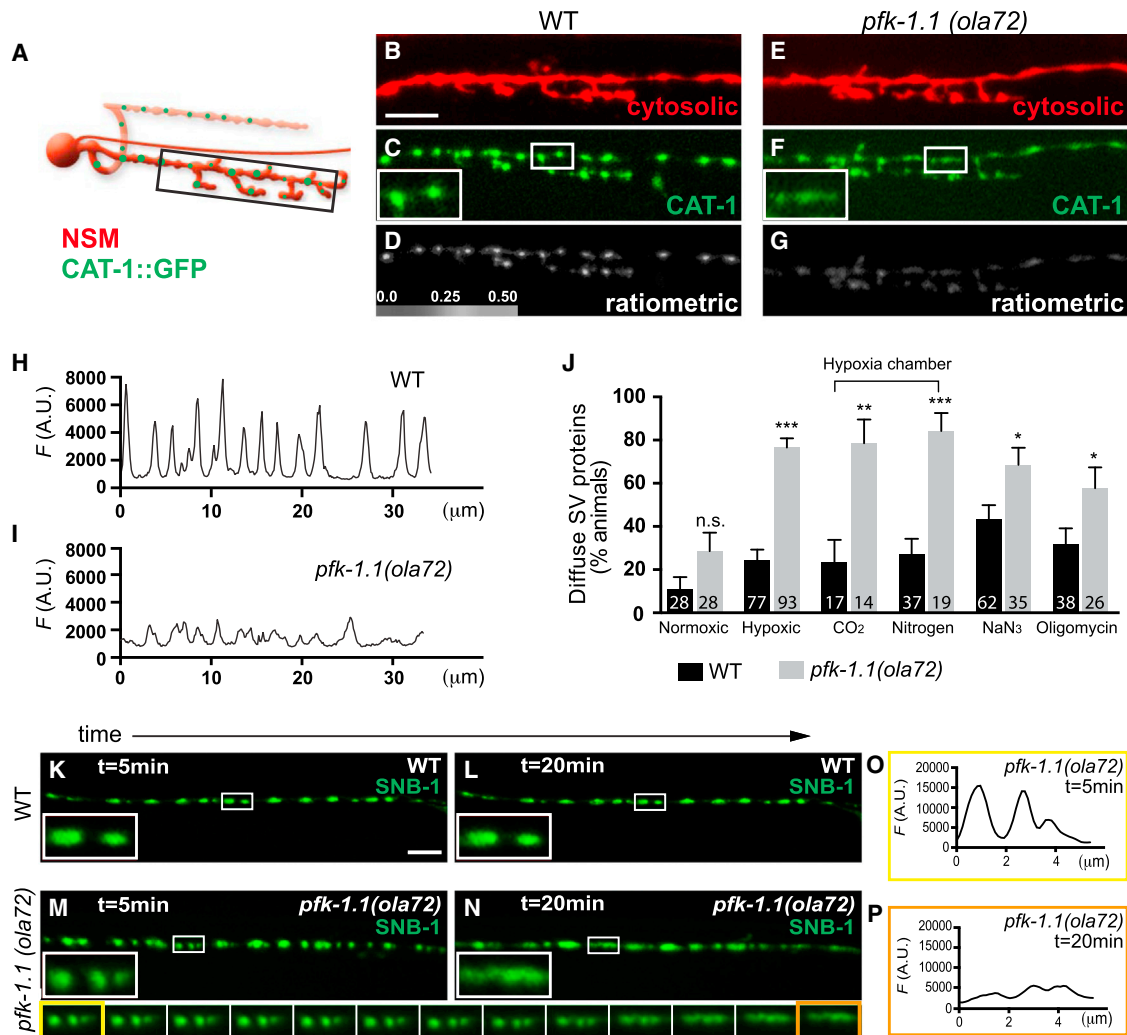


Figure 1. PFK-1.1 Is Required to Cluster Synaptic Vesicle Proteins under Hypoxic Conditions

(A) Schematic of NSM neuron (red) with synaptic release sites (green) (Axäng et al., 2008; Nelson and Colón-Ramos, 2013). Black box marks the ventral neurite imaged in (B)–(G). Illustration is a modification with permission from the Neuron pages of WormAtlas (Altun and Hall, 2016).

(B–G) The ventral neurite of NSM neurons (B and E) and its synaptic vesicle proteins (GFP-tagged vesicular monoamine transporter VMAT/CAT-1) (C and F) were simultaneously visualized in wild-type (B and C) and *pfk-1.1(ola72)* mutant animals (E and F) under hypoxic conditions (described in Supplemental Experimental Procedures). Ratiometric images of NSM represent the relative enrichment of CAT-1::GFP signal over cytosolic mCherry signal for wild-type (D) or *pfk-1.1(ola72)* mutant animals (G).

(H and I) Pixel fluorescence values along the ventral neurite of the wild-type (H) and *pfk-1.1(ola72)* (I), corresponding to images (C) and (F), respectively.

(J) Percentage of animals displaying a diffuse distribution of synaptic vesicle proteins in wild-type or *pfk-1.1(ola72)* mutant animals under varying conditions. Number of animals scored is indicated at the bottom of each column.

(K–N) Time lapse displaying the distribution of synaptic vesicle proteins in GABA neurons (imaged with synaptobrevin/SNB-1::GFP) of a single wild-type (K and L) or *pfk-1.1(ola72)* mutant worm (M and N) after 5 min (K and M) or 20 min (L and N) under hypoxic conditions. Insets correspond to zoomed-in (3×) images of the indicated regions. Bottom row corresponds to time-lapse images (1 min intervals) of the insets in *pfk-1.1(ola72)* (please also see Movie S1, a time-lapse movie of synaptic vesicles in *pfk-1.1(ola72)*).

(O and P) Line scan pixel fluorescence values for the first inset in the montage (O) (M; also yellow trim in bottom row) and the last inset (P) (N; also orange trim in bottom row).

Scale bar, 5 μm. Error bars: SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to wild-type animals under similar conditions.

greater mobility than proteins associated with synaptic vesicles (Bai et al., 2010; Kraszewski et al., 1996). We observed that synaptobrevin exhibited greater mobility in endocytotic 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

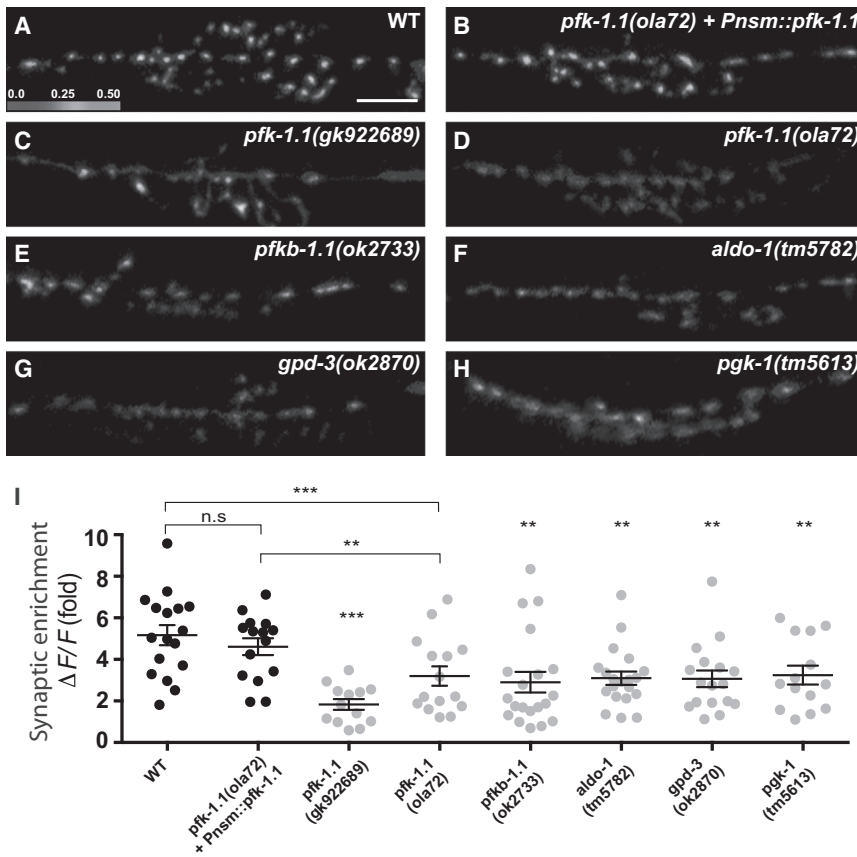


Figure 2. Glycolytic Proteins Are Required to Maintain Synaptic Vesicle Protein Clusters during Hypoxia

(A–H) Ratiometric images of NSM representing the relative enrichment of CAT-1::GFP signal over cytosolic mCherry signal for wild-type (A) *pfk-1.1(ola72)* mutant animals expressing a wild-type rescuing array of *pfk-1.1* cell autonomously in NSM (B), *pfk-1.1(gk922689)* (C), *pfk-1.1(ola72)* (D), *pfkb-1.1(ok2733)* (E), *aldo-1(tm5782)* (F), *gpd-3(ok2870)* (G), and *pgk-1(tm5613)* (H).

(I) Quantification of the distribution of the synaptic vesicle proteins for the examined genotypes as described (Dittman and Kaplan, 2006; Supplemental Experimental Procedures). The circles in the graph represent individual animals.

Scale bar, 5 μm . Error bars: SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to wild-type animals under similar conditions, unless otherwise indicated by brackets.

exocytosis would suppress the *pfk-1.1* mutant phenotype. Indeed, we observed that mutants for the synaptic vesicle docking protein UNC-13 partially suppressed the hypoxia-induced *pfk-1.1* phenotype (Figures 3G–3J). The observed partial suppression may be due to the hypomorphic nature of the *unc-13* allele, or to potential contributions to the phenotype from other, *unc-13*-independent pathways. The phenotype in *pfk-1.1; unc-13* double mutants, however, indicates that a defect in the synaptic vesicle cycle significantly contributes to the *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.

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 S2), preferentially localizing to synaptic-rich regions (Figure 4F) and within 0.2 μm 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 Knull, 1981; Campanella et al., 2008; Knull 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.

Aerobic 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. *C. elegans* GABA neurons can be stimulated through the bath application of levamisole, an agonist of acetylcholine receptors (such as *lev-8*) present in the GABA neurons (Towers et al., 2005). By applying levamisole under normoxic conditions, we examined whether *pfk-1.1* was required to support vesicle cycling during periods of strong neuronal stimulation. We observed that *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

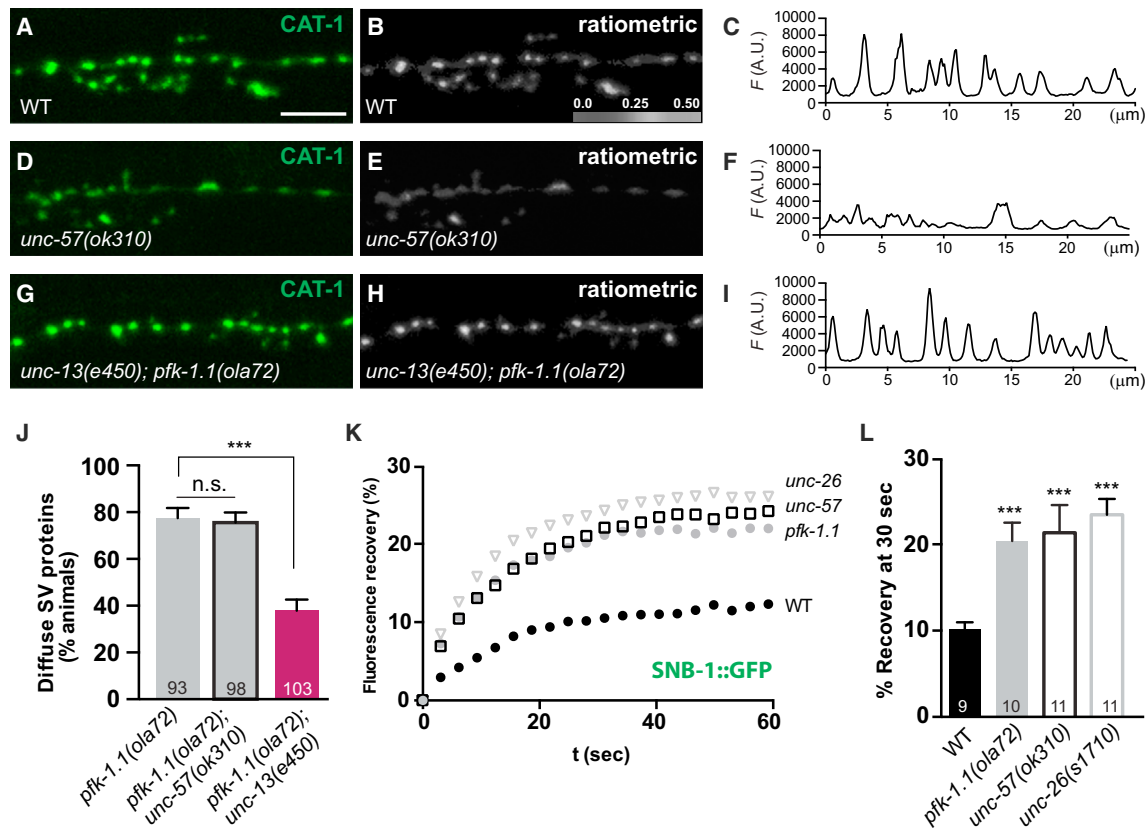


Figure 3. Glycolysis Is Required to Maintain the Synaptic Vesicle Cycle

(A–I) Synaptic vesicle marker CAT-1::GFP in NSM serotonergic neurons for wild-type after 10 min under hypoxic conditions (A), endocytosis mutant *unc-57(ok310)/Endophilin A* at normoxic condition (D), and *unc-13(e450);pfk-1.1(ola72)* double mutants after 10 min under hypoxic conditions (G). Ratiometric image of NSM representing the relative enrichment of CAT-1::GFP signal over cytosolic mCherry signal in the respective genotypes (B, E, and H) and the respective line scan pixel fluorescence values of the synaptic vesicle marker CAT-1::GFP (C, F, and I).

(J) Percentage of animals displaying a diffuse distribution of synaptic vesicle proteins in the NSM neuron after 10 min of hypoxia in *pfk-1.1(ola72)* (gray bar), *unc-57(ok310); pfk-1.1(ola72)* (gray bar with black outline), and *unc-13(e450);pfk-1.1(ola72)* (red bar) double mutants. Number of animals scored is indicated at the bottom of each column. *pfk-1.1(ola72)* is the same as shown in Figure 1J.

(K and L) Percentage of fluorescence recovery after photobleaching (FRAP) at synaptic varicosities of GABA neurons in *unc-26(s1710)* (triangles), *unc-57(ok310)* (squares), *pfk-1.1(ola72)* (gray circles), and wild-type (black circles) animals over time (K), and at 30 s post-photobleaching (L). Number of animals tested is indicated at the bottom of each column.

Scale bar, 5 μ m. Error bars: SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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 5D–5F). 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 Volkandt, 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 (Giegé et al., 2003). Therefore, we

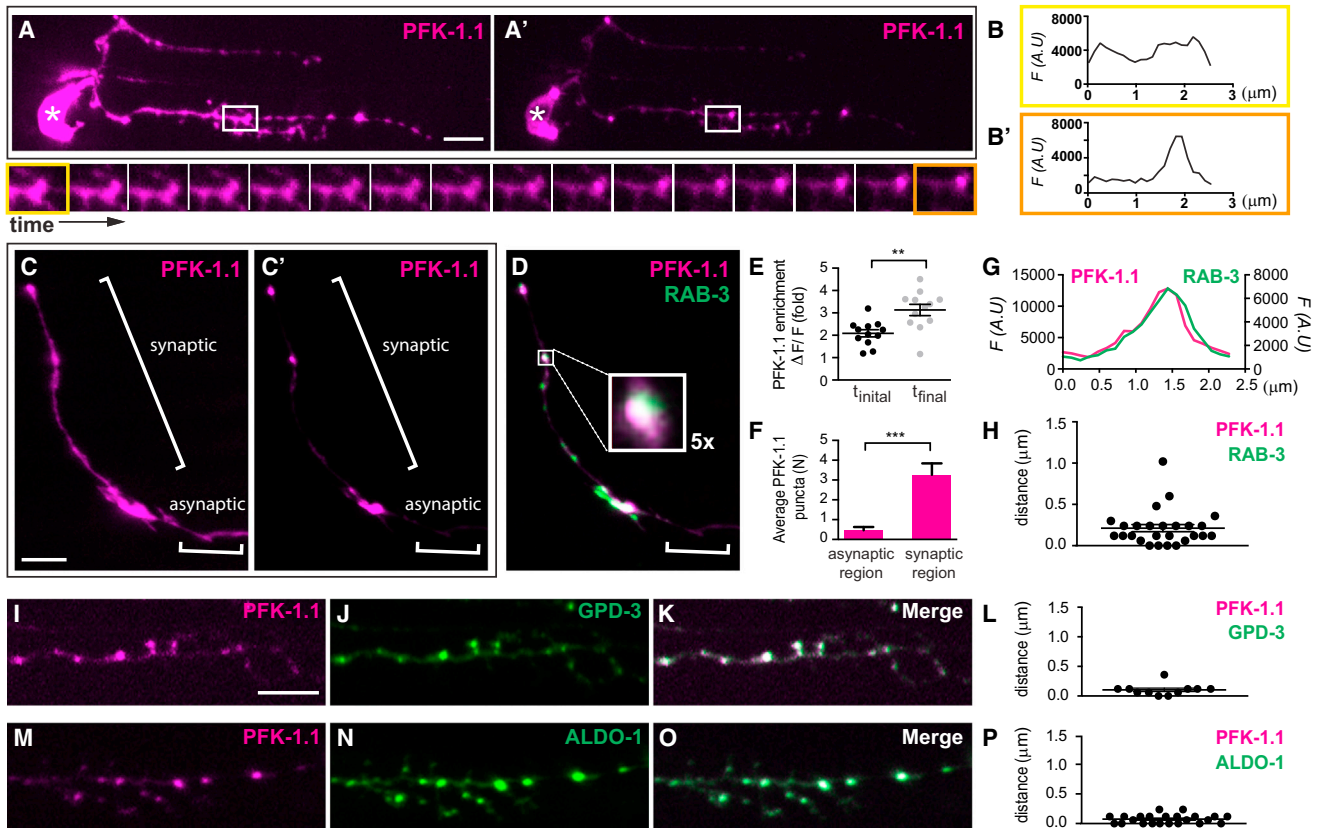


Figure 4. Glycolytic Proteins Dynamically Cluster Near Presynaptic Release Sites

(A and A') PFK-1.1::eGFP (pseudocolored magenta) localization in NSM after approximately 10 min (A) and 30 min (A') of exposure to hypoxic conditions (see Supplemental Experimental Procedures). Clustering dynamics of PFK-1.1 vary between individual animals, but clustering of PFK-1.1 is observed for most animals within 10 min of being exposed to hypoxic conditions. Here we filmed a neuron with slow clustering dynamics to capture the time-lapse image. Cell body of NSM neuron is indicated by asterisk. The time-lapse images of the inset in (A) and (A') (taken at 1 min intervals) are displayed below. See Movie S2 for time-lapse movie.

(B and B') Pixel fluorescence values for the first inset in the montage (B) (yellow trim in bottom row) and the last inset (B') (orange trim in bottom row).

(C–E) PFK-1.1::eGFP (pseudocolored magenta) and mCherry::RAB-3 (pseudocolored green) simultaneously visualized in AIY interneurons. PFK-1.1 in AIY after 20 min (C) and 40 min (C') of hypoxic conditions, and (C') visualized with synaptic vesicle marker RAB-3 (D); the brightness of (C) was increased more than in (C') in order to allow for visibility of the diffuse localization of PFK-1.1. Note that PFK-1.1 becomes increasingly clustered to presynaptic sites (denoted with brackets as “synaptic region”) and is excluded from asynaptic regions (Colón-Ramos et al., 2007; White et al., 1986). Inset in (D) corresponds to zoomed-in (5 \times) image of PFK-1.1 cluster localizing near a pool of synaptic vesicles. Enrichment of PFK-1.1 clustering in the AIY neurite over time in hypoxic treatment was quantified (N = 12) (see Supplemental Experimental Procedures) (E). t_{initial} is >20 min and t_{final} is 20–40 min of hypoxic treatment. Each circle represents an individual animal in the longitudinal study.

(F) Quantification of the number of PFK-1.1 clusters in the synaptic region and the asynaptic regions of AIY (N = 15).

(G) Pixel fluorescence values of PFK-1.1 and RAB-3 along the neurite for boxed region in (D) to compare the colocalized distribution of PFK-1.1 relative to synaptic vesicle proteins. Left y axis corresponds to PFK-1.1, and right y axis corresponds to RAB-3 pixel fluorescence values.

(H) Quantification of the distance between the maximum pixel fluorescence values of PFK-1.1 and the maximum pixel fluorescence values of RAB-3 in AIY neurons (n = 26).

(I–P) PFK-1.1::mCherry (pseudocolored magenta) (I and M) and glycolytic proteins GPD-3::eGFP (J) or ALDO-1::eGFP (N) were simultaneously visualized in NSM neurons (K and O) under hypoxic conditions. Quantification of the distance between the maximum pixel fluorescence values of PFK-1.1 and GPD-3 (L) (n = 11) and PFK-1.1 and ALDO-1 (P) (n = 23).

Scale bar, 5 μm . Error bars: SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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

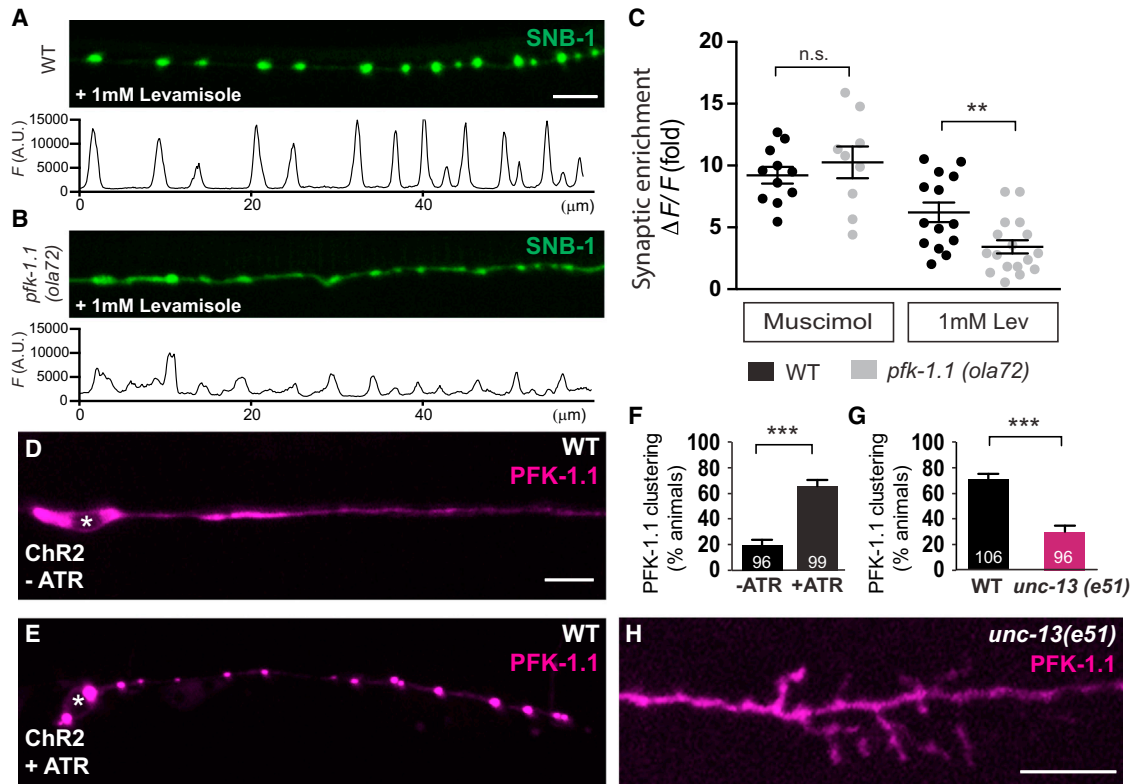


Figure 5. Neuronal Stimulation Induces PFK-1.1 Clustering

(A and B) Synaptic vesicle protein SNB-1::GFP in GABA neurons exposed to levamisole in wild-type (A) or *pfk-1.1(ola72)* mutant animals (B). GABA neurons express acetylcholine receptors that are stimulated by the cholinergic agonist levamisole (Towers et al., 2005), but not by GABA agonist muscimol (Schuske et al., 2004; C). Pixel fluorescence values of the synaptic vesicle proteins along the neurites are shown under (A) and (B).

(C) Quantification of the synaptic enrichment of the synaptic vesicle proteins SNB-1 ($\Delta F/F$) in the GABA neurons for wild-type (black circles) and *pfk-1.1(ola72)* (gray circles) under muscimol or levamisole treatment.

(D and E) Localization of PFK-1.1 (pseudocolored magenta) in wild-type animals expressing channelrhodopsin cell specifically in GABA neurons and stimulated with blue light in the absence (D) or presence (E) of rhodopsin cofactor all-trans-retinal (ATR). Cell body is indicated by asterisk.

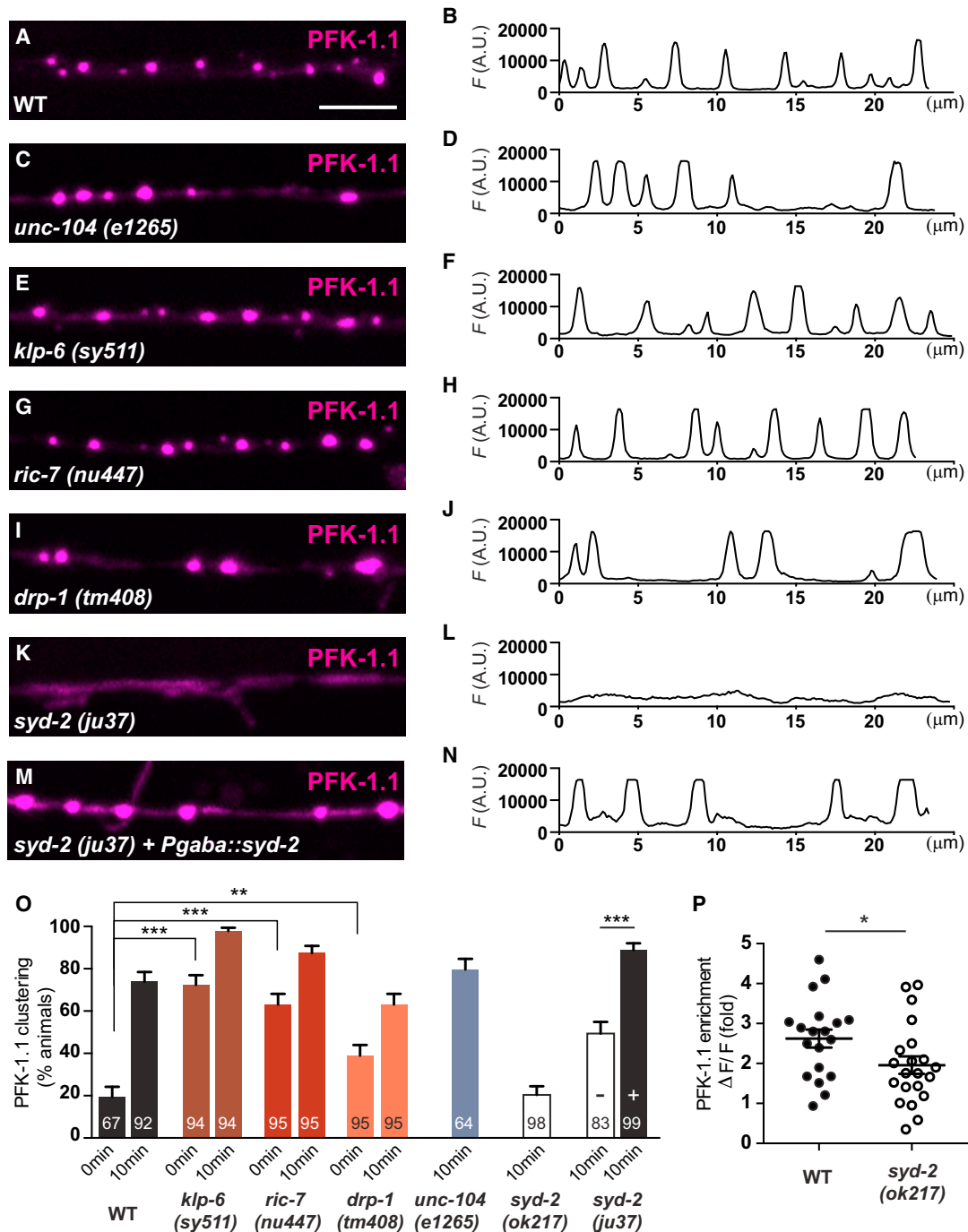
(F and G) Percentage of animals displaying PFK-1.1 clusters in GABA neurites expressing channelrhodopsin and stimulated with blue light in the absence or presence of ATR (F) and in NSM neurites under hypoxic conditions in wild-type (black bar) and *unc-13(e51)* (red bar) mutant backgrounds (G). Number of animals tested is indicated at the bottom of each column.

(H) Localization of PFK-1.1 in NSM neurons of *unc-13(e51)* animals exposed to 10 min of hypoxic conditions, quantified in (G).

Scale bar, 5 μ m. Error bars: SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between indicated groups.

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* mutant animals. We observed that in the *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-1.1 localization. Similar results were obtained for presynaptic protein SYD-1 (data not shown) (Hallam et al., 2002). Our findings demonstrate that presynaptic scaffolding proteins are cell-autonomously required in neurons for the clustering of PFK-1, and underscore a functional link



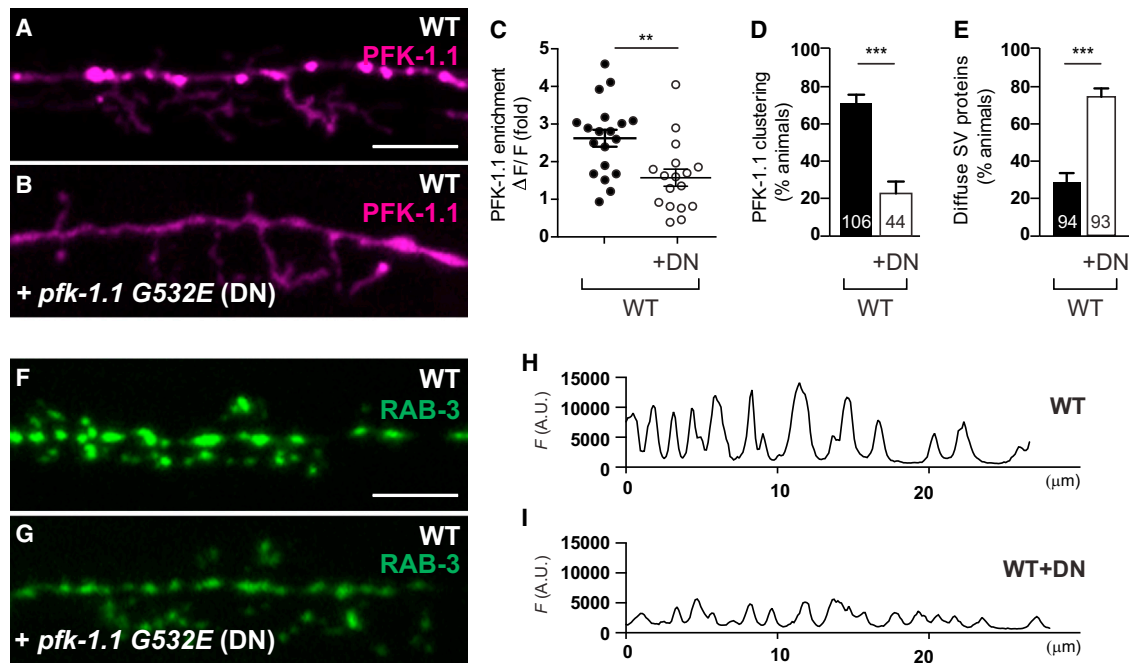


Figure 7. Localization of PFK-1.1 Is Necessary for Maintaining Vesicle Clusters under Energy Stress

(A and B) PFK-1.1::eGFP (pseudocolored magenta) in wild-type NSM neuron in the absence (A) or presence of PFK-1.1(G532E) (dominant negative, DN) (B). (C) Quantification of the enrichment of PFK-1.1::eGFP wild-type (black circles, N = 19) and PFK-1.1::eGFP wild-type coexpressed with the dominant-negative PFK-1.1(G532E) (+DN and white circles, N = 17). Wild-type control is the same as shown in Figure 6P.

(D) Percentage of animals displaying PFK-1.1 clusters or diffuse distribution of synaptic vesicle proteins in wild-type (WT) NSM neurons under hypoxic conditions when expressed without (black bar) or with PFK-1.1(G532E) (+DN and white bar). Number of animals scored is indicated at the bottom of each column. Wild-type control is the same as shown in Figure 5G.

(E–I) Synaptic vesicle proteins RAB-3 (pseudocolored green) in NSM neurons and its respective pixel fluorescence along the NSM neurite in wild-type animals (F and H) and in wild-type animals expressing the dominant-negative (DN) PFK-1.1 (G532E) (G and I) under hypoxic conditions. The respective penetrance is quantified in (E).

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., 1999; 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 \pm 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 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] of 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: oligo = 1.0 ± 0.047 versus control = 1.1 ± 0.037) (Figure 8G). By contrast, *pfk-1.1* mutants recovered more slowly (time constant > 35 s versus WT = 12 s) and failed to reach full recovery (0.60 ± 0.17 of wild-type plateau amplitude) (Figure 8G). 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 S5A). 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 S5B and S5C). 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 (Knull, 1978; Knull 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 (Laschet et al., 2004). Interestingly, localization of glycolytic proteins to presynaptic sites does not depend on the synaptic vesicle transport protein UNC-104/Kinesin 3/kif1A. 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

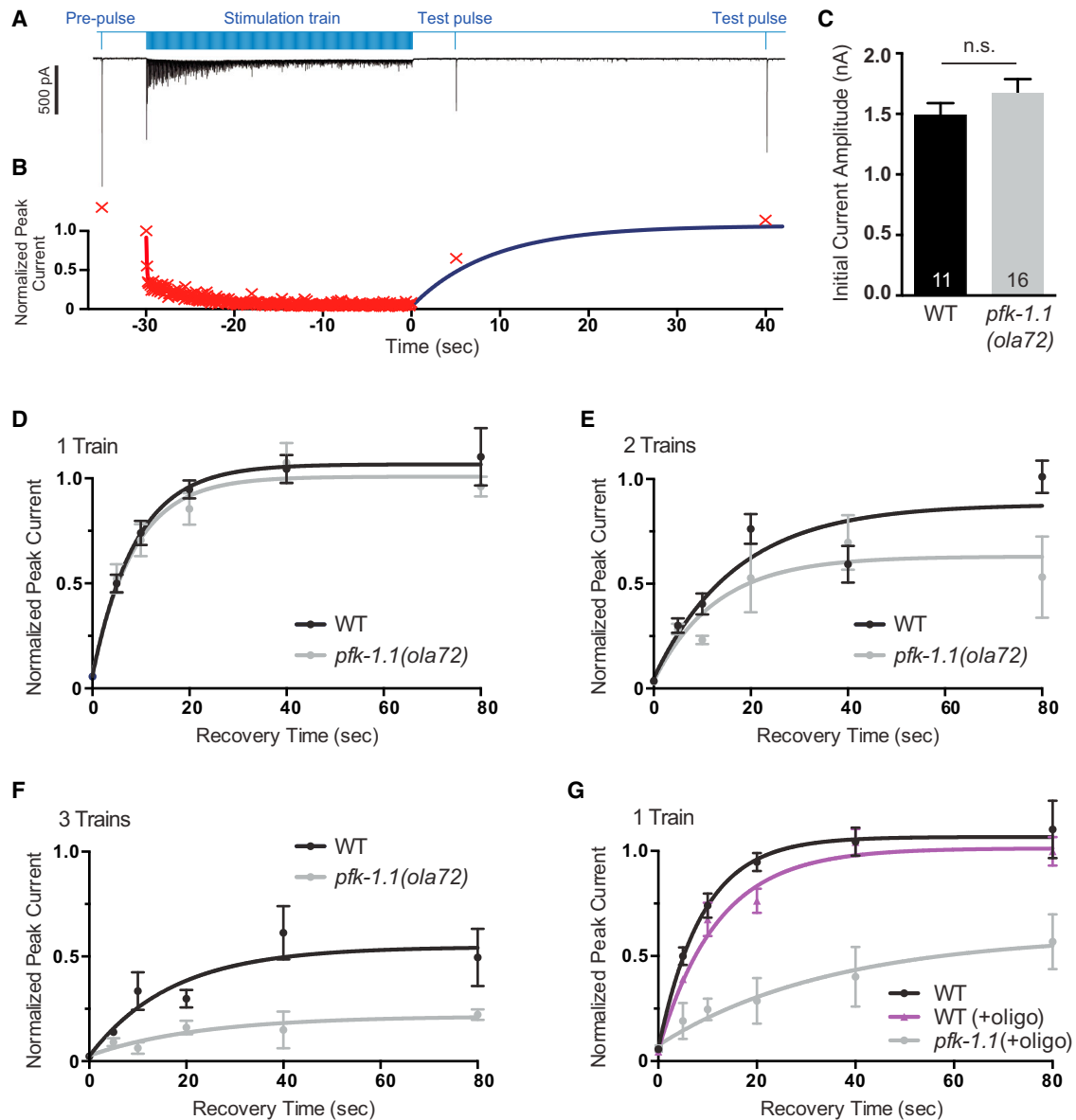


Figure 8. PFK-1.1 Is Required for Synaptic Recovery Following Fatigue

(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 (Burté 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 pre-synaptic 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 μ g/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

Worms were mounted on a slide and exposed to nitrogen gas, carbon dioxide, 10 mM sodium azide (NaN_3), 10 μ M 2-DG, or 1 μ M 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 (*oxIs352*) (Liu et al., 2009) was stimulated with blue light (~ 0.6 mW/mm²) for 5 min.

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, 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 μ 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 μ m interval, respectively). Synaptic enrichment was then calculated as $\Delta F/F$ as previously described (Bai et al., 2010; Dittman and Kaplan, 2006).

Electrophysiology

Worms expressing the channelrhodopsin I/II chimera ChIEF in acetylcholine neurons (*oxSI91[Punc-17::ChIEF]*) and lacking GABA inputs to the muscle (*unc-49(e407)*) 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 μ M 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>.

A video abstract is available at <http://dx.doi.org/10.1016/j.neuron.2016.03.011#mmc5>.

AUTHOR CONTRIBUTIONS

Conceptualization, J.C.N., S.J., and D.A.C.-R.; Methodology, J.C.N., S.J., L.R.-L., and D.A.C.-R.; Formal Analysis, S.J.; Investigation, E.G.B., E.M.J., J.C.N., F.G.T., K.U., L.C., S.J., and L.R.-L.; Writing – Original Draft, J.C.N., S.J., E.G.B., E.M.J., and D.A.C.-R.; Writing – Review & Editing, S.J. and D.A.C.-R.; Visualization, S.J. and D.A.C.-R.; Supervision, Project Administration, and Funding Acquisition, D.A.C.-R.

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