The Role of Mitochondrially Derived ATP in Synaptic Vesicle Recycling**

Received for publication, April 7, 2015, and in revised form, June 19, 2015 Published, JBC Papers in Press, June 30, 2015, DOI 10.1074/jbc.M115.656405

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Background: The ATP requirements of synaptic vesicle release are poorly understood.

Results: Mitochondrially derived ATP supports the function of boutons with and without mitochondria. Respiratory dysfunction selectively blocks the reinternalization of synaptic vesicles.

Conclusion: ATP diffuses rapidly in axons to support synaptic vesicle recycling. Mitochondrial dysfunction decreases synaptic energy and impairs function.

Significance: Understanding energy requirements will help determine how energy failure contributes to neurodegeneration.

Synaptic mitochondria are thought to be critical in supporting neuronal energy requirements at the synapse, and bioenergetic failure at the synapse may impair neural transmission and contribute to neurodegeneration. However, little is known about the energy requirements of synaptic vesicle release or whether these energy requirements go unmet in disease, primarily due to a lack of appropriate tools and sensitive assays. To determine the dependence of synaptic vesicle cycling on mitochondrially derived ATP levels, we developed two complementary assays sensitive to mitochondrially derived ATP in individual, living hippocampal boutons. The first is a functional assay for mitochondrially derived ATP that uses the extent of synaptic vesicle cycling as a surrogate for ATP level. The second uses ATP FRET sensors to directly measure ATP at the synapse. Using these assays, we show that endocytosis has high ATP requirements and that vesicle reacidification and exocytosis require comparatively little energy. We then show that to meet these energy needs, mitochondrially derived ATP is rapidly dispersed in axons, thereby maintaining near normal levels of ATP even in boutons lacking mitochondria. As a result, the capacity for synaptic vesicle cycling is similar in boutons without mitochondria as in those with mitochondria. Finally, we show that loss of a key respiratory subunit implicated in Leigh disease markedly decreases mitochondrially derived ATP levels in axons, thus inhibiting synaptic vesicle cycling. This proves that mitochondria-based energy failure can occur and be detected in individual neurons that have a genetic mitochondrial defect.

Mitochondria likely provide the main source of ATP that supports synaptic transmission, whereas anaerobic respiration from glycolysis contributes much less ATP (1). Yet, surprisingly, less than half of all synaptic boutons in adult rodent hippocampus or in cultured hippocampal neurons contain mitochondria (2, 3). This raises questions about where synaptic ATP originates, how it is distributed among boutons, and how much energy is needed to support synaptic transmission. Dysfunction of axonal mitochondria may also have a critical role in the pathogenesis of neurodegenerative diseases, including Parkinson disease and Alzheimer’s disease. Indeed, multiple neurodegenerative pathways are thought to converge on energy failure, and synaptic terminals degenerate early in many neurodegenerative diseases associated with impaired energy metabolism (4–6). These findings indicate that significant changes in bioenergetic function may occur that are restricted to or occur first in the neuronal processes. In addition, axonal mitochondria have unique properties and exist in distinct microenvironments; thus, it is critical that we first understand the bioenergetics of intact axons. But surprisingly, energy failure has never been proved to occur in dysfunctional or dying disease-affected neurons or in the intact neurons used in genetic disease models (6). Therefore, without knowing the normal energy requirements of neurons or how much energy depletion occurs in disease, we cannot know whether energy failure plays a causative role in degeneration.

Our understanding of neuronal bioenergetics has been limited because the established assays have insufficient resolution (6). Although respiration, glycolysis, and ATP can be measured in whole neuronal cultures, these approaches cannot accurately factor out ATP contributions from glia or provide subcellular spatial resolution. Synaptosome analyses continue to elucidate synaptic bioenergetic functions, but they are challenging in spe-
cific neuronal types (7) and are also subject to artifacts related to the isolation process. Mitochondrial membrane potential can be studied in intact neurons (8), but this is difficult in small axonal mitochondria and provides only indirect bioenergetic information. In addition, the mass and distribution of axonal mitochondria are just as important as their function (6).

To measure mitochondrially derived energy at the synapse, we designed sensitive ATP assays that suppress glycolysis so neurons must rely on mitochondria for energy. Using these assays, we identified the energy threshold needed to support synaptic vesicle cycling and showed that, when ATP levels drop below this threshold, endocytosis is blocked at or before the vesicle scission step. We showed that, to meet these energy needs, ATP rapidly disperses in axons, resulting in near normal ATP levels even in boutons lacking mitochondria. Finally, we showed that loss of a mitochondrial protein known to cause neurodegeneration (Leigh disease) also causes mitochondrially derived energy levels to drop enough in individual neurons to impair synaptic function, thus proving that mitochondrion-based energy failure can occur and be detected in individual neurons with genetic mitochondrial defects.

**Experimental Procedures**

**Molecular Biology**—All constructs were subcloned or cloned into the pCAGGS vector downstream of the chicken actin promoter (9). AT1.03YEMK and AT'R122K/R126K fluorescence resonance energy transfer (FRET) sensors (which use a variant of CFP (mseCFP) as the donor fluorophore and YFP (circularly permuted monomeric Venus) as the acceptor fluorophore, surrounding an ATP-binding protein) were the kind gifts from Hiromi Imamura (Kyoto University) and Hiroyuki Noji (Osaka University) (10). VGLUT1-pHluorin, mCherry-synaptophysin, mitoGFP, and the direct CFP-YFP fusion have been described (14, 15), and Cre recombinase (Cre) was from Addgene. Hiromi Imamura (Kyoto University) and Hiroyuki Noji (Osaka University) (10). VGLUT1-pHluorin, mCherry-synaptophysin, mitoGFP, and the direct CFP-YFP fusion have been described (14, 15), and Cre recombinase (Cre) was from Addgene. VGLUT1-pHluorin, mCherry-synaptophysin, mitoGFP, and the direct CFP-YFP fusion have been described (14, 15), and Cre recombinase (Cre) was from Addgene.

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ATP was used to prepare mouse hippocampal neurons were obtained from E9.5 of pregnant females. Neurons were dissociated using collagenase D (Roche Diagnostics), cultured in Neurobasal media supplemented with B27 and L-glutamine (Gibco Invitrogen), and plated on laminin-coated glass-bottomed microplates (Thoren Science). After 3 days in culture, neurons were transfected with plasmids encoding CFP5 (mseCFP) as the donor fluorophore and YFP (circularly permuted monomeric Venus) as the acceptor fluorophore, surrounding an ATP-binding protein) were the kind gifts from Hiromi Imamura (Kyoto University) and Hiroyuki Noji (Osaka University) (10). VGLUT1-pHluorin, mCherry-synaptophysin, mitoGFP, and the direct CFP-YFP fusion have been described (14, 15), and Cre recombinase (Cre) was from Addgene. Hiromi Imamura (Kyoto University) and Hiroyuki Noji (Osaka University) (10). VGLUT1-pHluorin, mCherry-synaptophysin, mitoGFP, and the direct CFP-YFP fusion have been described (14, 15), and Cre recombinase (Cre) was from Addgene.
were calculated by expressing CFP and YFP individually and then determining the ratios of $I_{\text{FRET}}/I_{\text{CFP}}$ and $I_{\text{FRET}}/I_{\text{YFP}}$, respectively.

**Immunocytochemistry**—Cells were fixed for 15 min in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and immunostained in PBS containing 5% fetal bovine serum and 0.2% Triton X-100. Total neurons were detected using an antibody to MAP2 (Millipore, catalogue no. MAB3418), astrocytes with GFAP (rabbit clone, Sigma, catalogue no. G9269), mitochondria with Tom20 (rabbit, Santa Cruz Biotechnology, catalogue no. SC-11415), and mCherry with DsRED (rabbit, Clontech, catalogue no. 632496).

**Results**

**Substrate Availability Determines Extent of Reliance on Mitochondria for ATP**—Physiologic brain glucose levels ($\approx 1$–1.5 mM (25, 26)) are far lower than those typically used to culture neurons and perform mitochondrial function assays ($\approx 25$–30 mM). Under these high glucose conditions, glycolysis likely compensates for any respiratory deficits and obscures our ability to detect whether and how any respiratory deficits affect neuronal function/survival. Therefore, to develop sensitive assays to measure mitochondrially derived ATP in neurons, we first delineated how the relative ATP contributions of aerobic and anaerobic respiration are influenced by substrate availability. Using a Seahorse instrument, we measured the rates of respiration and glycolysis in E18 hippocampal cultures grown for 11 days. In 30 mM glucose (a supra-physiological concentration often used in cell culture and imaging buffers) basal and maximal OCRs were low, indicating little aerobic respiration (Fig. 1). But when pyruvate (10 mM) was substituted for glucose for 30 min, respiration increased, as did the ratio of OCR to ECAR (Fig. 1D), indicating a greater reliance on aerobic respiration. Importantly, these data reflect bioenergetic changes in neurons specifically, as we both minimized glial content (using embryonic cultures grown in serum-free medium) and used parallel glia-only cultures to correct for the ATP contribution of the remaining glia. As such, our normalized glial OCR calculations show that glia only minimally contribute to the total OCR regardless of substrate (Fig. 1). Therefore, neurons derive more energy from mitochondrial respiration when exposed to pyruvate, but they rely more on glycolysis when exposed only to high glucose levels. So, we hypothesized that to detect the consequences of lower mitochondrial ATP levels, we should use substrates that promote aerobic respiration and minimize glycolysis.

To test this at the synapse, which is the first area to degenerate in several neurodegenerative diseases involving mitochondria (4, 5), we first examined how the substrate affects the ATP-dependent capacity of synaptosomes to maintain low calcium levels (7). We used fluorescence microscopy of the low affinity Ca$^{2+}$ probe fura-4F AM to measure the loss of Ca$^{2+}$ homeostasis in single synaptosomes. Most synaptosomes maintained low resting calcium levels (below the sensitive range of fura-4F; <100 nM [Ca$^{2+}$]) even without substrates in the medium, suggesting that endogenous substrates suffice (Fig. 1E). Glycolysis alone also maintained calcium homeostasis, but inhibiting all glycolysis (by iodoacetate; 250 $\mu$M), oxidative phosphorylation
**Aerobic and Glycolytic ATP Requirements of the Synaptic Vesicle Cycle**—Synaptic vesicle release may be impaired early in diseases that deplete energy (27), but the ATP requirements of the synaptic vesicle cycle are poorly understood. To investigate how much mitochondrially derived ATP is needed for different phases of the synaptic vesicle cycle, we assessed synaptic vesicle release when neurons were exposed to different substrates. For this and all subsequent live imaging experiments, we studied postnatal hippocampal neurons grown in serum-containing media (16–18). These cultures also include glia, and thus better incorporate glia-neuron interactions that likely influence energy metabolism in neurons (28). To monitor the synaptic vesicle cycle in individual boutons, we used the VGLUT1-pHluorin reporter, which targets a pH-sensitive GFP (pHluorin) to the lumen of synaptic vesicles. pHluorin does not fluoresce in an acidified vesicle, but it does fluoresce when a vesicle fuses and its contents are exposed to the alkaline extracellular environment (9). pHluorin fluorescence is then quenched after endocytosis of the vesicle and subsequent reacidification (Fig. 2).

Postnatal hippocampal neurons were incubated either in buffer containing 30 mM glucose to facilitate aerobic respiration and glycolysis or in buffer lacking glucose but containing 10 mM pyruvate to favor reliance on mitochondria. To determine whether the synaptic vesicle cycle could function in the different buffers for a range of activity levels, we exposed neurons to two commonly used electrical field-stimulation paradigms as follows: a longer but lower frequency stimulation (60 s at 10 Hz) that facilitates the preferential release of vesicles in the recycling pool (total pool of vesicles that can undergo activity-dependent synaptic vesicle cycling (Fig. 2, A–C) (29)), or a shorter but higher frequency stimulation (3 s at 30 Hz) that promotes the release of vesicles in the readily releasable pool (vesicles docked and “primed” at the active zone for immediate fusion upon stimulation (Fig. 2, D and E) (16, 30, 31)).

Synaptic vesicle cycling remained intact with either substrate (glucose or pyruvate) with either stimulation paradigm. But without glucose, the rate that the pHluorin signal returned to baseline was slightly impaired with repetitive 60 s at 10 Hz stimulation (Fig. 2A), indicating a small drop-off in endocytosis when glucose was absent. Therefore, because respiration could not fully compensate in this paradigm, some amount of glycolysis was required. These experiments were performed in the presence of glutamate receptor antagonists, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione and 50 μM DL-2-amino-5-phosphonovaleric acid, to eliminate endogenous network activity, but these antagonists had no detectable impact on our assays (data not shown) and were excluded from subsequent experiments.

Neurons do not store glucose in the form of glycogen (32) and cannot replenish their own glucose supply, so synaptic vesicle cycling that occurs in the absence of glucose but with pyruvate is likely supported by mitochondrially derived ATP. To test this, we applied rotenone (500 nM), which inhibits mitochondrial complex I. With rotenone, endocytosis was completely blocked for cells in pyruvate but not for cells in glucose (Fig. 2, B and E). Similarly, the ATP synthase inhibitor oligomycin (2.5 μg/ml) also blocked endocytosis, but only in the absence of glucose (Fig. 2C). This confirms that endocytosis depends on (oligomycin; 1 μg/ml), and creatine kinase (iodoacetamide; 250 μM) disrupted synaptosome calcium regulation, manifesting in a precipitous increase in [Ca2+](>6 μM). This suggests that ATP fell below the threshold required to maintain normal low calcium levels. So, because calcium regulation can be supported by either glycolysis or respiration, we must suppress glycolysis to determine how impaired mitochondrial ATP production affects calcium levels.
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mitochondrially derived ATP when glycolysis is impaired. In contrast, the rate of exocytosis was largely preserved throughout these trials. In particular, exocytosis was fully preserved with 30Hz*3s stimulation targeting the readily releasable pool (Fig. 2E), indicating that little energy is required to release vesicles, especially once primed.

Measuring ATP at the Nerve Terminal—To directly assess the ATP requirements of the synaptic vesicle cycle, we developed an approach to measure ATP in individual synapses. To do this, we obtained ATP FRET sensors (ATeams) (10) that are specifically sensitive to ATP (with no response to ADP, other nucleotide triphosphates, NADH, or dATP) (33, 34) and are resistant to physiologic changes in pH. In HeLa cells, expression of AT1.03YEMK (K_d = 1.2 mM) gave a robust FRET signal that rapidly decreased when aerobic respiration and glycolysis were inhibited with potassium cyanide (KCN, 1 mM) and 2-deoxyglucose (2DG, 10 mM), respectively (Fig. 3A). These interventions did not affect the FRET produced by the AT_{R122K/R126K} dead mutant (does not bind ATP) or the direct fusion of CFP and YFP, indicating that the sensor specifically measures ATP.

To calibrate the sensitivity of AT1.03YEMK to ATP levels in living cells, we did an in-cell calibration. We incubated permeabilized HeLa cells with increasing concentrations of exogenous ATP; the FRET progressively increased between 250 μM and 5 mM ATP (Fig. 3, B and C), which are physiologically relevant ATP concentrations. Because the FRET values plateaued >2 mM, they are most sensitive to ATP changes below ~2 mM. At baseline, AT1.03YEMK is near the top of its dynamic range in HeLa cells but is not fully saturated, as ATP levels drop instantaneously when we add respiratory/glycolytic inhibitors (Fig. 3A).

Based on their similar basal ATP FRET levels and similar FRET of the AT_{R122K/R126K} dead mutant (Fig. 3D), HeLa cells and synaptic boutons appear to have similar basal ATP levels (we estimate ~2–4 mM ATP). Although slightly higher, this is similar to the estimated ATP range recently reported by Ranagaraju et al. (35) (1.4 mM, 10th to 90th percentiles: ≈0.6–2.6 mM), who used a luciferase-based approach. Our additional permeabilization experiments in single synaptic boutons further confirmed that our probes are sensitive to physiologic ATP levels. A precise calibration was not possible though because the required cytosolic buffers and the permeabilization process made many of the synaptic boutons swell, disappear, or merge with adjacent boutons (Fig. 3E). However, these studies confirm that AT1.03YEMK (now simply called the ATP FRET sensor) is sensitive to physiologically relevant changes in ATP levels at the synapse and hence is an appropriate tool to study dynamic changes in ATP at the nerve terminal.

We next used the FRET sensor to monitor ATP in neurons given different substrates. At baseline, basal FRET values were stable in boutons over 25 min of imaging even when both glucose and pyruvate were absent (Fig. 4, A and B); neurons thus had enough substrates to maintain their basal ATP. But when glycolysis was fully blocked, ATP levels quickly dropped, and this was prevented by adding 10 mM pyruvate (a standard dose that enters cells and then mitochondria via monocarboxylate transporters (MCTs) (36)) to fuel respiration, indicating that respiration is required to support basal ATP when glycolysis is absent (Fig. 4C).

Threshold ATP Requirement for Endocytosis—Having developed an approach to monitor ATP levels in synaptic vesicles, we next examined how synaptic vesicle cycling affects presynaptic ATP levels, hypothesizing that neural activity, which increases energy demands, would decrease ATP levels. Indeed, in the presence of glucose and pyruvate, electrical stimulation (10Hz*60s) led to a small but rapid drop in ATP FRET levels that fully recovered within ~5 min (Fig. 5A). Stimulation in pyruvate alone produced a similar decrease in ATP with near complete recovery (Fig. 5B), but the lack of complete recovery suggests that some glycolysis is needed (as in Fig. 2A). A brief 30Hz*3s stimulation did not affect ATP levels when glucose and...
pyruvate were present, but in pyruvate alone it produced a surprisingly greater drop in ATP with no recovery (Fig. 5, A and B), suggesting that this stimulation paradigm either failed to trigger compensatory synthesis of ATP (35) and/or impaired mitochondria’s ability to synthesize ATP. Overall, these findings show that our ATP assay is sensitive enough to detect the energy requirements of physiologic stimuli, although we cannot exclude the possibility it would be even more sensitive if we could normalize for the total adenine nucleotide pool size (37).

Indeed, ATP levels can be maintained by redistribution of the adenine nucleotide pool (38), although we know very little about the concentrations and kinetics of interconversion of adenine nucleotides within individual neurons and their subcellular compartments (39).

To confirm that the drop in FRET specifically reflected energy requirements of synaptic vesicle cycling and was not an artifact of electrical stimulation, we examined ATP levels in the absence of calcium (no-calcium buffer with EGTA). Here, electrical stimulation did not affect ATP levels (Fig. 5C), which corroborates recent data suggesting that synaptic vesicle cycling is the greatest user of ATP at the synapse (35).

The pHluorin experiments (Fig. 2) show that, unlike the final stages of exocytosis, endocytosis has a high ATP dependence. Because blocking mitochondrial ATP production caused

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**FIGURE 3. Calibration of ATP sensors in cells.** A, FRET emission of HeLa cells expressing AT1.03YEMK (ATP FRET sensor, YEMK, black line), AT^R122K/R126K (R122K, a FRET sensor unresponsive to ATP (10), red line), or a direct CFP-YFP fusion control (blue line). Simultaneously inhibiting glycolysis with 2DG (10 mM) and oxidative phosphorylation with KCN (1 mM), indicated by the arrow, rapidly decreases relative ATP levels of ATP YEMK without affecting R122K. n = 4–6 coverslips/group, 3–6 cells/coverslip. Data are mean ± S.E. B and C, calibration of ATP FRET sensors in permeabilized cells. HeLa cells expressing AT1.03YEMK or the AT^R122K/R126K dead mutant were permeabilized with XF-PMP reagent (1 nM), and then ATP at defined concentrations was added. FRET was measured by microscopy, and the curve was fitted using a Hill equation. Data show mean ± S.D. Scale bar, 5 μm. D, comparison of raw FRET of ATP FRET sensor and dead mutant in intact HeLa cells and hippocampal boutons. E, hippocampal neuron boutons expressing the ATP FRET sensor were permeabilized by incubating in 1.25 nM XF-PMP reagent for 4 min and then bathed in 5 mM ATP. The FRET signal decreases markedly upon permeabilization and then returns upon exposure to ATP (arrows). However, during permeabilization, many boutons either detach or change shape/location (yellow arrows). In addition, the cytosolic buffer causes many boutons to swell rapidly, even before the permeabilization reagent is added. The fluorescence of the acceptor (Venus) protein in the ATP FRET sensor is unchanged. Scale bar, 5 μm.
pHluorin fluorescence to remain high after exocytosis (Fig. 2), energy depletion may have blocked endocytosis at or before vesicle scission or, in a subsequent step, prevented vesicles from becoming re-acidified. To distinguish between these possibilities, we first examined the ATP requirements of synaptic vesicle reacidification, a known energy-requiring process (40). However, bafilomycin (which blocks the reacidification of vesicles, Fig. 6A) did not affect the rate or extent of decrease in mitochondrially derived ATP produced by prolonged stimulation (10Hz*150s) (Fig. 6B), indicating that vesicle reacidification is not the primary ATP-consuming process.

To determine whether the block may instead occur proximal to reacidification at the vesicle reinternalization step, we next acutely added MES (pH 5.5) to see whether it quenched pHluorin fluorescence when endocytosis was blocked (Fig. 6C). We hypothesized that MES would not quench fluorescence if vesicles were blocked at the re-acidification stage because the vesicles would have already been internalized and therefore not accessible to MES. In contrast, MES would rapidly quench fluorescence if the block prevented vesicle scission. When we used rotenone to block endocytosis, MES rapidly quenched the increased pHluorin fluorescence, indicating that endocytosis requires high ATP at or before vesicle scission.

After establishing the stage at which synaptic vesicle cycling is blocked during energy failure, we used our ATP assay to define how much ATP is required for endocytosis (the ATP threshold). Defining this threshold would allow us to determine whether it is breached in models of neuronal dysfunction, which is critical for understanding whether energy failure truly impairs function and contributes to pathogenesis. We began defining this energy threshold by subjecting neurons to the same electrical stimulation patterns and non-glucose conditions that forced reliance on mitochondrially derived ATP in Fig. 2, B and E. Consistent with these results, the acute addition of rotenone decreased ATP levels faster in groups lacking glucose, especially with stimulation (Fig. 6, D and E), presumably because neurons could not adequately compensate with glycolysis. Therefore, after acutely blocking energy production, we found that the ATP threshold required to sustain endocytosis resides between the ATP level with and without glucose (Fig. 6, D and E). Based on our calibration in HeLa cells (Fig. 3), we estimate this ATP threshold to be ≈0.8 mM.
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Diffusion Keeps Mitochondrion-derived ATP above the ATP Threshold for Synaptic Transmission, Even in Boutons Lacking Mitochondria—At the nerve terminal, energy failure may result from either intrinsic deficits in mitochondrial function or changes in the mass and/or distribution of mitochondria at the synapse. But even under normal conditions, only ~39% of hippocampal boutons in our cultures contain mitochondria (109 boutons sampled from 10 coverslips), consistent with the ~40% estimated by prior EM studies in vivo (2, 3). Thus, we asked whether ATP levels are lower in boutons without mitochondria. If so, do they meet the ATP threshold to perform synaptic vesicle cycling?

To test this, we first compared mitochondrially derived ATP levels in boutons with and without mitochondria, identified using mito-FARRed fluorescence, and imaged in pyruvate without glucose to force reliance on mitochondrially derived ATP (Fig. 7A). Notably, roughly 30% of axonal mitochondria are moving in our rat neuronal cultures (41). Therefore, to determine the impact of synaptic mitochondria on ATP, we only compared those boutons with or without stationary mitochondria. Specifically, boutons were considered to contain a stationary mitochondrial if the mitochondrion was present in the bouton at the beginning and end of the imaging period. Indeed, among these boutons, all of the mitochondria did indeed remain stationary in the bouton throughout the 33-min imaging period (n = 25, five boutons/coverslip). Synaptic transmission was examined in sparsely transfected hippocampal cultures to unambiguously identify individual axons and their mitochondria. Synaptic vesicle cycling was equivalent in boutons with and without mitochondria (Fig. 7E). When we compared mitochondrially derived ATP levels within the same axon (a more sensitive approach), we found that ATP levels were

FIGURE 6. Acute block of respiratory function decreases mitochondrially derived ATP below the threshold level for endocytosis. A, acute treatment with bafilomycin (1 μM) inhibits the reacidification of synaptic vesicles after extended stimulation (10 Hz * 150 s), blue bar, in A and B), assessed with VGLUT1-pHluorin, and performed in pyruvate without glycolysis (2DG and IAA) to force reliance on mitochondrially derived ATP. n = 6 coverslips/group, with 10–16 boutons/coverslip. Data show mean ± S.E. ****, p < 0.0001 versus no bafilomycin control group by unpaired two-tailed t test for extent of endocytosis (amplitude endocytosis/ amplitude exocytosis)). B, inhibition of reacidification by bafilomycin does not prevent the decrease in ATP levels as compared with the no-bafilomycin control group, indicating that this step of vesicle cycling requires comparatively little energy.

FIGURE 7. Mitochondrially derived ATP diffuses from adjacent boutons to maintain synaptic function in boutons lacking mitochondria. A, individual synaptic boutons labeled by ATP sensor (Venus channel), with and without mitochondria, identified by mito-FARRed fluorescence. The axon has a bouton containing a mitochondrion (mito, arrow) next to two boutons without mitochondria (*). Scale bar, 2 μm. B, in total, ATP FRET levels did not differ significantly between boutons with and without mitochondria, despite being imaged in pyruvate (no glucose) to minimize glycolysis. NS (p = 0.52) by unpaired two-tailed t test. C, without glucose, ATP levels were also examined in adjacent boutons of sparsely transfected hippocampal cultures to unambiguously identify individual axons and their mitochondria. In ~60% of cases, boutons with mitochondria had slightly higher ATP (mito bouton higher ATP) than adjacent boutons without mitochondria in the same axon n = 15–16 coverslips (15–20 boutons/coverslip, p < 0.05 by binomial test). D, electrical field stimulation (10 Hz * 60 s) decreased ATP similarly in boutons with and without mitochondria. Data show mean ± S.E., n = 15–16 coverslips/group, 15–20 boutons/coverslip with and without mitochondria. E, VGLUT1-pHluorin fluorescence of neurons in pyruvate (10 mM; no glucose) subjected to electrical field stimulation (10 Hz * 60 s, blue line). Synaptic transmission was examined in sparsely transfected hippocampal cultures to unambiguously identify individual axons and their mitochondria.

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usually only slightly lower in adjacent boutons without mitochondria (Fig. 7C). Even after multiple stimulations to increase the energy requirements, ATP levels dropped similarly in boutons with and without mitochondria (Fig. 7D).

Thus, under normal conditions, ATP or ATP equivalents (42) diffuse rapidly into adjacent boutons to support synaptic function. These data suggest that boutons with and without mitochondria have similar amounts of mitochondrially derived ATP to support synaptic vesicle release. To confirm this, we compared synaptic vesicle cycling in boutons with and without mitochondria (again imaged in pyruvate without glucose to force reliance on mitochondrially derived ATP). As expected, synaptic vesicle cycling was identical in these boutons, and inhibiting respiration with rotenone blocked endocytosis similarly in boutons with and without mitochondria (Fig. 7E).

Importantly, we identified mitochondria by their co-expression of mito-mTagBFP, and all mito-mTagBFP-positive structures tested (30 out of 30) were immunopositive for the mitochondrial marker Tom20. This approach ensured that we considered only mitochondria within transfected neurons, which was essential for definitively determining that mitochondria were present within a given axon (43). To prevent false-negatives (i.e. to make sure all mitochondrial-negatives boutons truly lacked mitochondria), we optimized transfections so that the axons expressing VGLUT1-pHluorin also expressed our mitochondrial reporter (96% of pHluorin-positive axons also expressed mito-mTagBFP; n = 49 individual axons quantified). To further ensure that we accurately categorized boutons, we also examined sparsely transfected neurons. These experiments on single axons again showed that synaptic transmission was identical in boutons with and without mitochondria (Fig. 7F). Overall, we found that mitochondrially derived ATP levels in boutons without mitochondria exceed the ATP threshold required for synaptic vesicle cycling, and the small difference in mitochondrially derived ATP between these boutons has little functional consequence under the conditions tested. Thus, boutons without mitochondria have a similar capacity for synaptic vesicle cycling as those with mitochondria. Also, when energy requirements cannot be met, energy failure occurs similarly in boutons with and without mitochondria, at least when the boutons are normally distributed.

**Chronic Impairment in Mitochondrial Function Can Produce Energy Failure in Individual Neurons**—In neurodegenerative diseases, mitochondrial function is chronically impaired, and hence, the requirement for mitochondrially derived ATP for synaptic transmission may differ from that after an acute respiratory insult. However, despite being a central hypothesis in neurodegeneration (6), no study has proved that ATP decreases in individual neurons in any model of neurodegeneration. We do not know whether and when this ATP drop occurs, or whether it is enough to compromise function. To determine how a chronic deficit in mitochondrial function affects mitochondrially derived ATP, we cultured hippocampal neurons from floxed Ndufs4 mice (19). The NDUFS4 protein, NADH dehydrogenase (ubiquinone) iron-sulfur protein 4, is an essential subunit of mitochondrial complex I. Deleting this gene produces devastating neurologic consequences in humans (44) and mice (19, 45) and severely impairs respiration in mesencephalic neurons (46). However, cultured dopaminergic neurons that lack NDUFS4 due to Cre-mediated gene inactivation have normal survival (46, 47). Therefore, in standard culture conditions, even a severe mitochondrial deficit may be obscured, suggesting that standard culture paradigms are insensitive to the toxicity of mitochondrial-based energy failure. To test this, we co-transfected floxed Ndufs4 hippocampal neurons with Cre (to delete NDUFS4), mCherry-synaptophysin, and either the ATP FRET sensor or VGLUT1-pHluorin. We previously optimized our transfection conditions so >90% of axons that express these reporter probes also express Cre.

In standard high glucose conditions, deleting NDUFS4 in hippocampal neurons did not affect basal synaptic ATP FRET levels (controls, 2.74 ± 0.79; NDUFS4-depleted cells, 3.14 ± 0.34), suggesting that the neurons compensated with glycolysis. Surprisingly, in the acute absence of glucose, there was also no difference in response to an initial 10Hz*60s stimulation (Fig. 8A), suggesting that neurons with chronic respiratory defects may up-regulate their glycolytic capacity and/or endogenous glucose stores. However, after we added glycolytic inhibitors (2DG and iodoacetic acid (IAA)) to fully block glycolysis, ATP levels in NDUFS4-depleted boutons dropped more rapidly in response to a second electrical stimulation (Fig. 8A). Cre expression alone did not affect ATP levels in control neurons...
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(Fig. 8B), confirming that NDUFS4 loss is responsible for the drop in ATP. To determine whether mitochondrial-derived ATP drops enough in NDUFS4-depleted neurons to impair endocytosis, we examined synaptic vesicle cycling in floxed Ndufs4 mice. Indeed, NDUFS4-depleted boutons had impaired endocytosis (Fig. 8C), mimicking the effects of acute complex I inhibition on control neurons in the absence of glucose (Fig. 2). Therefore, mitochondrial-derived ATP levels are indeed compromised when NDUFS4 is deleted, and they drop enough to impair synaptic transmission. This establishes that mitochondrial-based energy failure can occur in individual neurons in a genetic model of neurodegeneration.

Discussion

To determine whether changes in mitochondrial bioenergetics contribute to neurodegeneration (6), we must first understand the normal energy requirements for neuronal function and survival and determine whether and how energy levels change when mitochondria are compromised. Using new assays that provide complementary measures of mitochondrial ATP at the nerve terminal, a region that is compromised early in neurodegeneration (4, 5), we define the energy thresholds needed to sustain different phases of the synaptic vesicle cycle, and we show that under normal conditions enough mitochondrial ATP disperses rapidly between boutons to support synaptic function, even in boutons that lack mitochondria. But we also show that acutely or chronically inhibiting the respiratory chain drops mitochondrial ATP levels below the threshold needed to sustain endocytosis, especially when energy consumption is increased by greater neural activity. Thus, a chronic mitochondrial deficit that arises from losing a key mitochondrial protein, NDUFS4, which is lost in the neurodegenerative disorder Leigh disease (44), can indeed cause energy failure in individual neurons.

Normal Requirement and Dispersal of ATP at the Nerve Terminal—In neurons, most ATP is likely dedicated to supporting synaptic transmission. However, the energy requirements of the synaptic vesicle cycle are poorly understood. Our studies show that endocytosis is far more sensitive to mitochondrial ATP levels than exocytosis. Furthermore, we found that re-acidification is not the key energy-requiring step, consistent with recent theoretical estimates of the ATP requirements of synaptic vesicle reacidification (48). Instead, the vesicle scission phase of endocytosis has high energy requirements, consistent with studies that showed ATP is required for the scission of coated pits into sealed vesicles in mammalian cell lines (49) and for membrane fission and/or retrieval in goldfish bipolar neurons (50). Indeed, attenuating endocytosis may help neurons preserve ATP when needed, as synaptic vesicle cycling likely uses much of the ATP at the synapse (Fig. 5 (35)). Further studies will be required to determine specifically how low ATP limits vesicle scission. In contrast to endocytosis, we show that vesicle release from the readily releasable pool requires little ATP, which is consistent with studies in permeabilized endocrine cells and goldfish retinal bipolar neurons, showing that once “primed,” vesicle release is independent of ATP but depends on Ca²⁺ (51). Although endocytosis is far more sensitive to decreased energy than exocytosis, some ATP is still needed to facilitate exocytosis, presumably in part due to the ATPase activity of the N-ethylmaleimide-sensitive factor (NSF), which is required before vesicle fusion (52).

To sustain the synaptic vesicle cycle, ATP must first reach the nerve terminals, which are often located far from the cell body. Although the cell body’s mitochondria may produce some ATP that diffuses to the nerve terminal as phosphocreatine (42), long distance energy transport probably does not contribute much energy at the synapse; in fact, it would make neurons unable to rapidly adjust their energy production to meet local needs. Instead, regional mitochondria in axons probably supply most of the energy at the synapse (53). Indeed, we show that when mitochondria are normally distributed and functioning, enough mitochondrial derived ATP can diffuse to maintain normal function even in boutons that lack mitochondria. This does not preclude the possibility that regional gradients of glycolytically derived ATP exist within axons to fuel specialized functions such as axonal transport (54), but such gradients are beyond the detection limit of our assay. These findings thus offer some of the first insights into how boutons that lack mitochondria function. Also, we show that when energy requirements are increased sufficiently, energy failure occurs to a similar extent in boutons with and without mitochondria, even under conditions where the boutons are forced to rely on their mitochondria for ATP. Nonetheless, more sustained increases in energy demands may still preferentially deplete ATP from boutons lacking mitochondria. Indeed, after multiple rounds of electrical stimulation, Sun et al. (55) found that the ATP/ADP ratio decreased more in boutons lacking mitochondria, despite having glucose present. In addition, we do not yet know whether regional energy failure between boutons also develops if the distance between mitochondria is pathologically increased (6).

Energy Failure at the Synapse in Neurodegeneration—Defects in mitochondrial bioenergetics likely influence the pathogenesis of many neurodegenerative diseases, including Parkinson disease, Huntington disease, and Alzheimer disease (6). Energy status at the synapse may be particularly important because in most neurons most mitochondria likely reside in axons (56), and degeneration begins in this compartment (4, 5). Even before synapses are lost, insufficient ATP may contribute to early disease-related changes in synaptic transmission (27, 57). However, energy failure has never been directly proven in affected neurons in these diseases or even in intact neurons in genetic models of these diseases, leaving the central tenet of the bioenergetic hypothesis unproven (6, 22).

The recent emergence of fluorescent sensors for energy metabolites allows us to interrogate energy status on a single-cell basis (10, 58); here, we show that this technology can be applied to individual living neurons and their synapses. Our results show that chronic, genetic loss of mitochondrial function can lead to energy failure, although energy failure will only occur when glycolysis is limited and neurons are forced to rely more heavily on their mitochondria, as likely occurs in vivo (6). In the brain, glucose levels are far lower than in standard neuronal culture (25, 26), and neurons in vivo likely derive a greater proportion of their energy from aerobic respiration. Much of this ATP may be generated from lactate produced by adjacent
glial cells and then imported into neurons via the astrocyte-neuron lactate shuttle, where it is converted into pyruvate (28, 59). However, recent evidence suggests that neurons (not astrocytes) directly metabolize much of the glucose entering the brain (60, 61), suggesting that glycolysis may actually contribute more to supporting neuronal energy needs than previously suspected. Importantly, we must better understand the role of energy failure in neurodegeneration because mitochondria have other functions; they buffer calcium, produce reactive oxygen species, synthesize lipids, and regulate apoptosis (62). How these other functions affect synapses is even less well understood than bioenergetics. If a given disease mutation does not violate a neuron’s energy threshold, then energy failure is probably not the main way the mutation produces degeneration. Conversely, if energy levels dip below the threshold, insufficient energy may help cause degeneration (6). As such, our assays may eventually be used to test therapeutic interventions that restore energy levels.

Author Contributions—D. P., L. S., B. A. M., A. A. G., M. D. B., R. H. E., and K. N. designed research. D. P., L. S., B. A. M., D. H., W. L., A. A. G., and K. N. performed research. D. P., L. S., B. A. M., D. H., A. A. G., H. K., and K. N. analyzed data. D. P., L. S., B. A. M., A. A. G., and K. N. wrote the paper.

Acknowledgments—We thank Richard Palmiter (University of Washington, Seattle) for the floxed Ndufs4 mice and for critically reviewing our manuscript. We thank Gary Howard and Celeste Brennecka for helping edit the manuscript, and the S. D. Bechtel Junior Foundation for their gift of a Seahorse Analyzer to the Gladstone Institutes.

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