

Flow of energy in the outer retina in darkness and in light

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Structural features of neurons create challenges for effective production and distribution of essential metabolic energy. We investigated how metabolic energy is distributed between cellular compartments in photoreceptors. In avascular retinas, aerobic production of energy occurs only in mitochondria that are located centrally within the photoreceptor. Our findings indicate that metabolic energy flows from these central mitochondria as phosphocreatine toward the photoreceptor's synaptic terminal in darkness. In light, it flows in the opposite direction as ATP toward the outer segment. Consistent with this model, inhibition of creatine kinase in avascular retinas blocks synaptic transmission without influencing outer segment activity. Our findings also reveal how vascularization of neuronal tissue can influence the strategies neurons use for energy management. In vascularized retinas, mitochondria in the synaptic terminals of photoreceptors make neurotransmission less dependent on creatine kinase. Thus, vascularization of the tissue and the intracellular distribution of mitochondria can play key roles in setting the strategy for energy distribution in neurons.

energy metabolism | phototransduction

A significant energy distribution problem can arise from the relative locations of mitochondria, ion pumps, and synapses in neurons. In photoreceptors, ion pumps occupy the intervening space between the centrally located mitochondria and the synaptic terminal. Ion pumping in dark-adapted photoreceptors consumes ~20× more energy than neurotransmission (1). Therefore, the pumps could intercept all the metabolic energy made by the mitochondria before it can reach the synaptic terminal. In the vascularized retinas of mice, rats, and humans (2–4) this problem is solved by the presence of additional mitochondria in the terminal. However, in the avascular retinas of zebrafish, salamanders, rabbits, and guinea pigs there are no mitochondria in the terminals (2, 4, 5), which creates a need to partition some of the energy made by the central mitochondria into a protected form that can bypass the ion pumps to support the essential energy demands of the synaptic terminal.

Energy consumption within retinal photoreceptors is compartmentalized and light-dependent. During illumination, phototransduction and light adaptation consume energy in the outer segment (OS). In darkness, energy is consumed by ion pumps in the inner segment and by glutamate release at the synaptic

terminal (1). Energy demands and O₂ consumption are far greater in darkness than in light (1, 6–8).

Metabolic energy is distributed in most cells as either ATP or phosphocreatine (PCr). There are 2 isoforms of creatine kinase (CK) in neurons, ubiquitous mitochondrial creatine kinase (uMtCK), and brain-type cytoplasmic creatine kinase (CK-B). uMtCK creates PCr from ATP at mitochondria (9), and CK-B can recreate ATP from PCr at sites of energy demand. In this way uMtCK and CK-B can collaborate to transfer metabolic energy between neuronal compartments (10, 11).

This paper describes how photoreceptor neurons solve the energy distribution problem. We found, unexpectedly, that CK-B in photoreceptors is sequestered in the synaptic terminal. We report evidence that metabolic energy flows as PCr to the terminal in darkness. In light, it flows as ATP to the OS.

Results

Distribution of Mitochondria in Photoreceptors Depends on Vascularization. A comparison of synaptic terminals in some of the types of retinas we studied, mouse, salamander, and zebrafish, is shown in Fig. S1. Mitochondria are abundant in mouse photoreceptor terminals but absent from salamander and larval zebrafish terminals. This observation confirms previous findings that mitochondria infiltrate photoreceptor terminals in the vascularized retinas of mice and rats (2–4), but they are absent from the terminals in the avascular retinas of salamanders and zebrafish (2, 4, 5).

CK-B and uMtCK Are the only Sources of CK Activity in Mouse Retina. We used knockout mice to identify the enzymes that contribute CK

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activity to the retina. The immunoblot in Fig. 1*A* confirms that uMtCK and CK-B are present in WT mouse retinas (12, 13). The blot also shows that CK-B is absent in retinas from CK-B^{-/-} mice (14) and that both isoforms are missing in retinas from CK-B^{-/-};uMtCK^{-/-} (“CK^{-/-} dko”) mice (15). We found that normal WT mouse retina homogenates have 2.0 nmol/min of CK activity per retina. Homogenates of CK-B^{-/-} retinas have ~5% and CK^{-/-} dko retinas have <1% of normal activity (Fig. 1*B*).

CK-B is Confined in Photoreceptors to the Synaptic Terminal. The upper left panel of Fig. 1*C* shows a section of a light-adapted mouse retina probed with an antibody to mammalian CK-B. Labeling in photoreceptors is strongest at the terminal and weakest in the OS. Immunoelectron microscopy provided additional evidence that CK-B is absent from the OS (Fig. S2*E*) (12). There is little difference in the distribution of CK-B in light vs. dark (Fig. 1*D*). Double labeling with an antibody to synaptotagmin, a presynaptic marker, showed that CK-B is present in both pre- and postsynaptic regions of the outer plexiform layer (Fig. 1*E*). Antibody specificity was confirmed by using CK-B^{-/-} retinas (compare upper and lower panels of Fig. 1*C*).

To determine if there is any CK-B in other parts of the photoreceptor besides the terminal, images were intentionally overexposed. Under conditions where the CK-B signal in the terminals is well into saturation, some CK-B immunoreactivity could be detected throughout the photoreceptor (Fig. S2).

Analyses of serial retina sections confirmed this distribution in rat and salamander retinas. Flattened rat retinas were frozen, and 10- μ m serial sections were cut and analyzed by immunoblotting (16) with antibodies that recognize specific isoforms of mammalian CK. The specificity of these antibodies is shown in Fig. 1*A*. Synaptotagmin (synaptic terminals), rhodopsin (OS), and uMtCK

(mitochondria) served as landmarks. The results (Fig. S3*A*) confirm that CK-B is absent from OSs and enriched in the photoreceptor synaptic layer of this vascularized retina. The distribution of CK activity in rat serial sections (Fig. S3*B*) also is consistent with this localization. Antibodies to CK from avascular retinas were not available, so we instead measured CK activity. Flattened dark-adapted salamander retinas were frozen, and 5- μ m serial sections were cut. Fig. S4*D* shows the distribution of CK activity in the salamander retina compared to the distributions of DNA (Fig. S4*B*), COX (Fig. S4*C*), and rhodopsin (Fig. S4*E*). A cross-section of a salamander retina is shown in Fig. S4*A* for orientation. CK activity in the salamander retina is lowest in the OS layer and highest in the ellipsoid and synaptic layers.

Model for Energy Distribution Within Photoreceptors. CK-B is abundant in the synaptic layer and absent from OSs. Therefore, the photoreceptor terminal, but not the OS, can convert PCr into ATP. This observation suggested a model for how energy is distributed in photoreceptors (Fig. 2). In light, energy flows from the cell body to the OS as ATP, but in darkness, it flows to the terminal as PCr. The model predicts that CK activity would not be required in light, whereas in darkness CK activity would be required for energy to flow to the synaptic terminal. Our model also predicts a more substantial need for CK activity in avascular retinas than in vascularized retinas, which have mitochondria within their synaptic terminals.

1 Fluoro 2,4 Dinitrobenzene (FDNB) Is a Specific Inhibitor of CK. We tested this model by using a cell-permeant inhibitor to alter CK activity. At low concentrations, FDNB specifically modifies a reactive cysteine on CK and inhibits the activity of CK (17, 18). Fig. S5*A* shows that 10 μ M FDNB inhibits >95% of

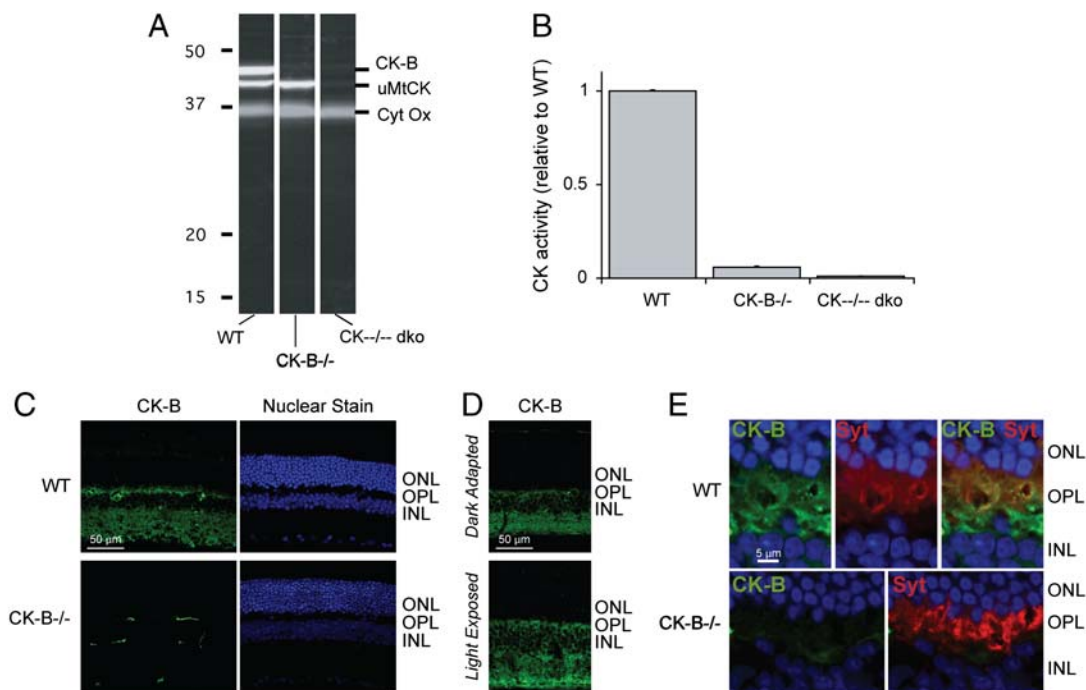


Fig. 1. Identification of the types of CK in mouse retina. (A) Immunoblot of retina homogenates from WT, CK-B^{-/-}, and CK^{-/-} dko mice. The blots were probed with antibodies to CK-B and uMtCK. The pattern of labeling demonstrates the specificity of the antibodies. (B) CK activity in retinal homogenates from WT, CK-B^{-/-}, and CK^{-/-} dko mice. The absence of CK activity in the dko retinas shows that no other active CK isoforms of CK are expressed at significant levels in retina. (C) CK-B antibody immunostaining (Top) in WT (Top) and in CK-B^{-/-} knockout (Bottom). There is intense labeling in the photoreceptor synapse layer. The signals in the CK-B^{-/-} retina come from the secondary antibody reacting with an unknown antigen in the retinal capillaries. The panels on the right show nuclear staining to demonstrate that there is no retinal degeneration in CK-B^{-/-} retinas. (D) Comparison of the distribution of CK-B in light- vs. dark-adapted mouse retinas. (E) Pre- and postsynaptic localization of CK-B in the photoreceptor terminal layer. Sections were double labeled with antibodies to CK-B (Green) and to a presynaptic marker, synaptotagmin (Red). The top three panels show the outer nuclear layer (ONL), outer plexiform layer (OPL), and inner nuclear layer (INL) of a WT mouse retina. The bottom two panels show labeling of a CK-B^{-/-} retina.

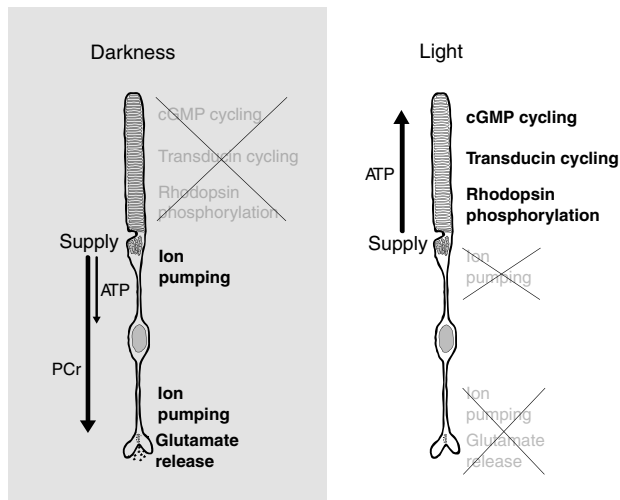


Fig. 2. Model for energy distribution within photoreceptors. In darkness, metabolic energy is consumed primarily in the inner compartments of the photoreceptor. ATP produced by the central mitochondria is consumed by ion pumps before it can reach the synaptic terminal. The mitochondria also use uMtCK to make PCr, which is not a substrate for the pumps. The PCr bypasses the pumps and diffuses to the synaptic terminal where CK-B, which is localized specifically to the terminal, converts PCr back into ATP to support synaptic transmission. In light, energy consumption shifts to the OS where ATP meets the energy demands of phototransduction. The model shown is for avascular retinas. In vascularized retinas, there are additional mitochondria at the synaptic terminal that can produce ATP directly within the terminal.

CK activity in mouse retina homogenates. The same amount of FDNB does not affect cGMP-gated channel activity (Fig. S6 A and B), cGMP levels (Fig. S6C), high-energy nucleotide levels (Fig. S7 A–C), O₂ consumption (Fig. S7D), or glucose uptake (Fig. S7E). These tests confirm that FDNB is a specific inhibitor of CK activity.

FDNB Does Not Affect Energy-Dependent Activities in the OS. The model in Fig. 2 predicts that energy-dependent functions in the OS should be insensitive to FDNB. Processes in the OS that consume energy include phosphorylation of rhodopsin, reduction of *all-trans* retinal, and phototransduction. FDNB does not affect any of these OS activities (Figs. S5 B–D and S7F).

Effect of FDNB on the Dark Current. If the model in Fig. 2 is correct, then energy-dependent activities in the terminal ought to be sensitive to FDNB. The dark current is a flow of ions into the OS, but it is sustained by ATP-dependent pumps in the inner segment and terminal (1, 19). The dark current is reduced ~50% by 10 μM FDNB in both salamander (Fig. S5E) and mouse (Fig. S5F) rods. Partial inhibition of pump activity is consistent with the model in Fig. 2. Pumps in the cell body may use ATP directly, whereas those in the terminal may be more dependent on PCr.

Effects of FDNB on Synaptic Transmission. Our model predicts that synaptic transmission would be especially sensitive to inhibition of CK, particularly in avascular retinas. The synaptic terminal requires energy to refill and recycle vesicles, and it contains the highest concentration of CK-B in the cell. We tested whether FDNB influences components of the electroretinogram (ERG) that depend on synaptic transmission. Larval zebrafish eyes were used because their retinas are avascular and because they produce robust on and off ERG responses (20). The larval eye is permeable to small molecules such as FDNB (21). Fig. 3A (Black Trace) shows ERG responses of dark-adapted eyes from six day postfertilization (dpf) larvae exposed to a 1-sec step of bright light. When light suppresses glutamate release, the synapse is cleared by a glutamate transporter (22). Removal of glutamate depolarizes

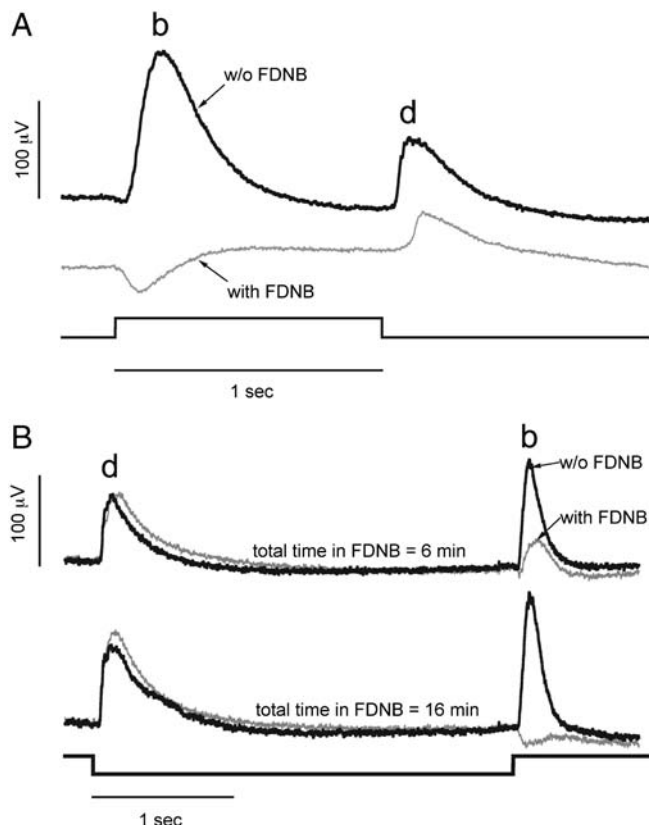


Fig. 3. Darkness enhances the effect of FDNB on synaptic transmission. (A) ERG responses of eyes from zebrafish larvae to a step of intense white light. The eyes were treated either with control solution (Top Trace) or with the same solution including 20 μM FDNB (Bottom Trace). When the eyes are dark-adapted the on response, “b wave,” and the off response, “d wave,” are eliminated by FDNB. (B) Light adaptation reduces the effect of FDNB. Light-adapted zebrafish either were treated with control solution (Black) or with the same solution including 20 μM FDNB (Gray). Eyes were exposed to constant illumination during isolation. Then they were exposed to a pulse of darkness every 30 sec. Responses to the pulses of darkness at 6 and 16 min after the beginning of FDNB treatment are shown. Off responses are less sensitive to FDNB when the eyes are maintained under nearly continuous illumination.

on bipolar cells (BPCs) and hyperpolarizes off BPCs, a response that contributes to the ERG signal known as the “b wave.” Returning the eye to darkness restores glutamate release, which hyperpolarizes on BPCs, depolarizes off BPCs, and contributes to the “d wave.” b and d waves represent combined BPC responses superimposed on field potentials from suppression and restoration of photoreceptor dark current (23). We used 2-amino-4-phosphonobutyrate and 6-cyano-7-nitroquinoxaline-2,3-dione to confirm that in the eyes of zebrafish larvae suppression of synaptic transmission leaves only the photoreceptor field potential contribution of the ERG (Fig. S8). We found that FDNB treatment in darkness similarly eliminates all of the components of both b and d waves associated with synaptic transmission (Fig. 3A, Grey Trace). Only the changes in field potential associated with the photoreceptor dark current remain. This result is consistent with FDNB choking off the flow of energy that supports glutamate release in darkness.

Light reduces energy demand by suppressing the dark current and glutamate release. We hypothesized that the decreased energy demand during illumination would eliminate or reduce the need for CK activity. We tested this idea by analyzing effects of FDNB on light-adapted ERG responses. We dissected zebrafish larval eyes in bright light and continued to illuminate them during FDNB treatment. After 5 min of constant illumination, we recorded responses to 3-sec pulses of darkness once every 30 sec. The traces in Fig. 3B show that d waves (off responses)

HCs Reliably Report Glutamate Release from Photoreceptors. Because we relied on HCs to report glutamate release, we made sure that FDNB does not affect the ability of HCs to respond to stimulation (24). Fig. 4C shows that 10 μ M FDNB does not influence HC currents induced by AMPA. Thus, HCs are reliable reporters of glutamate release from photoreceptors. HC patch pipettes in all experiments included 10 mM ATP to bypass any need HCs may have for CK.

Sustained Glutamate Release from Photoreceptor Terminals Requires CK Activity. Having established that CK is active in photoreceptors and that HCs reliably report glutamate release, we could test whether endogenous CK activity is required to sustain glutamate release. We used a nystatin-induced perforated patch electrode on the photoreceptor to control its membrane potential and to ensure that the photoreceptor relied on endogenous mechanisms for energy distribution. Depolarization of a cone from -70 to -10 mV induced glutamate release, which caused a transient inward synaptic current in the HC (Fig. 4D). In the absence of FDNB, the amplitude of these responses remained constant over 10 min (Fig. 4E). FDNB progressively attenuated the EPSCs in HCs (Filled Squares, Fig. 4F). Fig. S9A–C shows the corresponding experiments for depolarization of rods. Our findings show that CK activity is needed to maintain a flow of metabolic energy sufficient to sustain repetitive glutamate release at the photoreceptor terminal.

We also used paired cell recording to confirm that there are no relevant off-target effects of FDNB. We included 10 mM ATP in the whole cell patch pipettes on both the cone and HC, which ensured that neither cell would depend on PCr for energy distribution. FDNB should be ineffective under these conditions if it has no relevant target other than CK. We used an ATP-filled pipette to depolarize the cone and recorded postsynaptic currents in the HC. With ATP in the pipette 10 μ M FDNB had little effect (Filled Triangles, Fig. 4F), confirming the absence of off-target effects.

Role of Creatine Kinases in Vascularized Retinas. Mitochondria migrate to the photoreceptor terminals in vascularized retinas where O_2 is available from the inner retina (Fig. S1). These mitochondria could provide energy needed to support synaptic transmission and make photoreceptors in vascularized retinas less dependent on CK. We tested whether PCr and CK are as essential for synaptic transmission in vascularized mouse retinas as they are in the avascular zebrafish and salamander retinas.

We evaluated the role of CK in phototransduction and synaptic transmission in mouse retinas by comparing flash-induced ERG responses of WT and CK-B $^{-/-}$ mice. The a wave of the dark-adapted mouse ERG reports phototransduction in the OS, and the b wave reports synaptic transmission and bipolar cell responses. There is little difference between ERG responses of CK-B $^{-/-}$ (Fig. S9D) vs. WT mice. These findings are consistent with the synaptic mitochondria in these vascularized retinas providing sufficient energy to the terminal to support synaptic transmission under the conditions of these ERG analyses.

CK either could play no role at all in vascularized retinas or it could provide partial support for normal function of the synaptic terminal. If it plays a supporting role, then CK deficiency might cause retinas to compensate in some way for the loss of energy at the terminal. Previous analyses of other tissues showed that CK deficiency can evoke a compensation in metabolic capacity (14, 15, 25–30). We found evidence for such compensation in the retinas of CK-deficient mice (Fig. S9E). COX I levels are $48 \pm 5\%$ higher than normal in CK-B $^{-/-}$ retinas, and uMtCK is $38 \pm 5\%$ higher. COX I also is $24 \pm 5\%$ higher than normal in CK $^{-/-}$ dko retinas. It is possible that less compensation is needed in dko retinas because they do not convert any ATP into PCr.

Discussion

Our findings support a model in which PCr is used to solve the fundamental energy distribution problem in photoreceptors. In darkness, PCr from the central mitochondria of the photoreceptor flows past the highly active ion pumps to the synaptic terminal where it is made back into ATP. In light, energy flows as ATP to the OS. We proposed this model on the basis of our observation of the distribution of CK in the retina. We then confirmed its physiological importance by using both biochemical and electrophysiological measurements.

Distribution of CK in the Retina. A previous study detected CK-B in isolated bovine rod OS (13), but other studies of mouse (12) and chicken (31) retinas found CK-B only in photoreceptor inner segments and terminals. We used both immunological methods and biochemical activity measurements to confirm that CK is sequestered in the synaptic terminals of photoreceptors and absent from the OSs.

Energy Routing in Photoreceptors. The polarized distribution of CK in photoreceptors suggests that PCr enhances energy flow only in one direction, toward the synaptic terminal. The effects of the CK inhibitor FDNB are consistent with this suggestion. FDNB does not influence the OS activities we measured. In contrast, it blocks neurotransmission from photoreceptors in zebrafish and salamander retinas. The photoreceptor terminals in these avascular retinas have no mitochondria, so they are especially dependent on PCr from mitochondria in the central domain of the photoreceptor.

Solutions to the Neuronal Energy Distribution Problem. In darkness, ion pumps in a photoreceptor can consume as much as 10^8 ATP/sec (1). These pumps reside in the plasma membrane between the central mitochondria and synaptic terminal (19). There they consume ATP ~ 20 times faster than the terminal. If the rate of ATP production in the central photoreceptor is close to the rate at which the pumps consume it, then very little ATP can reach the terminal.

Our findings show that neurons solve this fundamental problem in distinct ways depending on morphology, environment, and availability of nutrients. Photoreceptors in avascular retinas divert a portion of mitochondrial ATP into a protected pathway. uMtCK makes PCr from ATP. The energy stored in PCr is protected because it cannot be used by the pumps. At the terminal CK-B converts the PCr back into ATP to support neurotransmission. Photoreceptors in vascular retinas solve the problem in a different way. Because O_2 is available from inner retina capillaries, mitochondria migrate to the terminal for on-site production of ATP.

Energy Requirements for Synaptic Transmission. Release of glutamate from photoreceptor terminals in darkness requires a continuous flow of energy to support recycling and refilling of vesicles. Each vesicle requires $\sim 10,000$ ATP to reload with glutamate (32) and ~ 500 vesicles fuse per second in darkness (33). These estimates suggest that the overall ATP consumption in the steady state could be as high as $\sim 5 \times 10^6$ ATP per second. Our direct measurements of CK activity in mouse retina homogenates and the distribution of CK activity show that there is enough CK even in a mouse photoreceptor terminal to make $\sim 10^8$ ATP per second, more than enough to meet this demand.

Additional Perspectives. Our findings provide a framework for understanding energy distribution in spatially polarized neurons. Additional studies will be needed to determine if regulation of either uMtCK or CK-B influences the efficiency and directionality of energy flow. Our study was extensive, but we focused it only on aerobic energy production. Further studies will be needed to

address the equally important contributions of glycolysis to photoreceptor energy metabolism in light and darkness (34, 35).

PCr and ATP production could be regulated so it is possible that inappropriate regulation could be a fundamental cause of photoreceptor pathology. Creatine also can have neuroprotective activity (36). PCr disperses throughout the photoreceptor (37) even though it is used mainly at the synaptic terminal. The entire cytoplasm is available to store PCr as an energy buffer. In principle, the reservoir of creatine and PCr in the cytoplasm could help to reduce the shock and stress of frequent and rapid changes in the magnitude and location of energy demand in photoreceptors between darkness and light.

Experimental Procedures

Please refer to *SI Text* for detailed descriptions of the methods.

Serial Sectioning/Immunoblots. Retina dissection and serial sectioning were as described (38).

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Photoreceptor Responses and Dark Current Measurements. Light-evoked currents from mouse and salamander rod photoreceptors were measured with suction electrodes from finely chopped pieces of retinal tissue as described (39).

Paired Whole Cell Recordings. Paired pre- and postsynaptic recordings from rods or cones with horizontal or off bipolar cells were obtained by using retinal slices from the aquatic tiger salamander as described elsewhere (40).

Electroretinography. Mouse ERGs were performed as described (41), and zebrafish ERGs were recorded from eyes isolated from six dpf zebrafish larvae as described by Wong et al. (21).

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