Mitochondria and neuronal activity

Oliver Kann and Richard Kovács

Institute for Neurophysiology, Charité-Medical University of Berlin, Germany

Kann O, Kovács R. Mitochondria and neuronal activity. Am J Physiol Cell Physiol 292: C641–C657, 2007. First published November 8, 2006; doi:10.1152/ajpcell.00222.2006.—Mitochondria are central for various cellular processes that include ATP production, intracellular Ca\(^{2+}\) signaling, and generation of reactive oxygen species. Neurons critically depend on mitochondrial function to establish membrane excitability and to execute the complex processes of neurotransmission and plasticity. While much information about mitochondrial properties is available from studies on isolated mitochondria and dissociated cell cultures, less is known about mitochondrial function in intact neurons in brain tissue. However, a detailed description of the interactions between mitochondrial function, energy metabolism, and neuronal activity is crucial for the understanding of the complex physiological behavior of neurons, as well as the pathophysiology of various neurological diseases. The combination of new fluorescence imaging techniques, electrophysiology, and brain slice preparations provides a powerful tool to study mitochondrial function during neuronal activity, with high spatiotemporal resolution. This review summarizes recent findings on mitochondrial Ca\(^{2+}\) transport, mitochondrial membrane potential (ΔΨ\(_{m}\)), and energy metabolism during neuronal activity. We will first discuss interactions of these parameters for experimental stimulation conditions that can be related to the physiological range. We will then describe how mitochondrial and metabolic dysfunction develops during pathological neuronal activity, focusing on temporal lobe epilepsy and its experimental models. The aim is to illustrate that 1) the structure of the mitochondrial compartment is highly dynamic in neurons, 2) there is a fine-tuned coupling between neuronal activity and mitochondrial function, and 3) mitochondria are of central importance for the complex behavior of neurons.

brain slice preparation; electrophysiology; NADH; mitochondrial calcium transport; temporal lobe epilepsy

MITOCHONDRIA ARE UNIQUE ORGANELLES providing the host cell with ATP by oxidative phosphorylation. Mitochondria are also central to intracellular Ca\(^{2+}\) homeostasis, steroid synthesis, generation of free radical species, and forms of apoptotic cell death. As a consequence, mitochondrial dysfunction has devastating effects on the integrity of cells and may thus be critically involved in aging, metabolic and degenerative diseases, as well as cancer in higher organisms and humans (218).

Most of the studies on mitochondrial function have been conducted in preparations of isolated mitochondria and dissociated cultures of excitable and nonexcitable cells. Data from these studies have been summarized and discussed in a variety of excellent general reviews on mitochondrial genetics, biochemistry, physiology, and pathology, characterizing mitochondria as a highly dynamic, sensitive, but potentially harmful entity (15, 24, 55, 80, 145, 179).

The central nervous system (CNS) has an extraordinary high metabolic rate, as it consumes about 20% of oxygen inspired at rest, while accounting for only 2% of the body weight (199). This immense metabolic demand is because neurons are highly differentiated cells that need large amounts of ATP for maintenance of ionic gradients across the cell membranes and for neurotransmission. Since most neuronal ATP is generated by oxidative metabolism, neurons critically depend on mitochondrial function and oxygen supply (1, 60, 144). Conversely, neuronal function and survival are very sensitive to mitochondrial dysfunction (62, 158). The second important aspect is the functional heterogeneity in neuronal segments, which is also reflected by the complex morphology of neurons, which can extend over hundreds of micrometers, depending on neuronal cell type and brain region. Accordingly, dendritic, somatic, axonal, and presynaptic segments of neurons might have quite different energy demands, which require local adaptation of energy supply, as well as local cellular signals interconnecting neuronal and mitochondrial metabolic activity. For these reasons, the detailed description of the interplay between mitochondrial function, energy metabolism, and neuronal activity is of critical importance for understanding of neuronal physiology and pathophysiology in various neurological diseases (101, 187, 193). However, the exploration of this interplay in intact animals is aggravated by a variety of factors, like restriction of investigations to cortices of cerebrum and cerebellum, limited control over stimulation parameters, complicated recording conditions (e.g., dye loading with fluorescence indicators), as well as artifacts resulting from breathing and changes in blood flow in vivo. To bypass these limitations, most of the recent studies in the field have been performed in vitro. The isolated nerve terminal (synaptosome preparation) is very suitable for exploring mitochondrial bioenergetics in a physiological milieu, and dissociated neuronal cell cultures...
provide eased access for patch-clamp, imaging, and pharmacological studies. However, the significance of the data for mitochondrial function in intact neurons of the postnatal brain is limited to a certain degree because synaptosomes represent a small fraction of neurons and neuronal cell cultures are derived from embryos. From the experimental point of view, the combination of brain slice preparations, new imaging techniques with high spatiotemporal resolution, and electrophysiology provides an alternative and powerful tool for studying mitochondrial function and energy metabolism during neuronal activity on the tissue level, as well as on the level of dendrites, axons, and somata. Slice preparations may experience brief hypoxic periods during preparation and rely on diffusion of nutrients and oxygen from the bath solution. Nevertheless, the great advantages in combining these methods are minimal invasiveness of imaging techniques, high-speed electrophysiological monitoring of neuronal activity, as well as preservation of neurons in their three-dimensional expansion and natural interaction with glial cells in brain slices, which is very close to the in vivo situation. Moreover, slice preparations allow experimental access to virtually any structure of the brain and can be made from embryonic, juvenile, and adult (aged) animals.

In this review, we will summarize and discuss recent findings on mitochondrial function and energy metabolism during neuronal activity, when stimulation paradigms are restricted to the physiological range. Because of the advantages mentioned above, we will focus on studies that were conducted in brain slice preparations and relate them to findings from in vivo, synaptosomes, or dissociated neuronal cultures, when suitable. We will then discuss how mitochondrial and metabolic dysfunction develops during pathological neuronal activity, focusing on temporal lobe epilepsy (TLE) and its experimental models.

**GENERAL ASPECTS**

**Structure, Distribution, and Bioenergetics of Brain Mitochondria**

Mitochondria are formed by two membranes that may have contact sites. The outer membrane is permeable for ions and small molecules, and its permeability is possibly well regulated. In the intermembrane space important enzymes like creatine kinase and holocytochrome c are located. The inner mitochondrial membrane is almost impermeable, thus forming a tight barrier between the mitochondrial matrix and the neuronal cytoplasm, and is equipped with a variety of ion channels and transporters, like the Ca\(^{2+}\) uniporter, K\(^+\)\textsuperscript{ATP} channels, and Na\(^+\)/Ca\(^{2+}\) exchanger, as well as mitochondrial enzyme systems like the electron transport chain (ETC). Mitochondria are thought to arise from the cell soma (47) and usually form a dynamic network (154, 225). Due to complex and perhaps special cytoskeletal transport mechanisms, individual mitochondria are highly mobile in anterograde and retrograde directions in neurons (88). Therefore, neuronal mitochondria can be positioned and retained in neuronal segments with high metabolic demand, like active growth cones and pre- and postsynaptic structures (128, 182).

Brain mitochondria primarily utilize pyruvate from cytoplasmic glycolysis to reduce nicotinamide adenine dinucleotides and flavin adenine dinucleotides by enzymes of the Krebs-Szent-Györgyi cycle (tricarboxylic acid cycle, TCA). NADH and FADH\(_2\) serve in energy transfer to the ETC, which consists of complex I (NADH ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (ubiquinol cytochrome c oxidoreductase), and complex IV (cytochrome c oxidase). Via these complexes, electrons are transferred from NADH and FADH\(_2\) to O\(_2\). The reactions of complexes I, III, and IV also imply the transfer of protons from the mitochondrial matrix to the intermembrane space, which establishes a potential difference ($\Delta$V\(_{\text{m}}\)) of 150–180 mV (negative with respect to cytosol) across the inner mitochondrial membrane. Thus, $\Delta$V\(_{\text{m}}\) sets the driving force for protons (together with $\Delta$PH) that actuate F\(_{1}\)F\(_{0}\)-ATP synthase (complex V) to generate ATP and for cytosolic Ca\(^{2+}\) ions to accumulate in the matrix via the mitochondrial Ca\(^{2+}\) uniporter (74, 151) (Fig. 1). The current concept on regulation of mitochondrial oxidative metabolism is primarily based on studies of isolated mitochondria or mitochondrial enzymes. It implicates regulatory functions of substrates like the ratio of ADP/ATP, as well as changes in the mitochondrial calcium concentration ([Ca\(^{2+}\)\(_{\text{m}}\]), which has been shown to stimulate the activity of TCA cycle dehydrogenases and to functionally modulate complexes IV and V (80, 99, 145). Recent studies applying physiological Ca\(^{2+}\)-mobilizing stimuli in nonexcitable cells and myocytes strongly support this concept (79, 178, 222).

**Mitochondrial Ca\(^{2+}\) Transporters and Channels**

Net mitochondrial Ca\(^{2+}\) transport depends on the relative rates of Ca\(^{2+}\) uptake and extrusion. The main Ca\(^{2+}\) uptake pathway is the mitochondrial Ca\(^{2+}\) uniporter, which is allosterically activated by Ca\(^{2+}\), giving rise to a Hill coefficient of 2 (15, 76). Although the exact molecular nature of the Ca\(^{2+}\) uniporter has not been determined (15), a recent patch-clamp study in mitoplasts suggests that it is likely an inwardly rectifying Ca\(^{2+}\)-selective ion channel (Ref. 109; see also Refs. 76, 134). Mitochondrial Ca\(^{2+}\) uptake was already observed in intact cells at cytosolic calcium concentrations ([Ca\(^{2+}\)\(_{\text{c}}\)]) as low as 150–300 nM (173). However, high local Ca\(^{2+}\) domains of tens of micromolar in the vicinity of voltage-operated Ca\(^{2+}\) channels or Ca\(^{2+}\) release sites of the endoplasmic reticulum (183) might be necessary for fast [Ca\(^{2+}\)\(_{\text{m}}\)]\(_{\text{transient}}\) (71, 164). During pathophysiological elevations in [Ca\(^{2+}\)\(_{\text{c}}\)], mitochondria virtually use the full respiratory capacity to accumulate Ca\(^{2+}\) (76). Since mitochondria have been found to more effectively translate oscillations than sustained elevations in [Ca\(^{2+}\)\(_{\text{c}}\)] into metabolic responses, the presence of an inactivating Ca\(^{2+}\) uptake mode has been suggested (Ref. 79; see also Ref. 204). Resting free [Ca\(^{2+}\)\(_{\text{m}}\)] has been found to be as low as [Ca\(^{2+}\)\(_{\text{c}}\)] in spite of the considerable electrochemical gradient for Ca\(^{2+}\) (8), which reflects the activity of Ca\(^{2+}\) extrusion mechanisms, as well as the high mitochondrial Ca\(^{2+}\) buffer capacity likely due to formation of Ca\(^{2+}\) phosphate complexes that have been suggested to represent a reversible Ca\(^{2+}\) store (45, 175). For translating small [Ca\(^{2+}\)\(_{\text{c}}\), transients to mitochondria, the Ca\(^{2+}\)-binding aspartate-glutamate carrier aralar has been proposed recently (166).

In excitable cells, the main mitochondrial Ca\(^{2+}\) extrusion pathway is the Na\(^{+}/Ca\(^{2+}\) exchanger, which is responsible for low resting [Ca\(^{2+}\)\(_{\text{m}}\]). This transporter was previously thought to exchange one Ca\(^{2+}\) ion with two incoming Na\(^{+}\) ions (20).
Other studies proposed the stoichiometry of three Na\(^+\)/H\(^+\) for one Ca\(^{2+}\) ion; thus the exchanger would mediate electrogenic Ca\(^{2+}\) extrusion (12, 98). Due to its low capacity, the transport rate of this exchanger can be surpassed by the Ca\(^{2+}\) uniport, leading to net Ca\(^{2+}\) accumulation in mitochondria. The mitochondrial H\(^+\)/Ca\(^{2+}\) exchanger is the second extrusion pathway, which is slow and saturates at a lower [Ca\(^{2+}\)]\(_{ic}\) than the Na\(^+\)/Ca\(^{2+}\) exchanger. Thus its contribution to fast mitochondrial Ca\(^{2+}\) efflux is transient low-conductance opening of the mitochondrial permeability transition pore (MPTP), which might save mitochondria from toxic Ca\(^{2+}\) loads (92, 230).

Neuronal Activity, Energy Metabolism and Neuronal-Astrocytic Interactions

Repetitive action potential firing causes accumulation of Na\(^+\), Ca\(^{2+}\), and Cl\(^-\) in the neuronal cytosol and the release of K\(^+\) to the extracellular space (86, 88). Elevations in the extracellular potassium concentration ([K\(^+\)]\(_{o}\)) are reversed by ATPase activity and K\(^+\) buffering of astrocytes (156).

To maintain ionic gradients and thus neuronal excitability, the activity of ion transport processes, namely Na\(^+\)-K\(^+\)-ATPases and Ca\(^{2+}\)-ATPases, increases. These processes cover about 60–80% of ATP consumption in the CNS. Another 10–20% is consumed by neurotransmission, which includes synthesis and packaging of neurotransmitters in vesicles and the transport to presynaptic endings (1, 61). The major energy substrate for mitochondrial oxidative metabolism is glucose (38, 198). Under physiological conditions, facilitated diffusion of glucose via the GLUT-1 transporter across the blood-brain barrier to the parenchyma is more rapid, compared with other oxidizable substrates such as lactate, keto acids, fatty acids, and amino acids (167). Although neuronal dendrites and axons have sparse contact with capillaries, neurons are well equipped to metabolize glucose. This is because brain glucose levels are evenly distributed between intracellular and extracellular compartments, and neurons possess high levels of glycolytic enzymes, as well as the fast glucose transporter, GLUT-3 (124, 171). By using lactate dehydrogenase-1, neurons might also derive pyruvate from lactate, which can be provided by astrocytic anaerobic glycolysis (139) (Fig. 1).

The interactions between neurons and astrocytes are not restricted to a putative energy supply with lactate. Astrocytes are also central for provision of precursors and other substrates to neurons, regulation of extracellular ion homeostasis, transport and metabolism of neurotransmitters, ammonia detoxification, and volume regulation (161, 188, 200). The underlying structural prerequisites of astrocytes are formation of an elaborated astrocytic network via gap junctions, close contacts to capillaries, and enwrapping of synapses.

MEASUREMENTS OF MITOCHONDRIAL FUNCTION IN BRAIN SLICES

Brain Slice Preparations

Acute brain slices. The most frequently used preparation to study fundamental principles in neurons and neuronal networks is the acute brain slice from mouse, rat, or even human brain...
specimens (127, 195). In this preparation, brain tissue is cut in slices of 300–500 µm thickness (101, 133, 150). To minimize ischemic (anoxic) neuronal damage due to arrest of blood circulation, the preparation of brain slices is carried out quickly in ice-cold artificial cerebrospinal fluid (ACSF). ACSF usually contains ion concentrations comparable to the cerebrospinal fluid in vivo (19, 85). Notably, for investigations of brain slices much higher glucose concentrations (10–26 mM) are used compared with the brain in vivo (0.35–2.6 mM; summarized by Ref. 126) because it is difficult to obtain healthy slices using lower concentrations. It has been speculated that high glucose levels get converted to lactate, which might support recovery from hypoxic events, e.g., during brain slicing (194). Oxygenation and pH adjustment of ACSF is achieved by a mixture of 95% O2 and 5% CO2 (carbogen). This is because many cells in the upper and lower 50 µm of the slice are damaged by the vibrating blade, and a sufficient oxygen supply for healthy neurons in the slice core ultimately depends on a steep O2 diffusion gradient. At 95% ambient O2 and under interface recording conditions (slice maintained on the ACSF surface), tissue oxygen tension (Po2) was determined, with values of around 700 mmHg at the surface and around 300 mmHg at a depth of 265 µm in acute hippocampal slices (400 µm thickness), which rapidly declined during repetitive electrical stimulation of the tissue (65). These values can be significantly lower in slices maintained under submerged recording conditions because of the limiting factors of O2 solubility, perfusion speed of ACSF, and a normal atmosphere above the bath chamber. These aspects on glucose and O2 supply have to be considered when interpreting data from brain slice preparations. Hyperoxic conditions, for example, might contribute to experimental artifacts in the uppermost cell layers of a slice. Nevertheless, hyperoxic conditions also exist when investigating isolated mitochondria or dissociated neuronal cultures at ambient Po2 levels. After the cutting procedure, brain slices are allowed to recover and equilibrate to ACSF for 1–2 h, which reverses neuronal and mitochondrial swelling (211).

Organotypic slice cultures. In organotypic slice cultures from hippocampus or cortex (112, 207), neurons and glial cells largely maintain morphology, histological architecture, and their natural interactions (“organotypic”) (228). In both rats and mice, organotypic slice cultures can be prepared from embryonic stages up to postnatal day 16. Brain slices (400 µm) are usually prepared from pups under sterile conditions and then maintained on Biopore membranes in interface conditions for up to 4 wk using standard incubators (5% CO2, 20% O2, 36°C). The medium also contains high glucose levels (10 mM and more) and a serum component (up to 25%) that supports recovery after cutting. Within days in vitro, slice cultures get thinner to 150–200 µm, corresponding to 10- to 12-cell layers (9), which reduces the diffusion distances for O2, ions, and drugs compared with acute slices. Under interface conditions with 20% ambient O2, Po2 values of 120 mmHg at the surface and 67 mmHg at a depth of 100 µm were determined at rest (176), the latter of which is closer to in vivo data obtained under normoxic conditions in the rat brain (217). Notably, slice cultures mature in vitro, and synaptic components like glutamate receptors are stably maintained (9), whereas aberrant synaptic reorganization occurs to a variable degree in long-term cultures (78).

Experimental Induction and Monitoring of Neuronal Activity

In brain slice preparations, neuronal activity can be reliably evoked by application of electrical stimulation, neurotransmitters, or pharmacology. For electrical stimulation, single pulses (0.1 ms) are repetitively applied at frequencies up to 100 Hz and with train durations in the range of seconds (21, 102). Alternatively, neurotransmitters and exogenous receptor ligands like glutamate or carbachol are dissolved in ACSF and applied using the superfusion system or a pressure device for brief, local application (103, 197). For induction of pathological epileptiform neuronal activity, a variety of tools exist. Omission of Mg2+ ions from ACSF results in hypereexcitability and manifestation of epileptiform activity in brain slice preparations because of removal of the Mg2+ ion blockade from N-methyl-D-aspartate (NMDA) receptors, removal of the surface discharge screening, and increased transmitter release probability (“low Mg2+ model”; Ref. 152). Further common pharmacological tools are reduction of synaptic inhibition by use of the GABA receptor antagonists, bicuculline and picrotoxin, and/or depolarization of neurons using 4-aminopyridine, or an elevated extracellular potassium concentration ([K+]o) in ACSF (69).

Patch-clamp recordings in the whole-cell or cell-attached configuration allow for precise monitoring of membrane currents or membrane potential changes in individual neurons (112, 150). Extracellular recording electrodes are used to monitor the evoked field potential from a large number of neurons neighboring the recording site. Shape and amplitude of field potentials allow judgment of the type and degree of neuronal activity and serve as a reference parameter to adjust, for example, the intensity for electrical stimulation (65, 69). An additional parameter is provided when using double-barreled, ion-sensitive microelectrodes. K+-sensitive electrodes, for example, record changes in [K+]o (138). Transient increases in [K+]o of 2 to 3 mM from basal levels occur during physiological neuronal activity in vivo (2, 84). By contrast, during pathological neuronal activity like seizures or spreading depression, [K+]o can increase up to tens of millimolar in vivo (52). Thus electrophysiological monitoring of [K+]o can be used as an accurate parameter to judge the degree of evoked neuronal activity in brain slice preparations (102, 125).

Fluorescence Monitoring of Mitochondrial Parameters in Brain Slices

A variety of tools and methods exist to monitor mitochondrial parameters. Here, we will briefly discuss the most common fluorescence parameters, when used in brain slice preparations. For technical details and properties of fluorescence probes, the reader is referred to manufacturers and other reviews (94, 158, 231).

NADH and FAD fluorescence have been studied in vivo and in brain slice preparations to monitor changes in cellular energy metabolism (21, 102, 103, 144). When excited with ultraviolet light (340 nm and 360 nm, for NADH and NADPH, respectively) the reduced forms, NADH and NADPH, fluoresce at 450 nm, while the oxidized forms are nonfluorescent (3). Investigators often refer to changes in NAD(P)H fluorescence because the emission spectra of NADPH and NADH overlap, and their redox states are coupled via the activity of the nicotinamide nucleotide transhydrogenase. However,
NADPH levels were found to be low in brain tissue (36, 104, 111). NAD(P)H fluorescence in brain slices is primarily governed by mitochondrial activity of the electron transport chain and the TCA cycle. Under certain conditions, it might be also influenced by extramitochondrial signaling and antioxidative processes, where NADH and NADPH serve as cofactors (14, 54, 110). Moreover, in astrocytes with high glycolytic activity, cytosolic NAD(P)H might significantly contribute to the overall NAD(P)H fluorescence (105). Thus NAD(P)H fluorescence and its changes in brain slice preparations are influenced by a variety of factors and have to be interpreted with care (190).

Electron transport flavoproteins and α-lipoamide dehydrogenase contribute to about 75% of the flavin fluorescence in neurons (90, 121). Since both of them are also closely linked to the mitochondrial NADH system, monitoring changes in FAD fluorescence gives insights into both the mitochondrial redox state (180, 197). Because here the oxidized form is fluorescent, changes in FAD fluorescence are opposite to NAD(P)H fluorescence. Although FAD fluorescence is weaker than NAD(P)H fluorescence, it has the advantage of an excitation maximum at 450 nm, allowing prolonged recordings in brain slice preparations, due to less phototoxicity.

For determining changes in $\Delta \Psi_m$, membrane-permeant cationic fluorescence indicators, like rhodamine 123, are used that show a Nernstian distribution at the polarized mitochondrial inner membrane and accumulate within the matrix. There are basically two experimental approaches for monitoring changes in $\Delta \Psi_m$ (157). When dye concentrations in the bath are in the micromolar range, mitochondria accumulate three to four orders of magnitude higher dye concentrations, leading to significant self-quenching of the fluorescence. Mitochondrial depolarization results in a release of the dye with subsequent unquenching in surrounding cytosol and the matrix, resulting in a net fluorescence increase. Conversely, if nanomolar dye concentrations are used for the staining, intramatrix quenching is negligible, and mitochondrial depolarization leads to a decrease of the mitochondrial fluorescence (231). Although the latter method is more tolerable for the cells (see below), brief loading periods with high dye concentrations have been found useful in brain slice preparations to reach fast dye equilibrium and to take advantage of the signal amplification due to unquenching (17, 114). The execution and interpretation of experiments with these cationic dyes in slice preparations require special attention because the geometry of cellular compartments is highly heterogeneous, the extracellular space is very small (7–16% of the tissue; Ref. 137), compared with dissociated cell cultures, and ACSF exchange is variable when comparing slice surface with core. These factors might significantly influence dye distribution and fluorescence signal kinetics (see also Ref. 158). In our hands, rhodamine 123 bulk loading in the micromolar range leads to bright mitochondrial and weak cytosolic staining, when applying confocal imaging in organotypic slice cultures. During neuronal activation, a significant increase in fluorescence occurs in the cytosol, reflecting dye release from mitochondria and subsequent unquenching. Although it is hard to resolve the extracellular space in confocal images, the contribution of changes in extracellular fluorescence is relatively low due to the volume ratio between cytosol and extracellular space. Once released into the cytosol, however, dye reuptake by repolarizing mitochondria competes with dye loss due to plasma membrane depolarization. Thus changes in plasma membrane potential might shape the recovery phase of the signal. It should be also stressed that rhodamine dyes are photosensitizers and potentially form reactive oxygen species (ROS) upon excitation, which might significantly influence mitochondrial function (91).

For determining mitochondrial free radical production, fluorescence probes like hydroethidine (HEt) and 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) are used. These molecules are nonfluorescent in their reduced form, but fluoresce after oxidation. The dyes show some preference for a specific free radical. For example, HEt was shown to be selectively oxidized by superoxide radicals (18), and DAF-FM has been used for detection of nitric oxide (NO) (26, 189). Unfortunately, the selectivity of the dyes that can be acquired in cell-free systems does not necessarily apply to the cellular environment, where compartmentalization and local redox conditions might influence the reaction. In case of several dyes (HEt, dihydrorhodamine), mitochondrial accumulation of the oxidation end-products and subsequent release due to depolarization of mitochondrial membranes might result in false-positive results (30). Therefore, studies on free radical formation in brain slices always require both positive and negative controls (189).

Mitochondria-specific, Ca$^{2+}$-chelating fluorescence probes, like rhod-2 and rhod-FF, are positively charged in their membrane-permeant, acetoxymethyl ester form and thus accumulate in mitochondria. After cleavage of the ester bond, the dye becomes fluorescent, Ca$^{2+}$ sensitive, and trapped in the organelles. Nevertheless, de-esterification of the dye already occurs in the cytosol, and therefore, a part of the fluorescence signal represents changes in [Ca$^{2+}$]c. Although the cytosolic part of the signal can be decreased by applying appropriate dye-loading protocols, control experiments with pharmacological means are required in brain slice preparations (102).

Another option for discrimination between cytosolic and mitochondrial fluorescence fractions is the use of spatial frequency filtering of recorded fluorescence images (71, 72, 112). This takes advantage of the fact that the cytosolic dye content gives rise to a smooth, slow, spatial frequency component, whereas mitochondria are represented at high spatial frequencies due to their small size. Another elegant way to decrease cytosolic fluorescence is the application of the dye in acetoxymethyl ester form through a patch pipette, which results in mitochondrial accumulation of the cleaved dye and dilution of the de-esterified dye in the cytosol by the large amount of leuco dye from the pipette (16). The dyes listed above measure free Ca$^{2+}$. Therefore, when considering total Ca$^{2+}$, it should be taken into account that the Ca$^{2+}$ buffer capacity of mitochondria is several orders of magnitude higher than the cytosol (8).

**MITOCHONDRIAL FUNCTION DURING PHYSIOLOGICAL NEURONAL ACTIVITY**

**Transient Increases in [Ca$^{2+}$]m During Synaptic Activity**

Neuronal activity is associated with transient elevations in [Ca$^{2+}$]c due to Ca$^{2+}$ entry via the plasma membrane and Ca$^{2+}$ release from intracellular stores. Ca$^{2+}$ entry is mediated by voltage-operated Ca$^{2+}$ channels, receptor-operated and store-operated Ca$^{2+}$ channels, or nonselective cation channels (4, 10,
203). Calcium release from endoplasmic reticulum occurs via inositol (1,4,5)-trisphosphate (IP3)-induced Ca2+ release (IICR) and Ca2+-induced Ca2+ release (CICR) (Fig. 1).

Repetitive electrical stimulation of brain slices and slice cultures has been used to investigate transient elevations in [Ca2+]m during synaptic activity. The major advantage of this experimental approach is that the resulting changes in synaptic activity affect a large population of neurons, thereby increasing the reliability of the fluorescence recording. Repetitive electrical stimulation for 10 s of the mossy fiber tract in hippocampal slice cultures elicited [Ca2+]m transients that were characterized by an elevated plateau phase, compared with [Ca2+]i transients during the stimulus train (102). Prolonged elevations in [Ca2+]m for up to tens of seconds after train termination were only evident when neuronal activity was elicited in the upper physiological range (increases in [K+]o of >2.5 mM), indicating slow mitochondrial Ca2+ extrusion. Increases in [Ca2+]m showed strong positive correlation with stimulus intensity and frequency (5 to 100 Hz), as well as elevations in [K+]o, suggesting a tight coupling of [Ca2+]m transients and the degree of synaptic activity.

Similar data were reported for the hippocampal CA3 region of acute brain slices showing that brief electrical stimulus trains elicited transient elevations in [Ca2+]m (17). However, in this study, the extracellular Mg2+ concentration was lowered to enhance excitability that led to spontaneous afterdischarges following the train and spreading waves in [Ca2+]m. A more exact assessment of stimulus-induced transient Ca2+ elevations in dendritic mitochondria of hippocampal area CA3 was achieved by using analytical electron microscopy in hippocampal slice cultures (174). [Ca2+]m was already significantly increased within 1 s after brief electrical stimulation (1 s, 50 Hz) and reached up to 10 times higher values at 30 s after stimulus, with a complete recovery after 180 s. At this time point [Ca2+]m in the endoplasmic reticulum was still high, supporting the hypothesis that Ca2+ extruded from mitochondria might be taken up by intracellular stores. The difference between the two approaches might be explained by the fact that fluorescent Ca2+ probes measure free mitochondrial Ca2+ that is several orders of magnitude lower due to the high Ca2+ buffer capacity of the mitochondrial matrix, while analytical electron microscopy evaluates both free and bound Ca2+.

Elevations in [Ca2+]m also occur in presynaptic structures during neuronal activation. Repetitive electrical stimulation of motor neurons (10–60 s, 50–100 Hz) resulted in an plateau elevation of [Ca2+]m in mitochondria in axon terminals to about 1 μM (45), which is in the appropriate range for activation of mitochondrial dehydrogenases (145), but well below the value necessary for opening of the MPTP (230).

In addition to studies applying electrical stimulation, there is evidence that spontaneous neuronal activity also significantly affects [Ca2+]m. Oscillations in [Ca2+]m have been observed during spontaneous oscillatory changes in membrane potential and action potential firing of neurons in brain slices from the mouse respiratory center (150). The amplitude of these [Ca2+]m oscillations correlated with the level of hypoxia that is the “physiological” stimulus for these cells. Moreover, changes in FAD and NAD(P)H fluorescence were also synchronized with [Ca2+]m oscillations, indicating tight coupling of mitochondrial redox state and Ca2+ level (see below).

Electrical stimulation of brain slices results in activation of voltage-operated Ca2+ channels and NMDA receptor-gated channels leading to high local [Ca2+]c domains. Whether elevations in [Ca2+]c also occur during activation of metabotropic receptors, as linked to the intracellular Ca2+-machinery, was explored in hippocampal slice cultures. Activation of metabotropic serotonergic, glutamatergic, and muscarinic receptors evoked transient elevations in [Ca2+]m in a dose-dependent manner. Moreover, by isolating IICR, CICR, and receptor-operated capacitative Ca2+ entry, it was demonstrated that these individual pathways were able to transiently elevate [Ca2+]m (103). These [Ca2+]m transients were also immediately integrated in a metabolic response. Though applying microfluorometric techniques without any spatial resolution, the fluorescence was recorded in both stratum pyramidale, which contains densely packed neuronal cell bodies, and stratum radiatum. Thus the data suggest that neuronal mitochondria even sense elevations in [Ca2+]c, as elicited by metabotropic receptor-mediated Ca2+ signaling (Fig. 1). This is in line with studies in individual neurons demonstrating that mitochondrial Ca2+ uptake buffers both IP3-mediated and caffeine-induced [Ca2+]c transients (123, 135).

The influence of the different Ca2+ sources on [Ca2+]m is technically easier to assess in dissociated neuronal cultures. In this preparation, glutamate concentrations have been estimated to peak at 1.1 mM in synaptic clefts, with a decay time constant of 1.2 ms during neurotransmission (41). A likely source for mitochondrial Ca2+ uptake during synaptic activity is Ca2+ influx through NMDA receptor-gated channels. Activation of NMDA receptors (100 μM NMDA) in primary cultures of striatal neurons elicited similar and time-locked transients in [Ca2+]c and [Ca2+]m, whereas mitochondrial Ca2+ increases lagged behind cytosolic Ca2+ increases when the latter was induced by depolarization with high extracellular [K+]o or by application of kainate or ionomycin (169). Comparably, NMDA and glutamate were most effective in filling mitochondrial Ca2+ stores in cultured cortical neurons, even at glutamate concentration as low as 3 μM (22). Application of 200 μM and 200 μM NMDA to cultured hippocampal neurons resulted in a rise in [Ca2+]c and [Ca2+]m as revealed by the chemiluminescence change of mitochondrially targeted aequorin (219). Interestingly, only application at 200 μM enhanced Ca2+ cycling across the inner membrane, which might explain the increased neurotoxicity at this concentration. Thus, it seems that neuronal mitochondria take up Ca2+ from different sources during synaptic activity. However, individual mitochondria may prefer a particular Ca2+ source based on the location in the cytosol (11, 45, 175).

Heterogeneity of [Ca2+]m in Neuronal Mitochondria

Ca2+ signaling in neurons is characterized by complex spatiotemporal patterns (4, 10). Mitochondria that are equipped with effective Ca2+ transport mechanisms and that are located at important strategic points in dendrites and presynaptic segments might contribute to this spatiotemporal complexity (16, 174, 185, 196, 223). Changes in [Ca2+]c of individual mitochondria have been monitored in hippocampal slice cultures by applying high-resolution confocal laser-scanning microscopy and offline spatial frequency filtering methods (112). [Ca2+]c was highly heterogeneous between individual mitochondria,
even under resting conditions without any external stimulation. This heterogeneity was present in each slice culture irrespective of the staining method (via patch pipette or bulk loading of slices) or the $K_a$ of the rhod-dye derivative used (0.5 μM and 19 μM). Interestingly, mitochondria with a granular appearance expressed brighter fluorescence, indicating elevated basal levels in $[Ca^{2+}]_m$, whereas the fluorescence was rather low in filamentous mitochondria. Colocalizing MitoTracker Green and rhod-2 fluorescence identified mitochondrial filaments that contained rhod-2 fluorescence hot spots of granular appearance, suggestive for high local $Ca^{2+}$ domains in mitochondria. This might indicate that either mitochondrial filaments are spatially discontinuous or diffusion barriers for $Ca^{2+}$ exist within mitochondrial filaments. A recent study on rat brain capillary endothelial cells demonstrated that the extension of mitochondria differs in terms of electrical continuity and distribution of $[Ca^{2+}]_m$. It was concluded that diffusion of $Ca^{2+}$ is limited to a single mitochondrion, whereas changes in $\Delta \Psi_m$ might spread over mitochondria forming an electrically continuous synctium (71). If this mechanism is also present in neurons, it would allow restriction of elevations in $[Ca^{2+}]_m$ and thus activation of TCA dehydrogenases in the locations of high $[Ca^{2+}]_m$ domains, whereas the cost of mitochondrial $Ca^{2+}$ uptake, namely mitochondrial membrane depolarization, would be distributed over the mitochondrial network. Thus ATP formation might be optimally enhanced at the place of highest demand (202).

Additionally, ultrastructural and confocal laser-scanning microscopy studies suggest that the distribution of the mitochondrial population in neurons and glial cells is largely heterogeneous and dynamically changing (44, 49, 149, 177). Mitochondrial filaments were found in vivo in the soma, dendrites, and axons of principal cells in dentate gyrus and areas CA1 and CA3 of the hippocampus. Interestingly, mitochondria do not seem to invade spines with the exceptions of the thorny excrescences in CA3 (177). This observation slightly differs from those made in vitro. This is possibly due to the fact that formation of such filaments from smaller granular mitochondria is a dynamic process (23, 49, 149). Mitochondria may undergo continuous fusion and fission cycles (37, 225), thus building up larger entities serving for more effective distribution of proton motive force (202). However, a balance shift toward the granular form (thread-grain transition) might be also an early sign of cell damage and apoptosis (201). Continuous fusion and fission cycles might also explain the recovery of the chemiluminescence of mitochondria-targeted aequorin during repetitive NMDA application to hippocampal neurons (11). It is possible that the aequorin pool, which is consumed during the first NMDA application in mitochondria closely situated to the plasma membrane, is replenished by mitochondrial transport and/or fusion with mitochondria from the bulk cytosol. In contrast to dendrites and somata, mitochondria in the axons and in presynaptic endings are rather granular in shape, which might influence their capability to act as a local $Ca^{2+}$ buffer (177, 196).

Another aspect of mitochondrial $Ca^{2+}$ buffering is related to the fact that mitochondria are highly motile organelles showing anterograde and retrograde transport in dendrites and axons, as well as small Brownian “wiggling” (149, 163). The velocity of the directed movements in neurons was largely dependent on temperature, with values about 0.36 μm/s (112), 0.6 μm/s (50), and 0.32–0.38 μm/s (153). Directed transport along microtubules is mediated by the dynein-kinesin system, whereas mitochondria also move along actin filaments by using myosin motors (88). Mitochondrial transport was shown to be influenced by $Ca^{2+}$ because local elevations in $[Ca^{2+}]_m$ led to an immediate stop of mitochondrial movement (182). This mechanism might enhance mitochondrial $Ca^{2+}$ uptake by retaining mitochondria at places of $Ca^{2+}$ release/influx and serve for energy supply in response to local demands (226).

Synaptosomal and somatic mitochondrial fractions show different sensitivities to excessive $Ca^{2+}$ challenge because synaptosomal mitochondria, which are primarily presynaptic, more rapidly undergo MPTP opening (27). Since axonal transport often implies long distances, axonal and presynaptic mitochondrial might represent an aged group in the mitochondrial population. Indeed, monitoring $\Delta \Psi_m$ showed that highly polarized mitochondria were transported in the anterograde direction while partially depolarized mitochondria were transported retrogradely along the axon (148).

**Involvement of Mitochondrial Ca$^{2+}$ Signaling in Neuronal Behavior**

Mitochondrial dysfunction and particularly mitochondrial ROS formation in the CNS is involved in acute insults like ischemia-reperfusion injury and in chronic neurodegenerative disorders like Alzheimer and Parkinson disease (39, 162). Much less is known on mitochondrial interactions with neuronal behavior under physiological conditions. Changes in $[Ca^{2+}]_m$ play a critical role in neuronal development, neurotransmitter release, and plasticity. In the previous section we discussed the tight coupling between cytosolic and mitochondrial $Ca^{2+}$ signals, and there is accumulating evidence that changes in $[Ca^{2+}]_m$ serve as a regulatory mechanism for adaptation of mitochondrial energy metabolism when neuronal activity is in the physiological range (see below).

Mitochondrial $Ca^{2+}$ uptake shapes the spatiotemporal pattern of cytosolic $Ca^{2+}$ signals and may thus influence neuronal behavior. It has been shown that mitochondrial $Ca^{2+}$ buffering decreases the peak amplitude of stimulus-induced $[Ca^{2+}]_c$ transients and slows $[Ca^{2+}]_c$ recovery by slowly recycling $Ca^{2+}$ into the cytosol (22, 42, 43, 68, 169, 219–221). By shaping the time course of $[Ca^{2+}]_c$ transients in neurons, mitochondria can exert control on processes that are dependent on the perfect timing and localization of $Ca^{2+}$ gradients, like release of synaptic vesicles. $[Ca^{2+}]_c$ elevation and subsequent synaptic transmission, as induced by high-frequency stimulation, seemed to be regulated by mitochondrial $Ca^{2+}$ uptake in motor neuron terminals (46, 70) and at the retinal amacrine cell synapse (146). Nevertheless, a recent study in a Drosophila mutant lacking mitochondria in synapses suggests that mobilizing the reserve pool of synaptic vesicles depends on the presence of ATP rather than on mitochondrial $Ca^{2+}$ buffering (214).

Mitochondria may also contribute to certain forms of synaptic plasticity. The phenomenon of post-tetanic potentiation is characterized by an increase in synaptic strength after high-frequency stimulation. The underlying mechanism is a short-term increase in synaptic release probability due to residual $Ca^{2+}$ in the presynaptic terminal following strong stimuli (51). This form of short-term synaptic plasticity can be also ob-
served at the neuromuscular junction. It was demonstrated that blocking mitochondrial, but not endoplasmic reticulum Ca$^{2+}$ uptake, prevented posttetanic potentiation. Mitochondrial Ca$^{2+}$ buffering kept [Ca$^{2+}$], below 1 µM during a tetanus, whereas the recovery of [Ca$^{2+}$], was largely prolonged due to subsequent extrusion of Ca$^{2+}$ from the mitochondria, resulting in an increase of synaptic release probability in this period (210).

The high-frequency stimulation in the previous examples results in high [Ca$^{2+}$], transients, and it is commonly accepted that mitochondrial Ca$^{2+}$ uptake is activated at such levels (174, 220). However, there is also evidence that mitochondria contribute to presynaptic Ca$^{2+}$ buffering, even after a brief stimulus train (4 pulses, 100 Hz) at the glutamatergic synapse of the calyx of Held. In this preparation, mitochondrial Ca$^{2+}$-buffering determined the fast recovery of postsynaptic currents after stimulus-induced synaptic depression (16).

In contrast, at the ribbon synapse of the retinal bipolar neurons, the plasma membrane Ca$^{2+}$ pumps were responsible for the Ca$^{2+}$ removal from the cytosol, and mitochondrial Ca$^{2+}$ uptake was observed only in about 30% of the cells. Concomitantly, mitochondrial ATP delivery was inevitable for proper functioning of the plasma membrane Ca$^{2+}$ pumps, suggesting involvement of presynaptic mitochondria in synaptic transmission (227). The difference between these two types of synapses might be the localization of mitochondria with respect to Ca$^{2+}$ entry sites (223).

Based on these examples it might be concluded that the general mechanisms of mitochondrial contribution to synaptic transmission are shaping of the spatiotemporal pattern of cytosolic Ca$^{2+}$ signals and accurate ATP delivery for restoration of ion gradients and vesicle pool cycling. However, the extent to which a certain mechanism influences neuronal behavior has to be determined for each type of synapse.

Another important consequence of Ca$^{2+}$ uptake is mitochondrial ROS formation, which has been shown for neuronal preparations in studies on excitotoxicity (18, 33, 57, 181). However, there is also evidence that endogenous glutamate release under physiological conditions leads to moderate increases in ROS formation, which modulates intracellular signaling cascades (89), synaptic transmission (7), as well as communication between neurons and glial cells (5).

**Changes in ΔΨ$_m$ During Physiological Synaptic Activity**

The driving force for mitochondrial Ca$^{2+}$ uptake is mainly represented by the mitochondrial membrane potential. Since both the Ca$^{2+}$ uptake and extrusion mechanisms are electrogenic (Refs. 12, 75, 98; but see Ref. 20), elevations in [Ca$^{2+}$]$_{m}$ associated with synaptic activity might result in a decrease in ΔΨ$_m$. In addition, several factors like substrates, free radicals, and changes in ATP/ADP ratio might significantly influence ΔΨ$_m$ during synaptic activity.

The same stimulation protocols, which were shown to elicit a [Ca$^{2+}$]$_{m}$ transient or even a spreading wave of [Ca$^{2+}$]$_{m}$, were also effective to evoke a spreading wave of mitochondrial depolarization in hippocampal slices (17). The fact that the amplitude of mitochondrial depolarization correlated with the intensity of synaptic activity and that ΔΨ$_m$ changes spread along anatomical pathways and were blocked by inhibition of the voltage-operated Na$^+$ channels or glutamatergic transmission, strongly indicate the dependence of changes in ΔΨ$_m$ on synaptic activity. The spatiotemporal pattern of changes in ΔΨ$_m$ correlated with that of the stimulus-induced [Ca$^{2+}$]$_{m}$ under the same experimental paradigm, suggesting that changes in mitochondrial Ca$^{2+}$ cycle might underlie the depolarization of mitochondrial membranes. In line with these findings, spontaneous oscillations in [Ca$^{2+}$]$_{m}$ in neurons of the respiratory center were associated with time-locked oscillations in ΔΨ$_m$ (150). It is noteworthy, however, that differences in ΔΨ$_m$ might be influenced by factors other than the regional variations in synaptic activity. It has been shown that mitochondria isolated from the CA1 region are more susceptible to an excessive Ca$^{2+}$ load than mitochondria from the CA3 region (143). There might be also age-dependent differences in the ability of mitochondria to cope with such a Ca$^{2+}$ load. Both mitochondrial Ca$^{2+}$ buffering capacity and ΔΨ$_m$ were decreased in forebrain neurons from aged rats (155). In line with these findings, recovery of ΔΨ$_m$ was slowed down in cerebellar brain slices from old rats after depolarization with a high extracellular K$^+$ concentration, suggesting increased vulnerability of aged mitochondria (224). The Ca$^{2+}$ dependence of the ΔΨ$_m$ was also shown in individual patched CA1 pyramidal cells in acute hippocampal slices (191). Depolarization steps resulted in a [Ca$^{2+}$], transient and a delayed loss in ΔΨ$_m$. This loss in ΔΨ$_m$ was dependent on Ca$^{2+}$ influx through voltage-operated Ca$^{2+}$ channels because it was suppressed in zero Ca$^{2+}$ ACSF.

In these studies strong stimuli were applied, which likely evoked neuronal activity above the physiological range. Nevertheless, we have also observed mitochondrial depolarization when applying moderate electrical stimulation or the muscarinic receptor agonist carbachol in slice cultures (Kann and Kovács, unpublished observation).

However, even this type of physiological stimulation results in neuronal activity, which is different from the activity which mitochondria might experience during spontaneous synaptic activity in slices. When using laser-scanning confocal microscopy for monitoring ΔΨ$_m$ in patched pyramidal cells, a considerable heterogeneity in rhodamine 123 fluorescence was observed. Filamentous mitochondria expressed rather low fluorescence compared with granular mitochondria and mitochondrial clusters (112). This is in line with findings from neuronal cultures showing mitochondrial differences in form and polarization by using the ratiometric mitochondrial probe JC-1 (149, 163). Despite the high level of background synaptic activity, spontaneous fluctuations in ΔΨ$_m$ were rarely observed in normal ACSF. However, Ca$^{2+}$ buffering via the patch pipette solution (1 mM EGTA) might have masked physiological, small-amplitude changes in [Ca$^{2+}$],, finally suppressing fluctuations in ΔΨ$_m$ (112).

Spontaneous fluctuations in ΔΨ$_m$ have been observed in neuroblastoma cells and in primary neuronal cultures (29, 136). Interestingly, these fluctuations were not dependent on synaptic activity because they persisted in the presence of tetrodotoxin (TTX) and the NMDA receptor blocker MK-801. Although they were also resistant to cyclosporin A, the contribution of MPTP to the ΔΨ$_m$ fluctuations cannot be completely excluded because cyclosporin A did not block Ca$^{2+}$-induced MPTP-opening in the presence of ATP in nonsynaptic brain mitochondria (28, 40). Alternatively, this phenomenon might represent changes in oxidative phosphorylation (81) or changes in electrogenic channel or transporter activity of the mitochon-
drial membrane. Indeed, prolonged multiple openings of mitochondrial ion channels have been observed in the presynaptic terminal of the squid giant presynaptic terminal during stimulus-induced synaptic transmission (95, 96).

In conclusion, these data suggest that even moderate changes in $\Delta \Psi_m$ can be induced by a broad spectrum of physiological stimuli. The underlying mechanisms may differ depending on the type of stimulus as well as on the preparation used. However, a close interplay between Ca$^{2+}$ cycling across the inner membrane and mitochondrial depolarization is proposed.

Since mild loss of $\Delta \Psi_m$ has been proposed to decrease mitochondrial ROS formation (165), there is an apparent contradiction between the Ca$^{2+}$-dependent increase in ROS formation, which we described in the previous section, and the Ca$^{2+}$-dependent decrease in $\Delta \Psi_m$. Indeed, mitochondrial ROS formation at its major sites, namely, complex III and complex I, was shown to be decreased or completely inhibited by depolarization of the mitochondrial membrane (158, 206, 216). In line with these findings, a neuroprotective role has been proposed for the mitochondrial uncoupling proteins (UCPs), the expression of which is upregulated after insults to the CNS (108). One alternative source of $\Delta \Psi_m$-independent superoxide radical formation might be $\alpha$-ketoglutarate dehydrogenase, which might also explain the upregulation of ROS formation in the presence of high [Ca$^{2+}$]m and NADH (39, 205). Moreover, once undergoing autooxidation, complex I can produce ROS in a vicious cycle, irrespective of $\Delta \Psi_m$ (117). In conclusion, Ca$^{2+}$-dependent, mild mitochondrial depolarization might exert an inhibitory effect on ROS formation that, however, might be surpassed by other targets of the Ca$^{2+}$ load.

**NAD(P)H and FAD Transients**

Changes in NAD(P)H fluorescence have been used to monitor adaptations in energy metabolism during neuronal activity in brain slice preparations (133, 190). Stimulus-evoked neuronal activity results in characteristic biphasic NAD(P)H fluorescence transients, namely, a brief initial “dip” component and a subsequent prolonged “overshoot” component. Such NAD(P)H transients can be elicited by repetitive electrical stimulation or application of receptor agonists (102, 103, 197). The typical time course of NAD(P)H transients is in the range of 3–4 min when a stimulus train of 10 s is applied at 20 Hz. Changes in P0$_2$, as well as application of substrates and drugs interfering with mitochondrial targets and metabolism, elicit slower baseline shifts in NAD(P)H fluorescence (65, 102, 133).

**Characteristics of tissue and cellular levels.** The ratio between “dip” and “overshoot” components varies between the cellular and tissue levels in the brain. In the early in vivo studies, NAD(P)H fluorescence transients primarily consisted of large “dip” components in the cerebral cortex upon direct electrical stimulation, evoked seizures, or spreading depression (97, 144, 184). Though the in vivo approach allowed optimal oxygen supply to the brain parenchyma, these studies were restricted to the cortical surface, thus reflecting changes in NAD(P)H fluorescence of apical dendrites and meninges. In brain slices, it was found that NAD(P)H transients almost exclusively displayed a “dip” component upon electrical stimulation or local glutamate application in the alveus (a hippocampal structure that predominantly contains neuronal fiber tracts), while biphasic NAD(P)H transients with an accentuated overshoot component occurred in s. radiatum (containing synapses, neuronal fiber tracts, and glial cells) (197). Therefore, the shape of NAD(P)H transients seems to depend on a variety of factors that include tissue P0$_2$ levels, the ratio between neurons (neuronal segments) and astrocytes, as well as cellular biochemical properties. Interestingly, applying high-resolution, two-photon laser-scanning microscopy in acute hippocampal slices, it was reported that stimulus-evoked dip and overshoot components of NAD(P)H transients were highly anti-colocalized in s. radiatum. Moreover, it was proposed that the dip represented a neuronal response, whereas the overshoot was primarily astrocytic, indicating a compartmentalization of oxidative and glycolytic metabolism between neurons and astrocytes (105). These findings may argue for the astrocyte-neuron lactate shuttle hypothesis (139). Nevertheless, biphasic NAD(P)H transients were described in individual sensory, hippocampal, and cerebellar neurons of dissociated cell cultures (56, 83, 193), similar to other excitable and nonexcitable cells (79, 178, 222). Moreover, biphasic NAD(P)H transients have recently been found to be unaffected by inhibition of glycolysis, as well as glucose uptake blockade, over a wide range of electrical stimulation in acute hippocampal slices (21). These experiments were conducted under conditions with provision of pyruvate as an alternative substrate and when adenosine-1 receptor antagonists prevented decreases in synaptic efficacy. The findings suggest that NAD(P)H transients reflect mitochondrial rather than glycolytic processes.

Ca$^{2+}$-dependent and -independent NAD(P)H transients. Adaptation of local energy demands in neurons requires intracellular signals integrating neuronal activity and metabolic responses of mitochondria. One likely candidate is intracellular Ca$^{2+}$ because 1) activated neurons experience transient intracellular Ca$^{2+}$ loads up to the micromolar range, and 2) the activity of TCA cycle enzymes, for example, is stimulated by Ca$^{2+}$ elevations (80, 145).

The tight temporal association between transient elevations in cytosolic and mitochondrial Ca$^{2+}$ concentrations and NAD(P)H (and FAD) fluorescence transients during neuronal activity has been shown in brain slice preparations (102, 103, 150, 197), which is supported by studies in dissociated neuronal cultures (56, 193). Moreover, significant reductions in the amplitude of NAD(P)H transients during electrical neuronal stimulation and under experimental conditions that prevent intracellular and mitochondrial Ca$^{2+}$ elevations, strongly support the hypothesis that Ca$^{2+}$ is a key integrator for neuronal activity and mitochondrial energy metabolism (56, 102). Nevertheless, a significant portion of NAD(P)H transients seems to be Ca$^{2+}$ independent in slice preparations (102, 197), similar to FAD transients in the in vivo cerebellum (180). The underlying mechanisms have not been clearly defined and are still under discussion. However, this finding has been particularly observed during prolonged neuronal stimulation that is per se associated with activity-dependent transient increases in [K$^+$]o (102), as well as under a condition where neurotransmitter release was not inhibited (197). Both factors may trigger Ca$^{2+}$-independent pre- and postsynaptic neuronal as well as astrocytic mechanisms, which include glutamate uptake and metabolism and/or activation of ATPases (for discussions see Refs. 21, 102, 105, 132).
In summary, the available data based on NAD(P)H and FAD fluorescence studies point to a complex regulation of energy metabolism in brain tissue. It is conceivable that mitochondrial Ca\(^{2+}\) accumulation as a local “extrinsic” signal adapts oxidative metabolism in mitochondria, which gets superimposed on an “intrinsic” regulation of NADH redox states (145). Candidates for such intrinsic regulation are the ratios of ADP/ATP, NAD\(^+/\)NADH, or CoA/acetyl-CoA (80, 99, 145). This might suggest an indication of coming metabolic demand for neuronal mitochondria that is mediated by Ca\(^{2+}\), as well as a feedback control that is ultimately mediated by ATP consumption.

Fine-tuned neurometabolic coupling. Some of the studies discussed above also provide clear evidence that neuronal activity and mitochondrial function are tightly coupled in neurons. Positive quantitative correlations between gradual activity and mitochondrial function are tightly coupled in discussed above also provide clear evidence that neuronal feedback control that is ultimately mediated by ATP monitored by transient increases in \([K^+]_o\) from 0.25 to 3 mM, indicating a tight coupling between neuronal activity and mitochondrial metabolic responses. These relations seemed to uncouple only under conditions of extended neuronal activity with short time intervals between stimulus trains and when the NAD(P)H “overshoot” had not yet recovered to baseline (102). This might be explained by ceiling levels of NAD(P)H fluorescence, possibly because of maximal reduction of the available nicotinamide adenine dinucleotides pool in the tissue.

From the studies discussed, there is also clear evidence for tight temporal relations between neuronal activity and mitochondrial function in brain slices. Almost immediately with onset of repetitive stimulation and rapid increases in \([Ca^{2+}]_{\text{im}}\), and \([Ca^{2+}]_o\), NAD(P)H fluorescence starts to decrease while the nadir of the resulting “dip” component is reached during or at termination of the stimulus train, depending on the stimulus parameters. This is in line with data demonstrating rapid Ca\(^{2+}\)-dependent mitochondrial NAD(P)H oxidation and \(O_2\) consumption that occurs within 0.2 s after onset of depolarization of cerebellar Purkinje neurons (83).

These tight quantitative and temporal relations between neuronal activity and mitochondrial function point to a fine-tuned “neurometabolic coupling” in the brain, which preserves neuronal excitability and neurotransmission in the acute phase during activation and as long as substrates are supplied to the parenchyma. In brain slice preparations, this prerequisite may be considerably masked due to the high concentrations of glucose in ACSF and/or the limiting prolonged diffusion distances for \(O_2\). However, in the in vivo brain sufficient substrate supply to the parenchyma is achieved by a highly developed network of vessels and capillaries with distances of 20–40 \(\mu\)m and a variety of complex regulatory mechanisms that may be attributed to the processes of “neurovascular coupling” and “neurobarrier coupling.” Neurovascular coupling designates the adaptation of blood vessel diameter and thus blood flow to the degree of neuronal activity (87, 215). The increase in regional blood flow matches the local needs of brain cells for nutrients after neuronal activation. This process requires functional links between neurons, astrocytes, and vascular cells that may form neurovascular units. Possible signaling factors are NO, adenosine, arachidonic acid metabolites, and elevations in [K\(^+\)]_o (32, 53, 160, 229). Neurobarrier coupling designates adaptations in the properties of glucose transport across the blood-brain barrier after neuronal activation, which may include changes in Michaelis-Menten parameters of the GLUT-1 protein, activity-dependent incorporation of GLUT-1 in blood-brain barrier membranes, and/or changes in GLUT-1 expression in response to chronic (developmental or pathological) changes in neuronal activity and/or plasma glucose concentrations (126). Neurovascular coupling is evident on the order of seconds after neuronal activation (140), while neurobarrier coupling may also imply long-term changes of at least hours or days at the blood-brain barrier. Thus both processes overlap in part with and follow up on neurometabolic coupling to synergistically fulfill the energy demands of neurons.

MITOCHONDRIAL DYSFUNCTION AND TEMPORAL LOBE EPILEPSY

Temporal Lobe Epilepsy and Experimental Models

Epilepsy is one of the most common acquired chronic neurological diseases that affects about 0.8% of the human population (82). Although treated with antiepileptic drugs, about 50% of epilepsy patients still experience seizures. Temporal lobe epilepsy (TLE) is a prevalent form of focal epilepsy, which is frequently resistant to drugs. In some patients TLE might be a progressive disease (172). A selected group of drug-resistant TLE patients benefits from surgical resection of the epileptogenic focus in the temporal lobe and hippocampus (59). In histopathology, hippocampal tissue from TLE patients is often characterized by neuronal cell loss and astrogliaosis (141). Other studies have described reorganization of neuronal networks (208) and functional alterations of receptors and ion channels (6), which might explain neuronal hyperexcitability and hypersynchrony in epileptogenic tissue. Nevertheless, the mechanisms underlying the pathogenesis of TLE are still unclear. In the initial phase of seizure activity, cerebral blood flow drastically increases up to 400–900% of control values, depending on the brain region (93, 147), which matches the increase in amplitude of local metabolic rates for glucose. After 1–2 h of recurrent seizures or status epilepticus, local cerebral blood flow decreases to 150–300%. This results in a relative hypoperfusion, while the metabolic demand remains high due to ongoing seizure activity. Based on these facts it has been suggested that the maladjustment between blood supply and energy metabolism plays a central role in seizure-induced neuronal cell death (93, 116, 209). Nevertheless, pathological neuronal activity might also induce mitochondrial dysfunction and ROS production in the early phase of seizure activity, which finally contributes to neuronal cell death (13). Moreover, functional neuroimaging studies in the interictal phase of epilepsy patients demonstrated a decrease in glucose utilization in seizure foci and adjacent brain structures (34, 118). Such chronic “hypometabolism” might be explained by dysfunction of mitochondrial oxidative and/or glycolytic energy metabolism.

Below, we will summarize evidence for acute and chronic alterations of mitochondrial function and energy metabolism as derived from experimental models of TLE. Studies on mito-
Mitochondrial function during epileptic processes have been conducted by applying primarily two experimental approaches. In the first approach, epileptiform activity is pharmacologically elicited in brain slice preparations from healthy animals. In the second approach, brain slices are made from animal models of TLE. In pilocarpine-treated, chronic epileptic rats, for example, symptoms, histopathology, and course of the disease resemble those occurring in TLE patients (35, 141).

Changes in $\Delta \Psi_m$ and $[Ca^{2+}]_m$ During Epileptiform Activity

As discussed above, there is evidence that $\Delta \Psi_m$ and $[Ca^{2+}]_m$ are influenced to a certain degree by stimulus-induced synaptic activity in brain tissue. Epileptiform activity, as induced in the low-Mg$^{2+}$ model in vitro, can be classified by interictal events, seizure-like events (SLEs), and late recurrent discharges, the latter of which is insensitive to antiepileptic drugs (115). Intercital activity did not cause detectable changes in $[Ca^{2+}]_m$, $[Ca^{2+}]_s$, and $\Delta \Psi_m$ at the tissue level, when studied with microfluorometric recordings in slice cultures. In contrast, SLEs elicited large $[Ca^{2+}]_m$ transients that were associated with elevations in $[Ca^{2+}]_s$ and loss in $\Delta \Psi_m$. The recovery of both $[Ca^{2+}]_m$ and $\Delta \Psi_m$ to values before SLEs outlasted synchronized neuronal activity by several minutes, a process that might acutely compromise energy metabolism (113, 114). When applying high-resolution confocal microscopy, small-amplitude fluctuations in $[Ca^{2+}]_m$ and $\Delta \Psi_m$ were associated with interictal activity and never did occur during control conditions in normal ACSF (112). However, it was hard to find a correlation between brief interictal discharges (~1 s, 0.2 to 0.7 Hz) and fluctuations in $[Ca^{2+}]_m$ and $\Delta \Psi_m$ that lasted for up to 20 s. Fluctuation in $\Delta \Psi_m$ occurred primarily in the dendrites but not in the soma. A possible explanation is that local $Ca^{2+}$ changes due to enhanced NMDA receptor activity and/or dendritic voltage-dependent $Ca^{2+}$ conductance are sensed locally by few mitochondria, whereas the distance to somatic mitochondria is too far from the $Ca^{2+}$ influx sites during interictal activity. By contrast, during SLEs a massive and synchronous loss in $\Delta \Psi_m$ occurred in neuronal dendrites and somata. Interestingly, amplitude and frequency of $[Ca^{2+}]_m$ fluctuations increased significantly on the level of individual mitochondria during SLEs while baseline $[Ca^{2+}]_m$ did not increase, indicating no net $Ca^{2+}$ accumulation. However, averaging rhod-2 fluorescence in a large population of mitochondria at a given time point revealed elevation in $[Ca^{2+}]_m$ similar to microfluorometric data from the tissue level. SLE-associated $[Ca^{2+}]_m$ but not $[Ca^{2+}]_s$ fluctuations were inhibited in the presence of Ru360 in the perfusion (112), indicating specific block of the $Ca^{2+}$ uniporter by Ru360 (see also Ref. 142). Intracellular application of either Ru360 or the mitochondrial Na$^+$/Ca$^{2+}$ exchanger blocker CGP-37157 via the patch pipette also prevented synchronized loss of $\Delta \Psi_m$, whereas cyclosporin A, an inhibitor of MPTP opening, had no effect when added to the perfusion (112). Though there is the possibility of cyclosporin A-resistant MPTP openings (28, 40), the fact that the selective block of the Na$^+$/Ca$^{2+}$ exchanger alone was sufficient to prevent changes in $\Delta \Psi_m$ indicates that enhanced mitochondrial Ca$^{2+}$ cycling across the inner membrane might dissipate $\Delta \Psi_m$ in a futile cycle. Lasting $\Delta \Psi_m$ dissipation might explain the decrease in ATP production as seen during seizures in vivo (64, 77), which might contribute to neuronal cell death. Thus the same mechanism by which synaptic activity regulates energy metabolism under physiological conditions might be responsible for the acute devastating effects of seizure activity on $\Delta \Psi_m$ (Fig. 2).

Mitochondrial ROS Formation During Epileptiform Activity

One possible consequence of enhanced mitochondrial Ca$^{2+}$ cycling might be an increase in production of ROS (Figs. 1 and 2). ROS formation was enhanced in isolated mitochondria exposed to elevated extramitochondrial $[Ca^{2+}]_m$ and [Na$^+$] (58), mimicking conditions that might occur during seizures. There is substantial indirect evidence for involvement of ROS in the seizure-induced cell loss from a variety of in vivo models of epilepsy that include fluorothyl-induced seizures (63), the kindling model of epilepsy (67), as well as the pilocarpine and kainic acid model of epilepsy (168, 170). Oxidative damage of mitochondrial enzymes has been found in chronic epileptic tissue from animal models (120, 131) and in resected human tissue (119). ROS formation is not only a consequence of sustained epileptic activity. ROS might also be a cause of epileptic activity because moderate oxidative stress in mitochondrial superoxide dismutase (SOD2) +/− mice has been attributed to spontaneously occurring seizures (130).

Mitochondrial ROS formation during epileptiform activity has been also demonstrated in hippocampal slice cultures using fluorescence indicators (66, 113). Based on the relative specificity of the chosen fluorescent probes (18), superoxide radical was the most likely formed ROS in these experiments. Other ROS like hydroxyl, alkyl, and peroxyl radicals, formed in subsequent reactions, might also contribute to neuronal injury (213). This is supported by the neuroprotective effects of the free radical scavenger α-tocopherol, which is a lipid peroxidation chain breaker, as well as by the observation of increased levels in the lipid peroxidation end-product malondialdehyde in epileptic tissue (66, 67, 113). While a decrease in the
mitochondrial content of the intrinsic antioxidant glutathione was demonstrated after kainic acid-induced seizures in vivo (129), the elevation of glutathione levels was neuroprotective in vitro and in vivo (66, 67).

NO is another free radical that might contribute to alterations in energy metabolism and neuronal injury during seizures. Studies applying electron spin resonance spectroscopy have demonstrated elevated NO levels after seizures in vivo, as induced by kainic acid (106) and pentylenetetrazole (100). Moreover, elevated levels of citrulline, the byproduct of NO synthesis, have been determined after acetylcholinesterase inhibitor-induced status epilepticus (77). Activation of NMDA receptors has been related to enhanced NO formation (31, 107), which is of particular importance for the low-Mg2+ model of epilepsy (152). Indeed, increased NO formation occurs during epileptiform activity in entorhinal cortex slices in this model, and NO synthase inhibition blocked epileptiform activity (189). This indicates a regulatory role for NO in the initiation of SLE by a hitherto unknown mechanism. The existence of a mitochondrial NO synthase is still controversial (Refs. 73, 212; but see Ref. 122). Nevertheless, this issue is very important because NO has been reported to block oxidative respiration, reversibly and irreversibly (25, 186). Thus, extensive NO formation might significantly disturb energy metabolism during epileptiform activity. Another study reported that neuronal tissue tolerates high NO levels (107). Nevertheless, in the presence of superoxide radical, NO forms highly reactive peroxynitrite, which might induce oxidative damage of mitochondrial and cellular structures like membranes and DNA. Degradation of the mitochondrial phospholipid cardiolipin leads to irreversible inhibition of the respiratory chain, whereas extensive DNA damage activates the nuclear protein poly(ADP-ribose) polymerase (PARP), resulting in depletion of cytosolic NAD and inhibition of glycolysis. Glycolysis is also inhibited due to the direct oxidation of glyceraldehyde-3-phosphate dehydrogenase by peroxynitrite. Thus NO and peroxynitrite, S-nitrosothiols, NO2-, and/or N2O3 may cause neuronal apoptosis by directly activating MPTP, leading to cytochrome c release and caspase activation during seizures (13, 48, 168) (Fig. 2).

Metabolic Dysfunction During Epileptiform Activity

When pathological epileptiform activity was elicited in the low-Mg2+ model in hippocampal slice cultures and acute slices of the entorhinal-hippocampal cortex, SLEs initially induced robust biphasic NAD(P)H transients similar to conditions with physiological stimulation. Nevertheless, with the increase in number of SLEs, severe alterations in NAD(P)H transients occurred, namely, a decrease and finally a complete loss of the overshoot component (113, 192). These alterations in NAD(P)H transients may reflect mitochondrial dysfunction that causes acute energy failure, probably as a consequence of the massive ROS production during SLEs. However, these findings have to be proved in other acute experimental models of epilepsy before final conclusions can be made.

Interestingly, similar alterations were observed when NAD(P)H transients, as elicited by electrical stimulation, were investigated in hippocampal slices from chronic epileptic rats and resected brain tissue of TLE patients (101). NAD(P)H transients showed smaller overshoot components in area CA1 compared with the subiculum in chronic epileptic rats, indicating region-specific alterations. In hippocampal tissue from TLE patients, NAD(P)H fluorescence transients showed marked initial “dip” components and lacked an overshoot component in most of the cases. These findings indicate severe mitochondrial and metabolic dysfunction in tissue from chronic epileptic rats and humans. In TLE patients, the dysfunction might be more pronounced because of seizure history over years or even decades. Similar alterations in stimulus-induced NAD(P)H fluorescence transients were also observed in hippocampal slices from kainate-treated, chronic epileptic rats (120). These studies from animal models and pathological human brain specimens provide evidence for a cellular correlate for hypometabolism, as observed in neuroimaging studies in epilepsy patients. The underlying mechanisms are still under discussion and include defects in mitochondrial enzymes like complex I and aconitase (119, 131), dysfunction of neuronal-astrocytic metabolic coupling (139), and/or abnormal Ca2+ cycling (120).

CONCLUSIONS AND PERSPECTIVES

Mitochondria in neurons form a compartment that is highly dynamic in structure and function. This involves mitochondrial transport in neuronal segments and strategic positioning of mitochondria at sites where ATP supply and Ca2+ handling is required. Moreover, a fine-tuned coupling between neuronal activity and mitochondrial function exists that is mediated by ions and substrates. Thus, mitochondria are critically involved in neuronal survival, neurotransmission, and plasticity. New high-resolution imaging techniques will help to extend our knowledge on the physiological interactions between neuronal and mitochondrial functions in different types of neurons, brain regions, and developmental stages of the CNS. Under pathological conditions, like epileptic seizures, the tight coupling between neuronal activity and mitochondria has devastating effects on mitochondrial function, implying mitochondrial Ca2+ overload, synchronous depolarization of the mitochondrial compartment, and enhanced ROS production. This might cause energy failure, release of pro-apoptotic factors, and oxidative damage of lipid membranes and DNA, even in the acute phase of seizure activity. Neuroprotective strategies in epilepsy research might focus on mitochondria-mediated secondary tissue damage. The development of pharmacological tools for protection of mitochondria and ROS scavenging might help to establish adjuvant forms of drug therapy for epilepsy patients.

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