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Mitochondrial Configurations in Peripheral Nerve Suggest Differential ATP Production

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Abstract

Physiological states of mitochondria often correlate with distinctive morphology. Electron microscopy and tomographic reconstruction were used to investigate the three-dimensional structure of axonal mitochondria and mitochondria in the surrounding Schwann cells of the peripheral nervous system (PNS), both in the vicinity of nodes of Ranvier and far from these nodes. Condensed mitochondria were found to be abundant in the axoplasm, but not in the Schwann cell. Uncharacteristic of the classical morphology of condensed mitochondria, the outer and inner boundary membranes are in close apposition and the crista junctions are narrow, consistent with their function as gates for the diffusion of macromolecules. There is also less cristae surface area and lower density of crista junctions in these mitochondria. The density of mitochondria was greater at the paranode-node-paranode (PNP) as was the crista junction opening, yet there were fewer cristae in these organelles compared to those in the internodal region. The greater density of condensed mitochondria in the PNS axoplasm and in particular at the PNP suggests a need for these organelles to operate at a high workload of ATP production.

Keywords

ATP production; condensed; cristae; crista junction; electron tomography; mitochondria; node of Ranvier; orthodox; peripheral nerve; Schwann cell; stereology

Introduction

Neuronal mitochondria display considerable structural diversity, particularly with the cristae (Frey et al., 2002; Johnson et al., 2007; Perkins and Ellisman, 2007; Perkins et al., 1997a; Perkins et al., 2001a). Evidence is accumulating that the topology of the cristae membranes is not random but rather is a regulated parameter (Barsoum et al., 2006; Davies et al., 2007; Frey and Sun, 2008; Griparic et al., 2004; Knott et al., 2008; Mannella, 2006a; Mannella, 2006b; Misaka et al., 2006; Ponnuswamy et al., 2005; Renken et al., 2002; Zick et al., 2008). Cristae topology affects the diffusion of metabolites and soluble proteins that can influence mitochondrial ATP generation and protein release accompanying apoptosis (Frezza et al.,

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2006; Mannella, 2008; Mannella et al., 2001; Olichon et al., 2003; Sun et al., 2007; Yamaguchi et al., 2008). Evidence is mounting that crista junctions regulate the dynamic distribution of proteins, lipids, and metabolites between mitochondrial subcompartments. Further progress is needed towards understanding the factors that control cristae membrane dynamics, mitochondrial energy states and their relationship with localized neuronal energy requirements.

The energy states of isolated mitochondria have been described carefully (originally reviewed by (Chance and Williams, 1956; Scheffler, 1999; Scheffler, 2008). These energy states are tied to the rate of respiration (Nicholls, 2007). The respiratory rate of the isolated organelle is determined by a driving force for oxidative phosphorylation (usually closely coupled to the concentrations of ADP and Pi), the presence of oxygen, and the availability of a substrate (e.g. malate, NADH, or succinate) (Alberts, 2002; Karp, 2002). A general consensus appears to favor the view that the control of respiration is mainly due to changes in ADP concentration inside the mitochondria (Scheffler, 1999). Energy state 4 is the prevalent state in vivo cells and tissues and is equated with a resting state. However, it is a high-energy or “charged” resting state because the ratio of [ATP/ADP] in vivo is very high (~100:1) in the cytosol. The high ratio means that state 4 mitochondria are analogous to a charged capacitor with stored potential. This potential may be discharged by the addition of ADP, provided that the mitochondrion is not uncoupled. In vitro, the addition of ADP to state 4 mitochondria transforms them to state 3 and a reversion to state 4 occurs when the ADP is converted to ATP. State 3 is the fully active respiratory state of isolated mitochondria and may under favorable conditions have a rate of oxygen consumption 10-fold or more higher than those in the resting state 4 condition (Fiskum, 1986). These states can be tied to mitochondrial structure.

There exists a long-standing body of work relating the structure of isolated mitochondria to their energy steady states (originally reviewed by (Benard and Rossignol, 2008; Munn, 1974). However, few studies have analyzed the link between mitochondrial morphology and energetics in situ, in particular as regards to cristae conformation and crista junction characteristics. Transitions from one metabolic state to another can be accompanied by characteristic ultrastructural changes, including the remodeling of cristae morphology, that occur not only in vitro but also in situ (Hackenbrock, 1968a; Hackenbrock, 1968b). In vitro, mitochondria can adopt many different structural conformations (Munn, 1974). However, in situ, the principal conformations of unperturbed, well preserved mitochondria are orthodox and condensed. During low respiratory activity (state 4), mitochondrial transmembrane electrochemical potential increases and the mitochondria in electron micrographs are in the orthodox conformation (Hackenbrock, 1968a; Hackenbrock, 1968b). In this configuration, the organelle is characterized by a relatively large matrix volume, concomitantly small intracristal volume, and with the inner boundary membrane (the non-cristae part of the inner membrane) closely apposed to the outer membrane (Hackenbrock, 1966; Lloyd et al., 2002). In contrast, during high respiratory activity (state 3), the mitochondria assume the condensed conformation (De Martino et al., 1979; Uhrík and Stampfli, 1981), characterized by a relatively small (condensed) matrix volume, often causing the inner boundary membrane to be pulled away from the outer membrane, and enlarged cristae. In this state, the intracristal space is expanded and many of the cristae compartments are interconnected (Renken et al., 2002). Figure 1 provides models of the classical orthodox and condensed configurations for comparison (following Munn, 1974) and to serve as a guide to interpret the results we present for peripheral nervous system (PNS) mitochondria.

The extreme length of PNS axonal processes suggests that the ATP availability is non-uniform in the axoplasm (Saks et al., 2007). As a consequence, the transport and positioning of mitochondria in the axoplasm may be essential for neuronal energy homeostasis (Chada

and Hollenbeck, 2003). What signals mitochondria to move? An obvious signal is local ATP need. ATP has a short diffusion distance (Yoshizaki et al., 1990). Hence, mitochondria must be positioned at sites of ATP consumption. Mironov and coworkers used single particle tracking experiments to monitor mitochondrial movement in neurons while simultaneously measuring the intracellular ATP levels (Mironov, 2007). They showed a direct correlation between energy usage (ADP level) in local areas of the neuron and mitochondrial movements.

In the work reported here, we show the abundance of condensed mitochondria in the spinal root axoplasm of healthy mouse tissue consisting of long PNS axons, supporting the concept that there is a need for the mitochondria in this neuronal compartment to operate at a high workload of ATP production. This structural configuration does not occur in the surrounding Schwann cells nor in the CNS axoplasm. Although research articles of the past 30 years have displayed micrographs of condensed mitochondria at the node of Ranvier and the surrounding vicinity (Berthold, 1996; Fabricius et al., 1993; Ghabriel and Allt, 1977; Mastalgia et al., 1976; Uhrik and Stampfli, 1981), rarely are they identified as such and little consideration is given concerning what their appearance might mean from a functional standpoint. Uncharacteristic of the classical morphology of condensed mitochondria, we observed that the outer and inner boundary membranes of the PNS organelle are in close apposition and the crista junctions are narrow. We discovered that there are fewer cristae in mitochondria at the paranode-node-paranode (PNP) region compared to the internodal region and that subdomains inside a mitochondrion can range from condensed to orthodox. The density of mitochondria is greater at the PNP as is the crista junction opening size. The crista volume ratio is higher in the axonal mitochondria than in the Schwann cell examples, yet the crista surface area ratio and the density of crista junctions are lower. We discuss why these findings suggest a differential energy usage in the PNP compared to the internode.

Material and Methods

Conventional preservation with aldehyde-fixation of spinal root samples from rat was accomplished as described previously (Perkins et al., 2001a) on four animals. The animal experimentation described here was approved by UCSD's Administrative Panel on Laboratory Animal Care (IACUC), which oversees the use of animals according to U.S. federal law. Spinal root samples from the same number of animals and prepared by high-pressure freezing and freeze substitution (HPF/FS) were described by Perkins and coworkers (Perkins et al., 2008). Conventional transmission electron microscopy (cTEM) of thin sections (~70–80 nm thick) was performed with a JEOL 1200 FX microscope operated at 80 kV. Forty-five images were recorded on film at 6,000, 10,000 or 20,000 magnifications. These negatives were digitized at 1200 dpi using a Nikon CoolScan system, giving an image size of 2689 × 4006 pixel array and a pixel resolution of 3.54 nm (6k magnification), 2.12 nm (10k magnification), or 1.06 nm (20k magnification). Another forty images were recorded at 3000 magnification with a newly installed 4K × 4k CCD camera on the same microscope giving a pixel resolution of 3.3 nm. All 85 images were used for a stereological analysis at nodes, paranodes, and internodes. Sixty-three of the images had nodes of Ranvier present and were used for the volume ratio analysis of nodes and paranodes. The other 22 images did not show nodes and were used for the internode analysis, using several axons per image. A 112 × 112 square grid (112 × 112 chosen for ease of use with Photoshop) was overlaid on each image and mitochondria and axoplasm lying under intercepts were counted. The relative volume of mitochondria was expressed as the ratio of intercepts coinciding with this organelle relative to the intercepts coinciding with axoplasm. The percentage contribution to nodal, paranodal, or internodal domains was then calculated. Electron microscope tomography of conventional and HPF/FS samples was performed as described previously (Perkins et al., 2008; Perkins et al., 1997b). A three-dimensional (3D) analysis of

substructural characteristics of mitochondria in 23 tomographic reconstructions was performed. The Student's t-test was used for all measures of significance. "p-values" were calculated and indicated at the corresponding locations in the figure captions.

Results

Upon examining electron tomographic reconstructions of well-preserved rat spinal root or saphenous nerves, we noticed unusual conformations of the mitochondrial inner membrane that were present whether prepared by conventional fixation means or by HPF/FS. To quantify these unusual conformational features, we added stereological analyses using cTEM, examining more than 100 axons. We found that the mitochondrial structure was not greatly different between samples prepared by conventional fixation means or by HPF/FS. No significant differences were measured for number of cristae per mitochondrion, mitochondrial volume ratio, abundance of the orthodox and condensed organelles, crista diameter, crista volume ratio, crista surface area ratio, crista junction opening size, or crista junction density for conventionally prepared samples in relation to HPF/FS samples (data not shown). Thus, the conventional fixation and HPF/FS data sets were combined for quantitative analyses. The biggest difference between the two sample preparation methods was the smoother appearance of membranes in the HPF/FS samples and this did not appear to affect significantly the substructural components (compare the mitochondrial membranes shown in figs. 2 and 3—HPF/FS—with fig. 4—cTEM).

Unlike mitochondria found in other tissues and cell types, including in the CNS, condensed mitochondria, with dilated cristae, are abundant in the axoplasm of the PNS as found in spinal root (fig. 2) and saphenous nerves (not shown). Orthodox mitochondria in the same region had the typical narrow cristae characteristic of this configuration (Perkins et al., 1997a; Perkins et al., 2001a). Whether condensed or orthodox, the mitochondria were almost invariably slender. As opposed to CNS mitochondria where the cristae are observed to run roughly transverse to the long axis of the organelle, the cristae in condensed mitochondria were invariably arranged longitudinally (fig. 2c). Mitochondria as long as 20 microns were observed consistent with previous observations (Peters, 1991). As these mitochondria are thin, it was common to see one or a few cristae arranged in parallel and extending the length of the organelle. The density of mitochondria is greater at the node and paranode than internode by a factor of about 3 (fig. 2d), yet much less than the factors of 10 and 24 reported for large ventral and dorsal spinal root axons in cat, respectively (Berthold et al., 1993; Fabricius et al., 1993). The density varied considerably between PNPs. For example, it was common to find nodes with no mitochondria present and just as common to observe nodes with several mitochondria in the compact axoplasmic region. To address the question of possible increased mitochondrial docking at the node or paranode, we measured mitochondrial association with microtubules in the axoplasm and found that most axonal mitochondria are in close proximity to microtubules (fig. 3i). However, there is a gradation of fewer mitochondria associating with microtubules the closer to the node the organelle is found, suggesting increasing docking in the PNP. Condensed mitochondria comprise roughly two-thirds of axonal spinal root mitochondria, with orthodox mitochondria constituting the remainder (fig. 2e). The relative abundance of condensed and orthodox mitochondria is approximately the same in the node, paranode and internode regions. As expected, condensed mitochondria have a much larger crista width than do orthodox mitochondria, with a mean about 3.5 times higher (fig. 2f).

Uncharacteristic of the classical morphology of condensed mitochondria that have been isolated (Hackenbrock, 1968a; Hackenbrock, 1968b; von Ahsen et al., 2000; Yamaguchi et al., 2008), yet typical of the PNS organelle, the outer and inner boundary membranes are in close apposition and have narrow, not elongated, crista junctions, a feature best analyzed

with the higher resolution 3D characterization provided by electron tomography (fig. 3 and supplemental movie 1) (Frey et al., 2006; Perkins et al., 1999; Perkins and Frey, 2000; Renken et al., 2002). As is typical of condensed axonal mitochondria in peripheral nerve, there are few, though usually large, cristae. Common for axonal mitochondria in the condensed configuration, cristae are separated by a relatively narrow matrix “bridge”, which is the natural gap between apposed cristae (fig. 3a). The crista junction opening (fig. 3a–g, j) is no larger in the condensed organelle than found in its orthodox counterpart. It is possible that the close apposition of the outer and inner boundary membranes could stem from a high proportion of contact sites. A wide range of these contacts is found in axonal mitochondria (fig. 3h): bridge contact sites of various fiber thicknesses, classical contact sites of various widths (defined in Perkins et al., 2001), and a feature only sometimes observed with mitochondria--outer and inner boundary membranes “zippered” over a substantial portion of the mitochondrial periphery. There are many more such contacts than shown boxed in fig. 3 and occur regularly around the periphery.

Surprisingly, we discovered that there are fewer cristae in mitochondria at the PNP region compared with the internode (fig. 4 and supplemental movie 2). 153 axonal mitochondria were examined to count the number of cristae per organelle. More cristae, and concomitantly more matrix bridges (fig. 4a–f), were observed in mitochondria at the internode (fig. 4g). With a greater number of cristae, the crista junction size decreased slightly in internodal mitochondria (fig. 4d, 6f).

Unexpectedly, we also discovered that subdomains inside axonal mitochondria could display different conformational states (fig. 5). This novel observation was made possible by the 3D aspect of electron tomography, examining slices of the reconstruction at different depths. The mitochondrion shown has predominantly condensed matrix characteristics on one side, both condensed and orthodox conformations in the middle, and only an orthodox conformation on the other side of the volume. We note that axonal mitochondria displaying “mixed” conformations are infrequent.

To determine whether the unusual structural characteristics of axonal mitochondria in the PNS are unique or might extend to the adjacent Schwann cells, we conducted an electron tomographic analysis of the Schwann cell paranodal lumen, which is often enriched with mitochondria. Mitochondria in this compartment have substantially different substructure than those in axons. These mitochondria all show an orthodox configuration, unlike those in neighboring axons (fig. 6 and supplemental movie 3). The cristae are comprised of both tubes and lamellae (fig. 6b). Secondly, the crista volume ratio was higher in the axonal mitochondria than in the Schwann cell mitochondria (fig. 6c). There was no significant difference whether the mitochondrion was found at the PNP or at the internode or whether the sample was prepared conventionally or by HPF/FS (data not shown). Three, in contrast to the volume ratio, the crista surface area ratio was lower in the axonal mitochondria than in the Schwann cell mitochondria (fig. 6d). Again, there was no significant difference whether the mitochondrion was found at the PNP or at the internode, or whether the sample was prepared with or without HPF/FS (data not shown). Four, the density of crista junctions is significantly higher in Schwann cell mitochondria compared to those in the spinal root axoplasm (fig. 6e). Five, the crista junction opening size is smaller in internodal mitochondria compared to PNP mitochondria (fig. 6f), but shows no significant difference between the axonal and Schwann cell organelle and between conventional preparations and those incorporating high-pressure freezing and freeze-substitution (data not shown). Six, the outer and inner boundary membranes were not pulled apart (fig. 6g) any more in the axonal mitochondria than in the Schwann cell mitochondria, even though the mean crista width was significantly greater in the former (fig. 6h). The finding that the internodal crista junction opening is smaller and the trend for the OM-IM distance and crista width to be smaller

(although not statistically significant) points to slightly less condensation of these mitochondria compared to nodal mitochondria.

To summarize our structural findings, we provide a model of the mitochondria studied in 3D in PNS axons and Schwann cells (fig. 7). The mitochondria in these axons are typically long and narrow, yet surprisingly are condensed. They have few cristae and unlike the classical condensed organelle (compare fig. 1), the inner boundary membrane and outer membrane are closely apposed along the periphery, typical of the orthodox organelle. There are also few crista junctions and they are the narrow tubular structure of relatively uniform opening diameter typically found in the classical orthodox mitochondrion, but not in the classical condensed organelle (compare fig. 1). Another defining feature of these mitochondria is the matrix bridge between cristae that likely forms because of the expansion of the cristae compartments and the corresponding condensation of the matrix. This model shows the axoplasm populated with a greater density of mitochondria in the nodal region, as was found (see fig. 2). In contrast to axonal mitochondria, the Schwann cell mitochondria do not deviate from the classical orthodox configuration (fig. 1, 7c) and are often clustered (compare fig. 6).

Discussion

The functional and structural heterogeneity of mitochondria in neurons appears to be the rule rather than the exception because these cells are polar, being composed of dendrites, somas, synapses, and axons—compartments with differing functions, macromolecular components, and energetic needs (Perkins and Ellisman, 2007). This heterogeneity is influenced by variations in the state of energy production through (1) availability of energy substrates, (2) pharmaceutical or genetic inhibition of oxidative phosphorylation, (3) DNA mutations, and (4) cell physiology (Benard and Rossignol, 2008). Because of energy considerations for action potential propagation in axons and especially the high metabolic load required to support ionic fluxes and phosphorylation and dephosphorylation of the molecular machinery concentrated at the node of Ranvier, we investigated the abundance and conformational states of mitochondria in peripheral nerve. We now discuss the bioenergetic implications with guidance from Mannella's (Mannella, 2006b) statement that the shape of the inner membrane influences mitochondrial function.

Positioning of Mitochondria in the Axoplasm

We found that the density of mitochondria is greater at the PNP compared with the internode in spinal root axons of rat and the density was highest in the constricted part, i.e., at the node of Ranvier itself, yet significantly less than the PNP/internode difference reported for cat (Berthold et al., 1993; Fabricius et al., 1993) or for the CNS (Berthold, 1996; Ghabriel and Allt, 1977; Mastaglia et al., 1976). Because of the absence of machinery required for protein synthesis in axons, macromolecular materials required for sustaining the function of axons and synapses are transported down the axon after synthesis in the cell body (Lindsey et al., 1981). It is not known if translocating neuronal mitochondria contain a complete set of enzymes already at their origin or collect the subset of enzymes required for certain specialized function carried out at their destination (Sonnewald et al., 2004). Axonal transport of mitochondria can slow transitorily upon reaching a node (Misgeld et al., 2007). It was speculated that this slowing might be caused by either a checkpoint mechanism governing trafficking of organelles or an active recruitment of mitochondria to this region with specialized function and high metabolic demand (Edgar et al., 2008). Consideration of mitochondrial transport is important not only for normal functioning but also because of the role the dysfunction of transport has in the progression of diseases (Guzik and Goldstein, 2004; Hirokawa and Takemura, 2004; Hollenbeck and Saxton, 2005). For example, abnormalities in mitochondrial dynamics—the fusion, fission, and movement of

mitochondria—have been identified in several neurodegenerative diseases, including Parkinson's disease (Knott et al., 2008) and the pathogenesis of inherited peripheral neuropathy (Baloh, 2008). The DNA and the outer and inner membranes of neuronal mitochondria are damaged in diabetic neuropathy (Leininger et al., 2006) owing in part to deregulation of mitochondrial fission and fusion proteins.

We observed recently that PNS mitochondria were linked to microtubules or neurofilaments (Perkins et al., 2008). Axonal mitochondria have several unique properties with respect to positioning (Bereiter-Hahn and Voth, 1994; Cai and Sheng, 2009; Forman et al., 1987; Hollenbeck and Saxton, 2005; Leterrier et al., 1994). First, they move by both anterograde and retrograde transport but their movements are separate from the vesiculotubular structure or multivesicular or multilammellar structures which carry the main bidirectional flows of axonal transport (Ellisman and Lindsey, 1983). Second, their movements are characterized by saltatory motility and prolonged stationary phases. Third, the net direction of movement is influenced by the physiological status of the axon. Fourth, mitochondria utilize both microtubules and actin-myosin based microfilaments for movement, and neurofilament association for stationary phases within axons. The “dual transport” model seems to be gaining acceptance and is that microtubules drive long-range axonal transport, whereas short-range positioning of mitochondria at nerve terminals, growth cones, and subcortical plasma membrane regions depends primarily on microfilaments (reviewed by Cai and Sheng, 2009). Hence, microtubules, not microfilaments, are expected to govern mitochondrial movement in the vicinity of nodes, and docking of these organelles there might simply involve detachment from the microtubule “highway”. Consistent with this model, we found that mitochondrial association with microtubules decreases in this order: internode>paranode>node (fig. 3).

The molecular signals that direct mitochondria to their positions include: calcium signaling (Cai and Sheng, 2009; Jeyaraju et al., 2009; Wang and Schwarz, 2009), high local ADP:ATP (Mironov, 2007), GTP hydrolysis by GTP-binding proteins (Bloom et al., 1993), actin-myosin-dependent nerve growth factor/TrkA/PI3-kinase signaling (Chada and Hollenbeck, 2004), several kinases (Morfini et al., 2004; Ratner et al., 1998), and the macromolecular composition of the paranode (Einheber et al., 2006). With the exception of the report of Einheber and colleagues, it is unclear which of these other molecular signals for mitochondrial positioning act specifically at the node of Ranvier or whether they influence the orthodox/condensed mitochondrial configuration.

Why Condensed Mitochondria?

We observed about twice as many condensed mitochondria as their orthodox counterparts in spinal root axons and this ratio did not vary significantly between node, paranode, and internode. This condensation was manifest in the greatly enlarged intracristal space, but not in an enlarged intermembrane space or expanded crista junction opening (Figs. 2, 3 and 6). What is the significance of condensed mitochondria in the axon and their absence in other neuronal regions and in the Schwann cell? Perhaps the greatest significance is that the condensed configuration is the manifestation of a more active functional state, as indicated by biochemical findings demonstrating a high oxidative capacity coupled with marked phosphorylation associated with the condensed organelle (De Martino et al., 1979). Because of the length of the axon and the ongoing transport, the number of mitochondria along any given segment is relatively small and so the workload per organelle is higher than in neuronal compartments replete with only orthodox mitochondria. Furthermore, the axonal mitochondria might be expected to have low bioenergetic capacity per mitochondrial volume because their crista surface area ratio (fig. 6d) is low (around 0.4) compared with central nervous system mitochondria in particular, ranging from 1 to 5 (Perkins et al., 2009). Hence, because cristae abundance is directly tied to ATP production via the ATP synthase

(reviewed recently by Benard and Rossignol, 2008; Mannella, 2008), the lower cristae abundance in axonal mitochondria might favor the transition to the condensed configuration to boost ATP production.

What might trigger an orthodox to condensed transformation? The trigger is probably the binding of ADP to the adenine nucleotide translocase rather than ramped up oxidative phosphorylation (Wookey, 1980). Other constituents influencing the trigger might be calcium and phosphate. It was proposed that the inner membrane topographies observed in condensed and orthodox mitochondria cannot be interconverted by passive folding and unfolding of the inner membrane (Mannella et al., 2001). Instead, transformation between these configurations would require membrane fusion and fission. In contrast, Benard and Rossignol (Benard and Rossignol, 2008) hypothesize that interconversion likely involves osmotic processes and may not necessitate the intervention of fusion or fission proteins. However, our observation of mitochondria with “mixed” conformation (fig. 5) seems to contradict that regulation is controlled by osmotic processes only. Moreover, fusion and fission machineries appear to influence mitochondrial respiration (reviewed by McBride et al., 2006) and so may be involved with conformational transformations.

We observed that the outer and inner boundary membranes were not pulled apart in situ as is commonly seen in isolated condensed mitochondria (von Ahsen et al., 2000; Yamaguchi et al., 2008). Could this be attributed to the constraints of the axoplasmic milieu on the organelle, including cytoskeletal components, which are abundant in the axoplasm of peripheral nerves that have been removed upon isolation? This close apposition of the peripheral membranes may be explained by the oncotic pressure hypothesis, which proposes that mitochondria are able to maintain a hyperosmotic matrix milieu because the outer membrane plays a special structural role in preventing osmotic swelling and lysis of the organelle (Kim et al., 2003). An alternative possibility is that tethering of these two membranes keeps them together. Support for this is found from tomographic images showing bridge contact sites consisting of fine filaments attaching to outer and inner boundary membranes and spanning the intermembrane space (fig. 3h; Mannella et al., 1999; Perkins et al., 2001). These narrow filaments might be broken during the isolation procedure leading to the membrane separation most often seen in vitro. The classical contact and “zippered” sites also observed (fig. 3h) are likely to hold these membranes together.

What advantage might the condensed configuration have on mitochondrial bioenergetics? From nuclear magnetic resonance studies of viscosity, support for considerable diffusional heterogeneity for small metabolites, including ADP and ATP, within isolated mitochondria was provided (Garcia-Perez et al., 1999; Lopez-Beltran et al., 1996; Sonnewald et al., 2004). These studies suggest that the free diffusion of ADP and ATP within the condensed mitochondrial matrix is unlikely. Whereas the matrix is dark, indicating molecular crowding, the intracristal space is light, indicating the opposite (Mannella et al., 1994; von Ahsen et al., 2000; Yamaguchi et al., 2008). It may be advantageous to have a crowded matrix to limit the diffusion of newly generated ATP away from the inner membrane where resides the adenine nucleotide translocase, which transports the ATP outside to the intracristal or intermembrane spaces. At the same time, it would be advantageous to have the intracristal space large (fig. 6h) and uncrowded to facilitate diffusion outside of the mitochondrion. Thus, condensed mitochondria appear to have an advantage in the rapid dispersal of a higher output of ATP.

We found that while the percentage of condensed mitochondria is no greater at the PNP than at the internode, the total density of mitochondria is greater at the PNP in agreement with prior studies on cat (Berthold et al., 1993; Fabricius et al., 1993). Therefore, it seems that the higher energy demand at the PNP is met by increasing the number (mass density) of

mitochondria there rather than by a shift from orthodox to condensed conformation, consistent with the general principle that mitochondrial mass density correlates with energy demands. In addition, the abundance of condensed mitochondria in the axoplasm not seen in the neighboring Schwann cell lumen argues that the density of mitochondria is sufficient in long PNS axons to satisfy ATP needs only inasmuch as a conformational transition is also triggered that allows for a substantial rate increase of mitochondrial ATP production. The difference in mitochondrial structure in adjacent cells reflects distinctly different environments and metabolic compartmentation in astrocytes and neurons (Sonnewald et al., 2004) and may also be influenced by the tremendous variation in protein and/or lipid composition of mitochondria from different cell types (Pagliarini et al., 2008).

We noted that though not a common occurrence, subdomains inside a mitochondrion could range from orthodox to condensed. Even though Haigh and coworkers (Haigh et al., 2007) provided evidence that individual mitochondria are equipotential throughout the entire organelle, they likely did not examine mitochondria possessing both condensed and orthodox components. Hence, one should not rule out the possibility that there are local differences in membrane potential within mitochondria showing a range of conformation.

Although the percentage of condensed mitochondria is no greater at the PNP than at the internode, it may be that the degree of condensation is slightly greater at the PNP compared to the internode. The differences in the cristae number (fig. 4), and the crista junction opening size (fig. 6), along with the trend for the OM-IM distance and crista width to be larger suggest that the mitochondria in the PNP region may be in a more condensed state than the mitochondria located at the internode. A transition to a more condensed state might manifest *in situ* via changes to crista junction size and cristae number (although a change in number would also require cristae membrane remodeling) that do not correspond to changes in the total cristae volume (fig. 6), considered a hallmark of the transition to the condensed configuration in isolated mitochondria.

Increased ATP Production by Condensed Mitochondria

By how much does a condensed configuration confer an increase in ATP production over orthodox mitochondria? It is thought that in healthy tissues, mitochondria are confined to only two respiration states—states 3 and 4 (Fiskum, 1986). State 3 respiration is the rate of oxygen consumption in the presence of substrate, phosphate, and ADP, whereas state 4 is a “resting” state after the ADP has been converted to ATP, i.e., high ATP/ADP ratio and low metabolic activity. Thus, state 4 respiration is measured as the rate of respiration before the addition of ADP. The orthodox to condensed transformation is induced by a low ATP/ADP ratio in the cytoplasm, brought about by the onset of a high rate of cellular metabolism (Wookey, 1980). Davis and Davis-van-Thienen (Davis and Davis-van Thienen, 1978) showed that as this ratio declines, the respiration of mitochondria approaches state 3, i.e., the active state. The respiratory control ratio (RCR), defined as the ratio of state 3 to state 4 rates of oxygen consumption (Fiskum, 1986) is the factor by which condensed mitochondria produce ATP over their orthodox counterparts under more-or-less normal conditions. Intact mitochondria *in vitro* can have RCR values as high as 15 that may be higher *in vivo* (Tzagoloff, 1982). It has been reported that ratios of 3–6 are common with malate or glutamate as substrates for rat liver mitochondria and ratios of 15 are not unusual with pyruvate as substrate for mitochondria derived from insect flight muscle (Whittaker and Danks, 1978). This ratio depends on the dissipation of the proton gradient across the mitochondrial inner membrane by 1) proton “leakage”, the antiport of protons, and the transport of other ions, 2) uncouplers, and 3) some residual ATP synthase activity. For brain mitochondria isolated from various rodents, the RCR varies from a low of 5.4 (Guinea pig) to a high of 14.7 (Gerbil). Rat and rabbit values lie somewhere between these extremes and

seem to cluster around 6–10 (Fiskum, 1986). The RCR for the PNS is likely similar to that of the brain because the “Energy-requiring processes and energy-producing pathways used in the peripheral nervous system appear to be the same as those in the central nervous system” (Pellerin and Magistretti, 2003). Thus, the amount of ATP available to drive ion transport and receptor-ligand binding at the PNP (Ellisman and Levinson, 1982; Mi et al., 1999; Sosinsky et al., 2005) would be several times greater with condensed mitochondria. However, we and others (Berthold et al., 1993; Edgar et al., 2008) noted that the variation in mitochondrial presence at the PNP is great and we observed no greater density of the condensed organelle there. Does this imply that nodes are heterogeneous in their energy needs, or possibly that where there are more mitochondria, a number of them are no longer viable, even with no obvious structural defect?

The Crista Junction

Although more structural details of the crista junction have become available in the last few years, it continues to be an open question as to what its full functional significance really is. Still only little is known about the molecular mechanisms mediating the formation and maintenance of this junction (Zick et al., 2008). Crista junctions may form spontaneously (Mannella et al., 2001; Ponnuswamy et al., 2005; Renken et al., 2002) and may not be a permanent structure (Perkins et al., 2001b). We found that there were relatively few crista junctions in axonal mitochondria in contrast to Schwann cell mitochondria. Why so few crista junctions? Having few crista junctions may trap supracomplexes of oxidative phosphorylation (Boekema and Braun, 2007) within the cristae membrane, already impeded in their diffusion across the crista junction because of their high molecular weight, as proposed by Zick and coworkers (2008). The crista junction opening size was essentially no different between PNP and Schwann cell mitochondria with the internode organelle having a smaller opening, consistent with a recent finding that the crista shape is not a reliable indicator of the size of the crista junction opening (Yamaguchi et al., 2008). With only speculative knowledge of the protein constituents of the crista junction, centering on OPA1 and complex V, current thought is that these junctions are not absolute barriers, but may be kinetic or diffusion barriers, or even gates for specific macromolecules (Mannella, 2006a,b; Vogel et al., 2006). A recent theoretical consideration was proposed to model how curvilinear membrane geometry occurring at the crista junction induces transient anomalous diffusion between the cristae and inner boundary membranes (Sukhorukov and Bereiter-Hahn, 2009). The observed differential distribution of protein complexes cristae and inner boundary membranes (Gilkerson et al., 2003; Vogel et al., 2006; Wurm and Jakobs, 2006) can be simulated by an asymmetric crista junction permeability. The preferential localization of protein molecules to cristae, such as cytochrome oxidase [34], could augment the impact of junctional and membrane topology on diffusion mobility of such molecules. The smaller crista junction opening size in internodal mitochondria is modeled to decrease the anomalous diffusion of proteins along the inner membrane, indicative of a more stable environment and may reflect the in-transit situation of these mitochondria. The modeling of Sukhorukov and Bereiter-Hahn does not include the protein composition of the crista junction, which may provide a diffusion barrier and may consist of differing concentrations of suspected junctional proteins such as OPA1, complex V, mitofilin (John et al., 2005), and ChChD3. Because of the changes in crista junction opening size and cristae topology upon disruption of OPA1 oligomers (Yamaguchi et al., 2008), the oligomeric state of OPA1 and complex V could be examined in PNS mitochondria for clues to the unique topology of their inner membranes, including the narrowed crista junction. Clearly, further attention is needed to understand the structure and function of the crista junction.

Conclusion

In summary, through the use of electron tomography and stereology, we provide observational and quantitative analyses of PNS mitochondria that have relevance to energy usage and disease states relating to compromised energy production. The significance of our work relates to the concept that there is a need for the mitochondria in peripheral nerve axoplasm to operate at a high workload of ATP production. Electron tomography provided a three-dimensional characterization that highlights the differences between previously published in vitro condensed mitochondria and the organelle found in situ. Differences in mitochondrial abundance and condensed state exist between nodal, internodal, and Schwann cell regions suggesting a differential energy usage in these regions, likely driven by ion channel activity. We tie the observation of abundant condensed mitochondria in the axoplasm to prior bioenergetic considerations relating to oxygen consumption and thus ATP production. This work helps to broaden our understanding of the factors that govern the variability of mitochondrial ultrastructure and the link to mitochondrial bioenergetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A and B Model of the classical orthodox mitochondrion. Based on Munn (1974), p. 326, Fig. 8.5a. Mitochondria have two membrane systems—outer and inner. With the orthodox organelle, the inner membrane can be topographically divided into two components—inner boundary membrane (IBM) and cristae membranes. The cristae can adopt a continuum of shapes, sizes, and orientations. The most common forms are tubes and lamellae depicted here. The cristae membrane connects to the IBM via narrow tubes of relatively uniform opening diameter termed “crista junctions” (arrowheads). The outer membrane is displayed in translucent blue, the IBM in translucent grey (A) and opaque grey (B), and the cristae in green. A cut-away view of (A) is shown in (B) with the IBM made opaque to highlight the small surface area occupied by the crista junction openings. **C and D.** Model of classical condensed and ultracondensed mitochondria. Compare Munn (1974), p. 326, Fig. 8.5c and d, respectively. Because the condensation of the mitochondrial matrix varies and this changes the compartment architecture, this model has two parts—ultracondensed (uc) and condensed (c). Typically, with the condensed organelle, the intracristal compartment is expanded as are the crista junctions (arrowheads). As the matrix becomes more condensed, the IBM is pulled away from the outer membrane and the crista junctions expand to the point that they are abolished leading to the ultracondensed state. In the ultracondensed organelle, the matrix can appear like a “sausage” snaking its way through the interior. The outer membrane is displayed in translucent blue. For the ultracondensed portion, the sausage-like matrix is shown in red. There is no IBM and the cristae are all interconnected and occupy the mitochondrial interior not occupied by the contracted matrix. The IBM for the condensed portion is shown in translucent grey (C) and opaque grey (D), and the cristae in yellow. A cut-away view of (C) is shown in (D) with the IBM made opaque to contrast the expanded crista junction openings in the condensed organelle compared to the orthodox organelle.

Figure 2.

Condensed mitochondria, with characteristic dilated cristae, are abundant in the axoplasm. Images from thin sections of rat spinal root prepared by conventional fixation and HPF/FS were analyzed for mitochondrial volume ratios, configuration, and crista width. **a.** Orthodox mitochondria from HPF/FS samples had the typical narrow cristae characteristic of this configuration. **b.** Mitochondrion in (a) expanded 3x to better visualize the cristae. The arrows point to two narrow cristae arranged roughly parallel and along the long axis of the organelle, typical of the orthodox mitochondria found in spinal root axons. **c.** Condensed mitochondria from HPF/FS samples had dilated cristae. Because the matrix is contracted to a smaller volume by the enlarged cristae, this configuration is termed “condensed”. Even though condensed, these mitochondria do not have the inner boundary membrane pulled away from the outer membrane, which is the classical morphology of condensed mitochondria in vitro. Same magnification as in (a). The inset shows the mitochondrion (*) expanded 3x. Arrows point to two cristae arranged in sequence with widths that are roughly half the diameter of the mitochondrion. **d.** The density of mitochondria is greater at the node and paranode than internode. Stereological measurements of PNPs and internodes “far” from a node, i.e., stretches of myelinated axon in low-magnification images where no node was seen, provided mitochondrial volume ratios. The box plot shows percent of axoplasmic volume occupied by mitochondria. The bottom of the box represents the lower quartile, the top of the box represents the upper quartile, the line through the box represents the median. The top and bottom of each whisker is $1.5 \times \text{IQR}$ (the interquartile range). Outliers are depicted with triangles above the whisker. The paranode had no outliers. A box plot was chosen to display the data because of the large variation in the volume density of mitochondria between node, paranode, and internode. The box plot shows the skew towards zero, i.e., a relatively large number of nodal and internodal axoplasm contained no mitochondria. Two metrics for variation (lower/upper quartiles and $1.5 \times$ interquartile range—whisker), and the outliers, of which the nodes and internodes had a few, corresponding to a high density of mitochondria. The number of measurements is shown inside each box. There is no significant difference in mitochondrial volume density between node and paranode, yet a large difference between these and the internode was found (**, $p < 0.01$). **e.** Condensed mitochondria comprise roughly two-thirds of axonal spinal root mitochondria, with orthodox mitochondria constituting the remainder. We found no difference in the relative abundance of condensed and orthodox mitochondria between node, paranode, and internode and so combined the measurements. Mean values are shown (error bar = SEM for 4 animals). **f.** Condensed mitochondria have a much larger crista width (**, $p < 0.001$). Mean values are shown (error bar = SEM).

Figure 3.

Condensed mitochondria in spinal root axons have a closely apposed outer and inner boundary membranes and narrow crista junctions. **a, b, c, d.** Successive 4.4-nm thick slices through the tomographic volume of a spinal root axon prepared by HPF/FS. The matrix (m) is darker (more condensed) than the intracristal space (i). As is typical of condensed axonal mitochondria in peripheral nerve, there are few, albeit large, cristae. This example has only two cristae (1 and 2). Uncharacteristic of condensed mitochondria that have been isolated, yet typical of the in situ organelle, the outer and inner boundary membranes are in close apposition (between white arrows). Common for axonal mitochondria in the condensed configuration, the cristae are separated by a relatively narrow “bridge” of matrix (black arrows). Two crista junctions were observed in these slices, one per crista. Boxed areas were expanded 3x and placed as insets top and bottom. The top crista junction is barely open in (a), open in (b) and (c), and closed in (d). The bottom junction is closed in (a) and (d) and open in (b) and (c). The numbered, white-boxed areas in (c) are shown enlarged in (h) and are examples of contact sites between outer and inner boundary membranes. A portion of a microtubule next to the mitochondrion is outlined. scale bar = 200 nm. **e. and f.** Top view and side view of the segmented volume that was surface-rendered. The outer membrane is shown in blue and made transparent to see the two cristae (green and cyan). The visible crista junctions are indicated by arrowheads. **g.** Segmented inner boundary membrane and side view of the surface-rendered volume. Seven crista junctions are seen in this view (numbered). The narrowness of the opening is similar to what is observed with orthodox mitochondria in situ, but less so with the isolated condensed organelle. See supplemental movie 1 for 3D perspectives. **h.** Contact sites between the outer and inner boundary membranes are abundant. Boxed areas in (c) are shown enlarged 3x to demonstrate the range of membrane contacts (arrowheads). 1. A bridge contact spanning the intermembrane space. 2. Two bridge contacts close to each other with the top bridge being about twice as thick as the bottom bridge. 3. A classical contact site. 4. A classical contact site that is about twice as long. 5. An area where the outer and inner boundary membranes appear to be “zippered”. **i.** Mitochondrial association with microtubules is greatest at the internode and least at the node. The percentage of mitochondria in close proximity to microtubules (e.g. see d), observed with both cTEM and electron tomography. The number of measurements is shown over each bar (* $p < 0.05$ —applies to node/internode only). Six animals were used. Error bar = SEM for $n = 6$. **j.** Crista junctions in condensed mitochondria are not enlarged. The mean crista width at its largest opening in tomographic reconstructions is compared in all axonal regions for orthodox and condensed mitochondria. The number of measurements is shown over each bar. Error bar = SEM.

Figure 4.

There are fewer cristae in mitochondria at the PNP region. **a.** Slice through a tomographic volume from a conventionally prepared sample of a mitochondrion at the PNP having only two cristae. A matrix bridge separates these large cristae (arrowhead). Scale bar = 200 nm. **b.** Top view and **c.** side view of the segmented volume from (a) that was surface-rendered according to membranous compartments. The outer membrane is shown in blue and made transparent to see the two cristae (green and cyan). The visible crista junctions are indicated by arrowheads. The structure is similar to the mitochondria prepared by HPF/FS, shown in figs. 1 and 2. **d.** Slice through a tomographic volume of a mitochondrion at the internode. Same scale as in (a). More cristae, and concomitantly more matrix bridges (arrowheads), were observed in mitochondria at the internode. A narrow crista junction typical of these axonal mitochondria is seen in this slice (arrow). **e.** Top view and **f.** side view of the segmented volume from (d). The outer membrane is shown in transparent blue to better visualize the 9 cristae. **g.** Measurements of mean cristae number per condensed mitochondrion at the nodal, paranodal, and internodal regions. (error bar = standard error). There are significantly fewer cristae per mitochondrion at the PNP compared to the internode (*, $p < 0.001$). See supplemental movie 2 for 3D perspectives.

Figure 5.

Subregions inside axonal mitochondria can display different conformational states. Three slices through a single mitochondrion at different depths of the tomographic reconstruction are shown. The slice shown at left was taken near one end of the mitochondrion and shows predominantly condensed matrix characteristics; the slice shown in the middle was taken near the middle of the reconstruction and displays both condensed (bottom) and orthodox (top) conformations; and the slice shown at right was taken near the other end of the reconstruction and shows only an orthodox conformation. Scale = 200 nm.

Figure 6.

As determined by electron tomography, paranodal mitochondria in Schwann cells have different substructure than those in axons. **a.** Slice through a tomographic reconstruction showing 10 mitochondria in close proximity in a Schwann cell. The mitochondria all show an orthodox configuration, unlike those in neighboring axons. The cristae are comprised of both tubes and lamellae. A surface-rendered volume (*) is shown in (b). Scale = 200 nm. **b.** Top and side views of a mitochondrial volume in (a) that was segmented and surface-rendered. The outer membrane is shown in blue and made transparent to see the six cristae present in this mitochondrion (various colors). The visible crista junctions are indicated by arrowheads in the side view. **c.** The crista volume ratio was higher in the PNP and internodal mitochondria than in the Schwann cell mitochondria (*; $p = 0.033$ PNP/Schwann; $p = 0.00069$ internode/Schwann). Mean values of the ratio of the sum of the cristae volumes to the mitochondrial volume are shown. The number of mitochondria measured in the tomographic reconstructions is indicated above each bar (same as panels d and e). **d.** In contrast to the volume ratio, the crista surface area ratio was lower in the PNP and internodal mitochondria than in the Schwann cell mitochondria (*; $p = 0.034$; PNP/Schwann; $p = 0.022$ internode/Schwann). Mean values of the ratio of the sum of the cristae membrane surface areas to the mitochondrial membrane surface area are shown. **e.** The density of crista junctions is significantly higher in Schwann cell mitochondria compared to those in the spinal root axoplasm. The total number of crista junctions per mitochondrion were counted and divided by the mitochondrial surface area derived from the tomographic volume to determine the crista junction density. Values are means (**; $p = 2.6 \times 10^{-5}$ PNP/Schwann; $p = 3.9 \times 10^{-8}$ internode/Schwann). **f.** The crista junction opening size is smaller in internodal mitochondria compared to PNP mitochondria (*; $p = 0.018$), but shows no significant difference between the axonal and Schwann cell organelle and between conventional preparations and those incorporating high-pressure freezing and freeze-substitution (data not shown). Mean values of the crista junction opening size are shown. The maximal width of the crista junction opening was measured and does not include the membrane width, i.e., only the opening itself was measured. See supplemental movie 3 for 3D perspectives. **g.** Even though condensed, axonal mitochondria have a mean intermembrane space width (OM-IM distance) no different than Schwann cell mitochondria with their orthodox configuration. Yet, their mean crista width (**h**) is significantly greater, as expected for condensed mitochondria (**; $p = 0.0012$ PNP/Schwann; $p = 5.3 \times 10^{-5}$ internode/Schwann). For f, g, and h, the inner distance between membranes was measured, i.e., the membrane width was excluded. (Error bar = standard error for all graphs).

Figure 7.

A. Model of mitochondria found in the PNS axon. The mitochondria are typically long and narrow, yet are condensed (see fig. 3). There are few cristae, with 2 shown here (c) that usually line up along the long axis (see fig. 3, 4). Unlike the classical condensed organelle (compare fig. 1), the inner boundary membrane (IBM) and outer membrane (OM) are closely apposed along the periphery (paired arrowheads), reminiscent of the orthodox organelle. The OM and IBM are translucent to visualize the cristae, shown here as large, elongated compartments common in PNS axonal mitochondria. **B.** Cut-away view of the model. The cristae have few crista junctions (arrows), which are the narrow tubular architecture of relatively uniform opening diameter typically found in the classical orthodox mitochondrion in contrast to the classical condensed organelle (compare fig. 1). A defining feature of these mitochondria is narrow matrix bridges between cristae (paired arrowheads; see fig. 3, 4). **C.** Model of the PNS node of Ranvier. A cut-away view shows the Schwann cell in white with 3 regions demarcated: “node”, “paranode”, and “internode” and the axonal shaft in light blue. The axoplasm is populated with the modeled mitochondria of A and B with a greater density in the nodal region (arrow), as measured (see fig. 2). The Schwann cell mitochondria have the classical orthodox configuration (see fig. 1) and are often clustered (arrowhead; see fig. 6). The modeled orthodox organelle of fig. 1 is used for this display.