1 **Morphology of Mitochondria in Spatially Restricted Axons Revealed by Cryo-**
2 **Electron Tomography**

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4 Tara D. Fischer[1,2†], Pramod K. Dash[1,3], Jun Liu[1,‡], and M. Neal Waxham[1*]

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6 **Author Affiliations:**
7 [1]Department of Neurobiology and Anatomy, McGovern Medical School, University of
8 Texas Health Science Center at Houston, Houston, TX 77030

9

10 [2]The University of Texas MD Anderson Cancer Center UTHealth Graduate School of
11 Biomedical Sciences; The University of Texas Health Science Center at Houston,
12 Houston, Texas 77030

13

14 [3]Vivian L. Smith Department of Neurosurgery, McGovern Medical School, University of
15 Texas Health Science Center at Houston, Houston, TX 77030

16

17

18 Present Address:
19 [†]Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological
20 Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892
21 [‡]Department of Microbial Pathogenesis & Microbial Sciences Institute, Yale School of
22 Medicine, New Haven, CT 06519

23

24 ***Corresponding Author:** M. Neal Waxham. 6431 Fannin St., MSB 7.254, Houston, TX
25 77030. (713) 500-5621. m.n.waxham@uth.tmc.edu

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Abstract

Neurons project axons to local and distal sites and can display heterogeneous morphologies with limited physical dimensions that may influence the structure of large organelles such as mitochondria. Using cryo-electron tomography (cryo-ET), we characterized native environments within axons and presynaptic varicosities to examine whether spatial restrictions within these compartments influence the morphology of mitochondria. Segmented tomographic reconstructions revealed distinctive morphological characteristics of mitochondria residing at the narrowed boundary between presynaptic varicosities and axons with limited physical dimensions (~80 nm), compared to mitochondria in non-spatially restricted environments. Furthermore, segmentation of the tomograms revealed discrete organizations between the inner and outer membranes, suggesting possible independent remodeling of each membrane in mitochondria at spatially restricted axonal/varicosity boundaries. Thus, cryo-ET of mitochondria within axonal subcompartments reveals that spatial restrictions do not obstruct mitochondria from residing within them but limited available space can influence their gross morphology and the organization of the inner and outer membranes. These findings offer new perspectives on the influence of physical and spatial characteristics of cellular environments on mitochondrial morphology and highlights the potential for remarkable structural plasticity of mitochondria to adapt to spatial restrictions within axons.

Introduction

Neurons are architecturally complex cells that can extend axonal projections with elaborate arborization for several hundreds of millimeters (and in some cases, meters) to form synaptic connections with local and distal targets [1-3]. Depending on the target, presynaptic compartments can either be found at the end of axons (terminal boutons) or tracking along axons as intermediate swellings (*en passant* boutons or varicosities), such as in the unmyelinated CA3->CA1 axons of the hippocampus [3-7]. The bead-like presynaptic varicosities are morphologically heterogeneous, displaying diameters that can range from 1-2 µm connected by thin axon segments that can have diameters less than 100 nm [4, 5, 8, 9].

Tracking through the axons is a well-developed system of microtubules that mediate motor-driven anterograde and retrograde transport of signaling cargoes, protein complexes and organelles critical for function and homeostasis at distant synapses [10, 11]. Transport of these intracellular components creates a spatially and temporally dynamic environment within the axon that can contain a variety of organelles and cargoes of different shapes, sizes, and number [12, 13]. As axon segments interconnecting varicosities can be remarkably thin and occupied by various structures, whether physical adaptations to available space are required for the motility of large organelles, such as mitochondria, with diameters ranging between 100-500 nm, poses an interesting question [14, 15]. Mitochondria within axons and synaptic compartments are particularly critical for development, function, and plasticity. During transport, mitochondria are known to make frequent stops, or saltatory movements, at presynaptic compartments to provide local ATP synthesis and calcium regulation required for proper neurotransmission [16-20]. Given the morphological complexity of axons, restricted physical dimensions and available space could potentially influence the subcellular localization, distribution, and transport of mitochondria required to meet local energy needs and for supporting synaptic transmission. Although mitochondria are morphologically dynamic organelles that can exist in a variety of shapes and sizes, how mitochondria adapt to the physical constraints presented in axons has not been previously examined.

Advances in microscopy and imaging techniques have played a pivotal role in revealing the three-dimensional architecture of neurons and their intracellular environments at resolutions reaching the nanometer scale [21, 22]. In the current study, we employed cryo-electron tomography (cryo-ET) to visualize three-dimensional spatial

87 relationships and organellar structure within cultured hippocampal axons and
88 varicosities. The unique native state preservation afforded by cryopreservation and the
89 resolution of cryo-ET revealed that axon morphology and physically restrictive
90 intracellular dimensions present a previously unrecognized influence on the morphology
91 and ultrastructure of mitochondria residing at the boundary between large varicosities
92 and small axonal subcompartments.

93

94 **Results**

95 **Preparation of primary hippocampal neurons for cryo-ET**

96 　　　　Primary neuronal cultures from E18 rat hippocampi were grown on holey carbon
97 grids prior to cryo-preservation. At 10 days post plating, primary hippocampal neurons
98 have extended dendritic and axonal processes and have established presynaptic
99 varicosities and initial excitatory synaptic connections [23]. **Figure 1A** and **B** show
100 representative bright field images of neurons cultured on a Quantifoil grid where
101 widespread elaboration of processes is evident. **Figure 1C** and **D** demonstrate images
102 of companion grids that were fixed and immunolabeled with antibodies to
103 calcium/calmodulin dependent protein kinase II alpha (CaMKIIα) and synapsin 1 to
104 visualize the excitatory neuron population and presynaptic varicosities, respectively.
105 CaMKIIα antibodies largely label soma and processes, while the synapsin 1 antibody
106 shows distinct puncta representing enrichment of synaptic vesicles at presynaptic
107 varicosities.

108 　　　　For cryo-ET, fiducial gold markers were applied to prepared grids to aid in image
109 alignment during data acquisition and image processing, and then cryopreserved by
110 plunge freezing in liquid ethane. Cryopreservation conserves the near native state of the
111 preparation permitting an assessment of spatial relationships between organelles
112 present in different neuronal compartments free of fixation- or stain-induced artifacts.
113 Low magnification images were first collected (**Fig. 1E**) and montaged to provide maps
114 for targeting areas of interest for data collection. A higher magnification image reveals
115 the distribution of presynaptic varicosities and axonal processes (**Fig. 1F**). Varicosities
116 and axons can be seen residing on both the carbon and overlying the grid holes. To
117 provide maximum contrast, tomographic data collection was targeted to cryopreserved
118 structures within the grid holes. In these preparations, the increased thickness of the
119 soma and proximal dendrites prevented sufficient electron beam penetration for imaging
120 of these structures. In contrast, the sample thickness surrounding the axonal processes

121 and varicosities was ideal, permitting a detailed assessment of the spatial relationships

122 of cytoskeletal structures and organelles within these subcompartments.

123

124 **Organelle populations of presynaptic varicosities in primary hippocampal neurons**

125 **are heterogeneous**

126 Areas were randomly chosen, and tilt series were collected from varicosities and

127 axon segments overlying grid holes to visualize cytoskeletal and organellar structures.

128 **Figure 2A** shows a slice through a representative tomographic reconstruction with

129 various resident organelles and structures visible, including two mitochondria, a multi-

130 vesicular body (MVB), microtubules, endoplasmic reticulum (ER) and a collection of

131 vesicles. Supplementary **Figure S1** shows 2D images of identified organelles and other

132 structures observed within the population of varicosities analyzed. To determine the

133 three-dimensional (3D) relationship between the different structures, segmentation was

134 accomplished of the tomographic reconstruction of both the presynaptic varicosity and

135 the adjoining axon (**Fig. 2B; Movie S1**). The reconstruction demonstrates microtubules

136 (light blue) forming a continuous set of tracks traveling from one end of the varicosity to

137 the other. Microtubules are well organized and relatively straight in axons, however, they

138 exhibit greater curvature in the varicosity, while again gathering together and

139 straightening when passing through the adjoining axon segment. The ER (yellow) also

140 forms a reticulated and continuous structure spanning the entire length of the varicosity,

141 consistent with other reports on the ubiquitous presence of ER in axons and synaptic

142 terminals [21]. Mitochondria, MVB, and other sac-like compartments, exhibit more

143 random distributions within the varicosity, while vesicles appeared to be somewhat

144 clustered. To determine if the presence of each of these identifiable organelles was

145 consistent across varicosities, we analyzed their distribution and characteristics in an

146 additional 77 tomographic reconstructions. ER and microtubules were present in 100%

147 of presynaptic varicosities, vesicles were present in 97%, mitochondria in 82%, sac-like

148 compartments in 38%, MVBs in 21%, lamellar bodies in 9%, and autophagosomes in 4%

149 (Table 1).

150

Table 1. Organelle population representations

	Presence within population (%) n = 77
Mitochondria	81.8
Number/Varicosity	
0	18.1

1	66.2
>1	15.5
Gross Morphology	
Round	26.3
Slightly Tubular	30.2
Tubular	43.4
Internal Morphology	
Thin, tubulated	23.6
Thick, unstructured	76.3
Endoplasmic Reticulum	100
Contact with Mito	38.4
Near Mito	89.7
Vesicles	97.4
Number/Cell	
1-25	2.5
25-50	64.9
50-100	10.3
>100	6.4
MVBs	20.7
Lamellar Bodies	9.0
Unidentified Membrane-bound Compartments	37.6
Autophagosome	38.9

151

152   Variations in distinct sub-features of the observed organelle populations also

153   emerged in 3D at high resolution. Specifically, the ubiquitous ER was observed in close

154   apposition to almost every other organelle within the varicosities, consistent with ER-

155   membrane contact sites described by others [21].   The vesicular population was

156   heterogeneous in number, ranging from 1 to >100 vesicles per varicosity.   75% of

157   varicosities contained <50 vesicles, 10% had 50-100, and 6% contained more than 100

158   (Table 1).   Vesicles were observed in clusters with electron-dense filamentous

159   connections (data not shown), consistent with previous reports of proteinaceous

160   (synapsin, Bassoon, and/or ERC2) tethering of synaptic vesicles [12, 13]. Mitochondrial

161   cristae structure within the 3D tomographic datasets was variable, but could be broadly

162   segregated into two distinct populations based on ultrastructural features (**Fig. S2**). A

163   population of mitochondria displayed the canonical thin, tubular cristae morphology (**Fig.**

164   **S2A**), while a second population displayed thick, unstructured cristae (**Fig. S2B**).

165   In contrast to the heterogeneous appearance and distribution of organelles in

166   presynaptic varicosities, the adjoining axonal segments were more consistent in

167   composition.   The most obvious components were microtubules that were seen as

168   continuous elements, gathered at the sites of axonal narrowing at both ends of the

169 varicosity. Microtubules did not display variability in diameter (~20 nm), however they
170 did vary in number among different processes and occupied a significant portion of the
171 available volume within the axon. As noted, microtubules are the essential tracks
172 required for motor driven organelle transport and are critical for maintaining synaptic
173 homeostasis and neuronal signaling. The corresponding microtubule occupation of
174 axonal space leaves the qualitative impression that the available volume in axons to
175 accommodate large organelles, such as mitochondria, might be an under-appreciated
176 constraint affecting transport. The magnitude of this spatial constraint can be visually
177 appreciated in **Movie S2**.
178

179 **Mitochondria display distinct morphological features at spatially restricted**
180 **axon/varicosity boundaries**

181 Previous EM studies have reported an average axonal diameter between 0.08
182 and 0.4 μm for unmyelinated cortical axons and an average varicosity diameter of ~1-2
183 μm [1, 5, 7, 8]. Varicosities and axons in our cryopreserved hippocampal preparations
184 exhibited slightly smaller Feret diameters, with an average of 649 and 81 nm for
185 varicosities and axons, respectively (Table 2). Additionally, the average Feret diameter
186 of mitochondria observed in hippocampal varicosities was ~250 nm. These dimensions
187 (summarized in **Table 2**) further reinforce the idea that significant spatial constraints
188 exist that may influence mitochondrial structure in axons.
189

Table 2. Two-dimensional morphological measures

	n	Average	Range
Varicosity			
Diameter	200	649 $\pm$241 nm	257 nm – 1.65 μm
Axon			
Diameter	195	81 $\pm$27 nm	33 – 204 nm
Mitochondria			
Length	67	647 $\pm$305 nm	255 nm – 1.68 μm
Diameter	71	291 $\pm$87 nm	160 – 547 nm

190

191 For more in-depth investigation of this issue, high magnification tomographic data
192 were collected targeting mitochondria residing at the boundary where the varicosity
193 narrows into the small axonal segment. While such precise mitochondrial positioning
194 was rare in the cryo-preserved neuronal population, the static events that were captured
195 revealed distinct mitochondrial morphologies at the boundaries between varicosities and

196 axons. **Movie S3** and **S4** demonstrate a mitochondrion partially residing in both a
197 neuronal varicosity and the adjoining axon segment among the other organelles
198 occupying space in these compartments. The portion of the mitochondrion residing in
199 the varicosity is 305 nm in Feret diameter, while the portion of the mitochondrion residing
200 in the axonal segment is narrowed to only 70 nm in diameter at its tip. Thus, the
201 mitochondrion displays a major morphological change with an approximately 77%
202 reduction in diameter within the axon. **Figure 3A** shows a snapshot of the segmented
203 mitochondrion as well as additional examples of mitochondria captured at the
204 varicosity/axonal boundary (**Fig. 3B and C**). **Figure 3C** shows a reconstruction that
205 revealed two mitochondria residing in the varicosity and adjoining axon segment, with a
206 portion of a third residing mainly in the axon. **Movies S3 – S10** demonstrate several
207 tomographic reconstructions of mitochondria displaying similar morphologies at the
208 varicosity/axon boundary. **Figure 4** and **Movie S9** show a particularly revealing
209 example, in which a mitochondrion was captured bridging a short (100 nm in length)
210 axon segment between two varicosities. The short narrow space produced a barbell
211 shaped mitochondrion with a diameter between 200-300 nm in both varicosities while
212 the portion spanning the axon segment was only 19 nm in diameter. To illustrate the
213 ultrastructural features of the mitochondrion spanning the two varicosities, the inner
214 mitochondrial membrane (IMM) and cristae were segmented in addition to the outer
215 membrane (OMM; **Fig. 4C**). The resolution of this tomographic reconstruction was not
216 sufficient to determine whether the IMM was continuous through the short axon
217 segment, however **Movie S10** demonstrates an additional example of a mitochondrion
218 spanning a short axon segment between two varicosities, in which the IMM appears
219 continuous in the constricted section of the axon. Analysis of eight mitochondria
220 exhibiting these drastic morphological features revealed an average of 84% (n = 8, SD =
221 6%) reduction in the Feret diameter of the mitochondrial area in the varicosity relative to
222 the adjoining axon segment. These captured events highlight the potential adaptability
223 of mitochondrial morphology to accommodate the available space within an axon. Note
224 that in all of the segmented tomographic reconstructions in **Figure 3 and 4**, the
225 presence of microtubules and additional organelles, such as the ER, further restricts the
226 available volume to mitochondria within axons.

227

228 **Mitochondrial membranes display unique organizations within spatially restrictive**
229 **axons**

230   Mitochondrial ultrastructure is thought to be dynamic with remodeling of the inner

231   membrane and formation of specified subcompartments, termed cristae [24-26]. Given

232   the atypical gross morphologies of mitochondria we observed between varicosities and

233   the adjoining axonal segments, we questioned if the inner mitochondrial membrane also

234   displays distinct structural features in axons with limited physical dimensions. To

235   address this issue, the outer and inner membranes of mitochondria captured at the

236   boundary between varicosities and adjoining axons were segmented, separating the

237   outer membrane, and two regional components of the IMM, the inner boundary

238   membrane (IBM) and the cristae. A conservative approach was taken during manual

239   segmentation of cristae (i.e., only clearly discernible cristae membranes were included).

240   Segmentation of each of these mitochondrial components revealed discrete

241   morphologies between the inner and outer membranes. Most notably, two distinctions

242   were observed between the outer membrane and the adjacent IBM at the narrowed

243   mitochondrial tip in the adjoining axon segment. First, in some instances the IBM was

244   observed to maintain apposition to the outer membrane at the narrowed mitochondrial

245   tip entering the axon (**Fig. 5A**). Both the inner (orange) and outer (green) membranes

246   can be seen narrowing as they enter the restricted axonal space. Interestingly, cristae

247   (pink) are largely absent from this narrowed portion of the mitochondrion (~69 nm in

248   diameter) residing in the axon. Second, the outer membrane was observed to separate

249   from the inner membrane, leaving a space free of the inner membrane and matrix of the

250   mitochondrion (**Fig. 5B**). Thus, it appears the IMM does not always remain in apposition

251   with the OMM within the rather dramatic tubulation evident of the outer membrane

252   residing within the restricted space of the axon. Two out of the eight representations of

253   mitochondria displaying these atypical morphological features in our dataset also show

254   the OMM separated from the IMM. **Movie S11** shows the segmented model of the three

255   mitochondria in **Figure 3C and 5C**. The left mitochondrion can be seen to display the

256   OMM separated from the IMM, while the right mitochondrion displays the OMM and IMM

257   in juxtaposition, demonstrating the occurrence of both events in one varicosity/axon

258   boundary area.

259

260   **Discussion**

261   Although the transport of mitochondria within axons has been widely studied, the

262   potential morphological adaptation of these large organelles to restricted physical

263   dimensions and available space within axons has not been discussed [18, 27, 28]. The

264   current study provides insight into spatial environments within presynaptic varicosities
265   and thin axons of cryopreserved hippocampal neurons, unperturbed by fixation or stains
266   via 3D cryo-ET. Distinct morphological characteristics of mitochondria were revealed at
267   the boundaries between large varicosities and axon segments with limited physical
268   dimensions (~ 80 nm). To our knowledge, this is the first study to describe such atypical
269   mitochondrial morphologies apparently influenced by the limitations of physical space
270   within thin axons. Additionally, the 3D reconstruction and segmentation of mitochondrial
271   ultrastructure revealed distinct morphological features between the inner and outer
272   mitochondrial membranes at spatially restricted axonal/varicosity boundaries, suggesting
273   possible differential regulation of each membrane during these morphological
274   adaptations.

275   Axon morphology can be widely variable depending on brain region. *En passant*
276   boutons are common in axons of the hippocampus and cortex, giving rise to
277   heterogeneous axon morphologies with presynaptic varicosities distributed along thin
278   unmyelinated axons [3-7]. As axons and synaptic varicosities are dynamic,
279   heterogeneous environments that can be occupied by organelles and molecules differing
280   in size and number, it is important to consider whether available space within the axon
281   may constrain motility or transport of large cargo, such as mitochondria. Using the
282   advantages of cryo-ET, we were able to capture the static spatial environments in thin
283   axons and presynaptic varicosities of cultured hippocampal neurons to examine
284   organelle characteristics and distribution. Mitochondria, in particular, at an average of
285   250 nm in diameter, presented a clear spatial challenge to inhabit axons that are, on
286   average, three times smaller (~80 nm). In the 3D segmentation of mitochondria residing
287   at the boundary between the larger presynaptic varicosity and thin adjoining axon
288   segments, we observed mitochondria displaying atypical morphological features.
289   Mitochondria displayed a normal morphology within the varicosity and a narrowed
290   tubulated portion, creating a "tip" that existed in the narrowed space within the axon.
291   Interestingly, in some cases this narrowed portion of the mitochondrion was smaller than
292   the inner boundary of the axonal plasma membrane, suggesting that additional material,
293   not apparent in the tomograms, might further constrain the available space within the
294   axon. The ability of mitochondria to display morphological diameters near 20 nm when
295   challenged with limiting available space within axons is surprising and suggests
296   mitochondrial morphology may be more adaptable in nature than previously considered.
297   While it is important to emphasize that our methodology does not address temporal

298   dynamics of mitochondria within axons, these observations highlight the potential for the

299   adaptability of mitochondrial morphology and present interesting questions to the

300   mechanisms that may be involved.

301   Movement of Intracellular cargo and transport within axons is mediated by

302   microtubule-associated motor proteins that create the driving force necessary to pull

303   organelles through the cytoplasm in axons and varicosities [11, 28]. Specifically, kinesin

304   and dynein motor proteins exert a mechanical force on the mitochondrion to drive

305   polarity-directed movement within axons through interactions with outer mitochondrial

306   membrane and adaptor proteins, such as Miro and Milton [29]. We speculate that

307   directional forces induced by motor proteins may drive the morphological features of the

308   mitochondrial membrane, as observed in the current study. Kinesins, in particular, are

309   known to induce membrane deformation or tubulation in *in vitro* reconstituted

310   membranes   [30, 31]. A recent study also described similar thin tubulation of

311   mitochondria that is mediated by KIF5B, a member of the kinesin family [32].

312   Interestingly, the influence of mechanical forces on mitochondrial membrane dynamics

313   was recently demonstrated by the recruitment of mitochondrial fission machinery and

314   subsequent division at sites of induced physical constriction [33]. In fact, it is also

315   possible that recruitment of such machinery would lead to the production of fission

316   intermediates of the mitochondria of reduced size that would facilitate their movement

317   through axons. If so, appropriate machinery would have to be present in adjacent

318   varicosities for the reassembly of mitochondria. Although the distinct morphological

319   features of mitochondria in axons observed in our static, cryopreserved tomographic

320   reconstructions cannot address dynamics for transport, whether force-driven microtubule

321   interactions play a role is an interesting possibility. Additionally, it is also possible that

322   neuronal activity might influence the structure of varicosities, axons or mitochondria that

323   would impact the magnitude of this problem. In this context, a recent report analyzing

324   varicosities and axons in hippocampal slices, showed that high-frequency stimulation of

325   axons, increased the size of varicosities and axons [34], although the peak effects on

326   size were relatively modest (~5% increase in varicosity/axonal diameter). Thus, further

327   investigation into the precise mechanisms involved in the regulation of space within

328   axons and mitochondrial morphological adaptations within the available space is

329   warranted.

330   Mitochondrial function is highly dependent on the unique architecture of the inner

331   mitochondrial membrane (IMM), in which the respiratory complexes along with ATP

332 synthase are concentrated in formations of distinct compartments, termed cristae [26,

333 35-37]. Classic and more recent studies examining inner membrane morphology and

334 cristae formation have shown distinct ultrastructural features in states of high cellular

335 energy demands, however whether inner membrane structure changes occur

336 correspondingly with gross morphological changes (excluding those involved in

337 mitochondrial fission or fusion) is not well defined [24-26, 38-41]. Therefore, we

338 questioned whether the significant reduction in size of mitochondria within the physically

339 restrictive axonal space also affected their internal structure. Segmentation of cristae

340 and the inner boundary membrane (IBM), distinct components of the IMM, revealed

341 intriguing differences in the relationship between internal structure and the outer

342 mitochondrial membrane (OMM). In some cases, the IBM remained in close apposition

343 with the OMM at the narrowed tip of the mitochondrion in the axon. Interestingly, this

344 area was also void of cristae, whereas cristae structure remained unperturbed in the

345 portion of the mitochondrion that resided in the varicosity, suggesting possible

346 differential regulation of cristae compartments with adaptation to the limited space within

347 axons. Conversely, a few mitochondria displayed a dissociation of the inner and outer

348 mitochondrial membrane, in which the outer membrane was no longer in apposition with

349 the IBM, but distinctly separated in the narrowed tip of the mitochondrion creating a

350 "matrix-free" space. The IBM and cristae remained intact and unperturbed within the

351 portion of the mitochondrion residing in the varicosity. The visualization of the 3D

352 ultrastructure of mitochondria displaying these morphological features within spatially

353 restricted axons suggests a potentially new instance of structural remodeling of the IMM.

354 Mechanistically, this observation generates several interesting questions to the

355 regulation and dynamic nature of mitochondrial ultrastructure, as well as the functional

356 status of mitochondria in different axonal compartments. Although mechanisms for

357 regulating inner membrane morphology remain incompletely defined, recent research

358 has provided insight into some mechanisms involved in the regulation of cristae structure

359 and IMM morphology [25, 37, 42, 43]. In addition to Opa1, the primary IMM fusion

360 protein, ATP synthase and the mitochondrial contact site and cristae organizing system

361 (MICOS) exhibit membrane bending functions and are proposed to determine curvature

362 of the cristae membrane [37, 38]. MICOS components have also been implicated in

363 regulating cristae morphology, as well as inner-outer membrane tethering, which could

364 be a potential mechanism driving the differences in inner-outer membrane apposition or

365 dissociation observed in the present study [44, 45]. Moreover, distinct events of IMM

366 constriction independent from the OMM have also recently been observed that are
367 driven by increased mitochondrial matrix calcium levels [46-48]. Thus, there is some
368 evidence at the cellular level for differential morphological regulation of the inner and
369 outer membranes. The 3D reconstructions of the OMM, IBM, and cristae in the cryo-
370 preserved axon, and the observed dissociation between membrane morphologies in the
371 current study lends support for the notion that the OMM and IMM can be remodeled
372 independently. Functionally, the observed changes in cristae and IMM morphology also
373 pose the question as to whether the lack of cristae or adaptation of the IMM influence
374 the functional capacity of these mitochondria. Although the technical limitations of
375 measuring mitochondrial function in relation to mitochondrial structure are challenging,
376 these will be critical investigations in uncovering the dynamic and adaptive nature of the
377 IMM and regulation of mitochondrial function in axons.

378   The importance of mitochondrial dynamics and synaptic localization to support
379 neuronal function is well recognized (for reviews see [17, 18, 49]). Mitochondria must
380 traffic through axons and populate distal synapses to mitigate local energy depletion and
381 maintain calcium homeostasis required for vesicle release and recycling [16, 50, 51].
382 Additionally, mitochondria undergo active fission, fusion, mitophagy, and maintain
383 contact with other organelles to communicate, functionally adapt, and maintain quality
384 control within the local environment. Thus, disruptions in mitochondrial motility and
385 dynamic behaviors can be detrimental to synaptic communication, plasticity, and
386 survival, and have been implicated in several neurodegenerative processes (reviewed in
387 [17, 29, 52]).   The current study highlights the potential implications for spatial
388 restrictions dictated by axon morphology to influence mitochondrial morphology, and
389 potentially motility within the axon. These findings offer new perspectives on the physical
390 and spatial influence of the cellular environment on mitochondrial morphology and
391 highlights the remarkable structural plasticity of mitochondria to adapt to the limited
392 available space within axons. Given the importance of membrane structure for
393 mitochondrial function and the necessary transport of mitochondria for maintaining
394 synaptic health and neurotransmission, these findings have far reaching implications for
395 mitochondrial and neuronal biology.   Future research will be essential to gain
396 mechanistic insight to regulation of structural changes at mitochondrial membranes and
397 the influence of morphological adaptations of mitochondria during axonal transport.
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409   **Materials and Methods**

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411   Neuronal Culture and Cryopreservation

412   Primary neuronal cultures were prepared from E18 rat hippocampi. All protocols

413   involving vertebrate animals were approved by the Institutional Animal Care and Use

414   Committee prior to initiating the studies. Briefly, pooled hippocampi were digested with

415   papain for 20 min at $37^{o}C$ and then triturated with a 10 ml pipet. Cells were counted and

416   diluted in Opti-MEM containing 20 mM glucose to a density of 1.5×10^{5}/ml. 200 mesh

417   gold grids covered with Quantifoil 2/1 carbon were placed in 35 mm glass bottom Mat-

418   Tek dishes and were treated overnight with 100 ug/ml poly-D-lysine. The dishes were

419   then washed 3x with sterile water before plating cells at a density of 1.5×10^{6}/dish. After

420   letting the cells attach for 1 hr at $37^{o}C/5\%CO_2$, the media was exchanged for Neurobasal

421   A supplemented with 2% B-27 (Life Technologies), GlutaMAX (Thermo Scientific,

422   Waltham, CA), and penicillin-streptomycin (Sigma, St. Louis, MO) and incubated for 10 d

423   at $37^{o}C/5\%CO_2$.

424   To cryopreserve intact neurons, the grids were lifted from the Mat-Tek dishes

425   and 5 uL of Neurobasal media containing BSA coated 10 nm gold fiducials was applied.

426   Fiducial gold facilitates tracking during image acquisition of tilt series and alignment of

427   image frames during post-acquisition processing. After manual blotting, the grids were

428   plunged into liquid ethane cooled with liquid N_2. The entire process between removal of

429   the grid from the culture dish and plunge freezing was on average ~30 s, but never more

430   than 60 s. Cryo-preserved grids were stored in liquid N_2 until use.

431

432   Immunocytochemistry

433   Immunostaining of neurons grown on Quantifoil grids was accomplished by fixing

434   the neurons at 10 d post-plating in freshly prepared 4% paraformaldehyde in 0.1 M

435   phosphate buffer, pH 7.4, for 10 min at room temperature. The fixative was removed

436   and reaction quenched with a 5 min incubation in 50 mM glycine in 0.1 M phosphate

437   buffer, pH 7.4. Neurons were permeabilized with 0.5% TX-100 in 0.1 M phosphate

438   buffer, pH 7.4, for 15 min and then non-specific sites blocked with Blocking buffer (2%

439   normal goat serum, 1% bovine serum albumin, 0.1% TX-100 in 0.1 M phosphate buffer,

440   pH 7.4) for 30 min. Primary antibodies were diluted 1:1000 in Blocking buffer and

441   incubated for 1 hr at room temp. Primary antibodies included a monoclonal antibody to

442   CaMKIIα (created in our lab; [53]) and a rabbit polyclonal antibody to synapsin 1

443   (Synaptic Systems Inc.). Grids were then washed 3x, 5 min each, with Wash buffer

444   (0.2% normal goat serum, 0.1% bovine serum albumin, 0.01% TX-100 in 0.1 M

445   phosphate buffer, pH 7.4). Grids were then incubated in 1:500 dilution of Alexa 488

446   labeled goat anti-mouse IgG and Alexa 568 labeled goat anti-rabbit IgG diluted in

447   Blocking buffer for 30 min at room temperature. Grids were washed 3x 5 min each in

448   Wash buffer, once in 0.1 M phosphate buffer, pH 7.4 and then mounted in Fluoromount

449   anti-fade mounting compound. Bright field and fluorescent images were collected with a

450   10x, or 40x magnification using a 0.9 NA water immersion lens on a Zeiss inverted

451   microscope using an Andor Zyla 4.0 CMOS camera. Exposure time, shutter and filter

452   wheel (Sutter Instrument Co.) were controlled through Metamorph software (Molecular

453   Devices).

454

455   Cryo-electron Tomography

456   For tomographic data collection, single-axis tilt series were collected from -50° to

457   +50° in 3° increments at approximately -8 □m under focus on an FEI Polara G2 operated

458   at 300 kV equipped with a Gatan K2 Summit direct electron detector operated in photon

459   counting mode. Data collection was performed in a semi-automated fashion using Serial

460   EM software operated in low-dose mode [54]. Briefly, areas of interest were identified

461   visually and 8 x 8 montages were collected at low magnification (2400x) and then

462   individual points were marked for automated data collection. Data were collected at

463   either 8.5 or 4.5 Å/pixel. Movies of 8-10 dose-fractionated frames were collected at each

464   tilt angle and the electron dose spread across all images was limited to a total dose of <

465   100 e$^-$/Å^2 per tilt series. There is a "missing wedge" of information in the reconstructions

466   due to the inability to collect tilt series through a full 180° (+/-90°) of stage tilting.

467   Additionally, as the stage is tilted the electron path through the sample increases,

468   decreasing the contrast and quality of the high tilt images. For our system, tilting the

469   stage +/- 50° was found to be an optimal compromise. The missing wedge leads to

470   anisotropic resolution producing elongation and blurring in the Z-dimension and

471   tomographic reconstructions need interpreted acknowledging this limitation.

472

473   Tomographic Reconstruction and Segmentation

474   Each tomographic data set was drift corrected with MotionCorr2 [55] and stacks

475   were rebuilt and then aligned using IMOD [56, 57]. Tomograms of the aligned stacks

476   were then reconstructed using TOMO3D [58, 59]. Contrast was enhanced using SIRT

477   reconstruction implemented in TOMO3D.

478   Reconstructed tomograms were further processed using the median, non-local

479   means, and Lanczos filters in Amira (FEI, ThermoFisher Scientific) for manual and semi-

480   automated segmentation. Segmentation was accomplished by manually tracing

481   membranes for each Z slice of the tomographic data set. Membranes were identified and

482   segmented with reference to visualization in all three dimensions (X, Y, and Z). The

483   brush tool was primarily used in manual segmentation. When possible, masking

484   approaches were also used in combination with density thresholding for semi-automated

485   segmentation. After segmentation, smoothing tools were employed for the manual

486   tracings and surfaces were rendered for model construction. All measurements (length

487   and diameter) were performed in either Amira using the 3D measurement tool or in

488   IMOD.

489

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502   **Figure Legends**

503

504   Figure 1. Growth and characterization of primary hippocampal neurons on EM grids.
505   Hippocampal neurons were isolated from E18 rats and plated on poly-D-lysine coated
506   Quantifoil 2/1 gold grids. A) Low magnification bright field image showing the typical
507   distribution of neuronal soma and processes after 10 d in culture. B) Higher
508   magnification image of the area from the white box in panel (A) with neuronal processes
509   highlighted by white brackets.   (C) Wide-field fluorescence image showing
510   immunolabeling of the neuron specific protein CaMKIIα in green and the presynaptic
511   vesicle associated protein synapsin 1 in red. The blue color is from a bright field overlay
512   of the same area that also highlights the bars of the EM grid. (D) Higher magnification
513   image in a different area of the same immunolabeled grid highlighting the punctate
514   staining of synapsin I (red; arrowheads) along processes typical of *en passant*
515   varicosities in hippocampal axons. Blue is again from where the grid bars and holes in
516   the Quantifoil grid are apparent. Scale bars in panels A-C = 100 μm and in panel D = 50
517   μm. (E) Low magnification montage of one area in a cryopreserved grid of hippocampal
518   neurons 10 d post-plating showing the typical distribution of axons and synaptic
519   varicosities. Scale bar = 10 μm.   F) Higher magnification representation from (E; white
520   box) showing the axon and varicosity distribution overlying carbon (slightly darker areas)
521   and grid holes. Examples of varicosities lying within grid holes are marked with white
522   asterisks while examples lying on the carbon are marked with red asterisks.   Axon
523   segments interconnecting the varicosities are highlighted with black brackets. Scale bar
524   = 2 μm.

525

526   Figure 2. Tomographic reconstruction of a typical pre-synaptic varicosity and adjoining
527   axon segment. A) 2D slice from the tomographic reconstruction showing the distribution
528   of organelles in the varicosity PM: plasma membrane, MT: microtubules, Mito:
529   mitochondrion, ER: endoplasmic reticulum, Ves: vesicle, MVB: multi-vesicular body.
530   Scale bar = 200 nm. B) Segmented representation of the entire 3D tomogram volume
531   shown in (A) revealing the relative size and spatial distribution of the organelle
532   environment in the varicosity and axon segment. Plasma membrane (dark blue),
533   microtubules (light blue), mitochondrial outer membrane (dark green), endoplasmic
534   reticulum (yellow), vesicles (dark purple), multi-vesicular body (light purple), unidentified
535   membrane-bound compartment (pink). Scale bar = 200 nm.

536

537 Figure 3. Mitochondria display atypical morphological features in physically restrictive

538 axons. A-C) Three different 3D segmented reconstructions showing representative

539 examples of mitochondria residing partially in the varicosity and adjoining axon

540 segments, demonstrating different morphological states at the transition from the

541 varicosity into the restricted space of the axon segment.   Additional organelles

542 occupying the axon space are also segmented. For ease of visualization, not all of the

543 organelles and structures in the varicosity are shown. The plasma membrane (dark

544 blue), microtubules (light blue), mitochondrial outer membrane (dark green),

545 endoplasmic reticulum (yellow), vesicles (dark purple) are highlighted.

546

547 Figure 4. Structural features of a mitochondrion captured spanning two varicosities. A)

548 10 nm slice through a 3D tomographic reconstruction showing a mitochondrion spanning

549 two closely spaced varicosities connected by a short (~100 nm) axon segment.   An

550 expanded region of the red box shown in (A) reveals the tubulated nature of the portion

551 of the mitochondrion within the axon segment. Microtubules can be seen running

552 parallel to the tubulated portion of the mitochondrion. B) shows a surface rendered

553 version highlighting the plasma membrane (purple), microtubules (blue) small segment

554 of ER (yellow) and mitochondria (green). C) is the same mitochondrion as in (B)

555 displaying distinct segmentation of the outer membrane (green), inner membrane

556 (orange), and cristae (pink).

557

558 Figure 5. Mitochondrial membranes display distinct morphological features at the

559 boundaries of varicosities and axons with limited physical dimensions. A-C) To highlight

560 the membrane organization of the mitochondria shown in Figure 3A-C, the cristae and

561 inner and outer membranes of the 3D reconstructions were segmented. A) Mitochondrial

562 inner and outer membranes remain in close apposition within the narrowed portion of

563 this mitochondrion resident in the axon. B) Mitochondrial outer membrane is separated

564 from the inner membrane, creating a distinct "matrix-free" compartment in this portion of

565 the mitochondria residing in the axon. C) Three mitochondria near the varicosity/axon

566 junction show distinct internal membrane organization. The left mitochondrion shows a

567 portion of outer membrane separated from inner membrane while the top right

568 mitochondrion is narrowed near the axon junction, but the inner and outer membranes

569 remain in apposition. A short tip of a third mitochondrion is partially captured at the edge

570   of the tomogram that also shows inner and outer membranes together. Plasma

571   membrane (dark blue), mitochondrial outer membrane (dark green), mitochondrial inner

572   boundary membrane (orange), cristae (pink).

573

574

575   Supporting Figure Legends

576

577   Figure S1. 2D images from tomographic reconstructions of different membrane bound

578   organelles observed in the varicosities of cryopreserved hippocampal neurons. A)

579   mitochondrion, B) MVB and vesicles (arrowheads). C) ER and an unidentified

580   membrane-bound compartment (arrowhead), D) Autophagosome, E) Lamellar body.

581   Scale bar = 200 nm.

582

583   Figure S2. Different populations of mitochondrial cristae ultrastructures in varicosities. A)

584   Representations of mitochondria displaying thin, tubulated cristae. B) Representations of

585   mitochondria displaying thick, unstructured cristae. Populations were identified based on

586   full 3D tomographic reconstructions. Scale bar = 200 nm.

587

588   Movie S1. Animation of the 3D tomographic reconstruction and overlay of the

589   segmented structures in Figure 2. Scale bar = 200 nm.

590

591   Movie S2. Animation demonstrating space within an axon encountered by a

592   mitochondrion presented in the X dimension. 3D tomographic reconstruction

593   corresponds to Figure 3B and 5B. Scale bar = 200 nm.

594

595   Movie S3. Animation of the 3D tomographic reconstruction and overlay of the

596   segmented structures in Figure 3A. 3D tomographic reconstruction also corresponds to

597   Figure 5A. Scale bar = 200 nm.

598

599   Movie S4. Movie of the tilt series of tomographic data set corresponding to Figure 3A

600   and 5A. Scale bar = 200 nm.

601

602   Movie S5. Movie of the 3D tomographic reconstruction corresponding to Figure 3B and

603   5B. Scale bar = 200 nm.

604

605   Movie S6. Movie of the tilt series of tomographic data set corresponding to Figure 3C

606   and 5C. Scale bar = 200 nm.

607

608   Movie S7. Movie of a mitochondrion at spatially restricted axonal/varicosity boundary.

609   Scale bar = 200 nm.

610

611   Movie S8. Movie of an additional mitochondrion at spatially restricted axonal/varicosity

612   boundary. Scale bar = 200 nm.

613

614   Movie S9. Movie of the 3D tomographic reconstruction corresponding to Figure 4. Scale

615   bar = 200 nm.

616

617   Movie S10. Movie of a 3D tomographic reconstruction displaying a mitochondrion

618   spanning a short axon segment and residing in two varicosities. Scale bar = 200 nm.

619

620   Movie S11. Animation of the 3D tomographic reconstruction and overlay of the

621   segmented membranes of mitochondria in Figure 3D. Scale bar = 200 nm.

622

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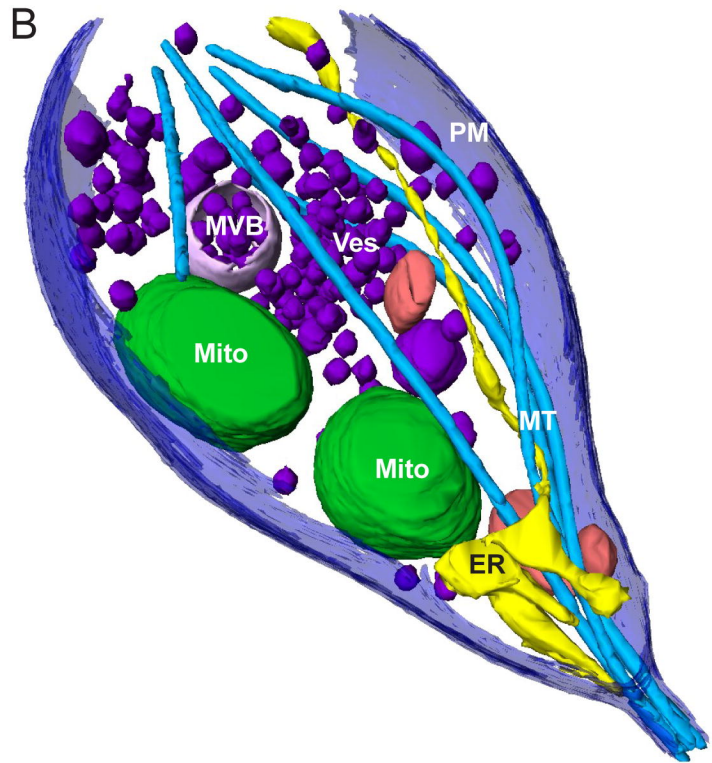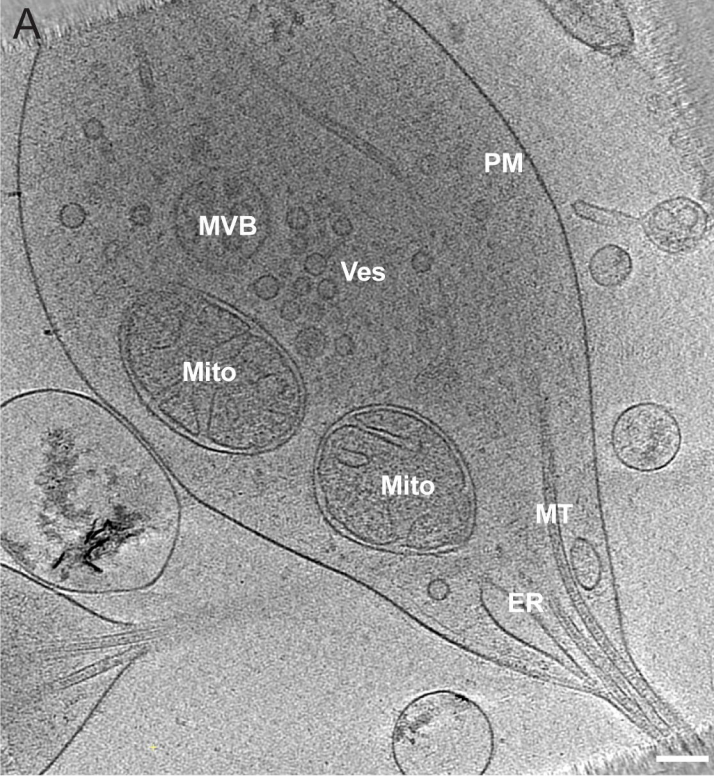
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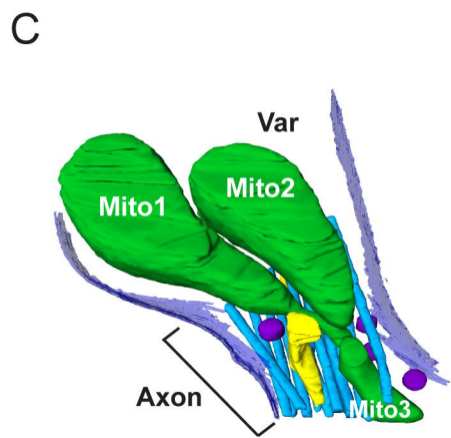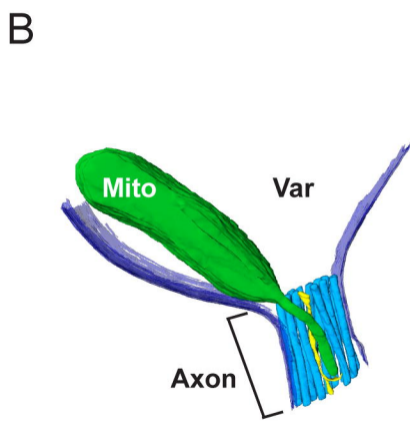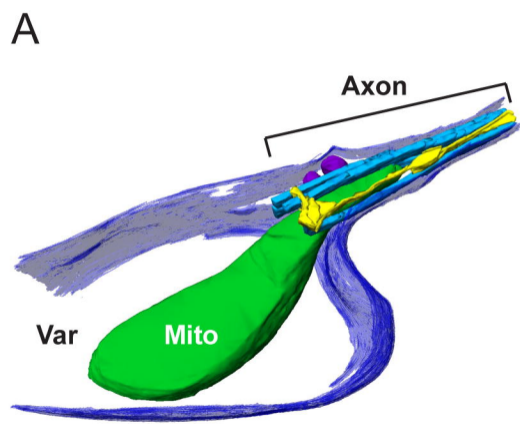
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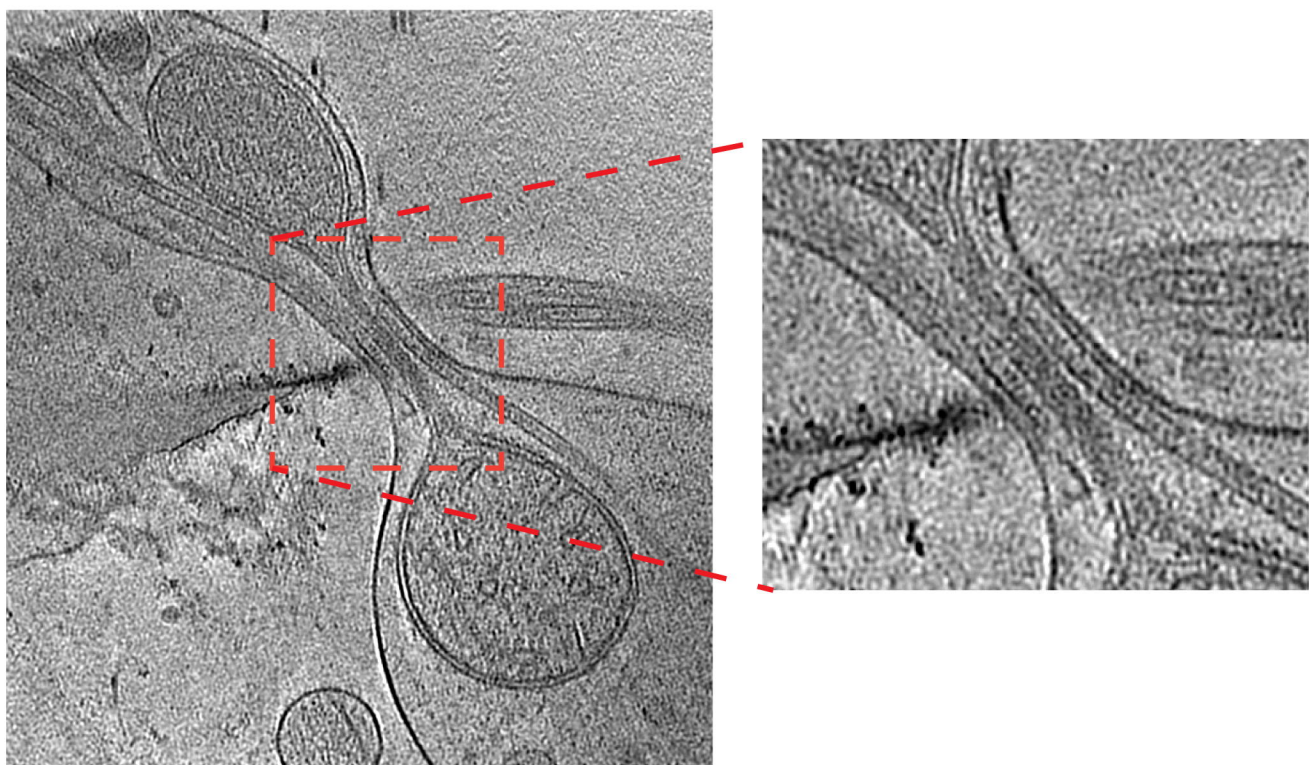820   PubMed PMID: 21172911.

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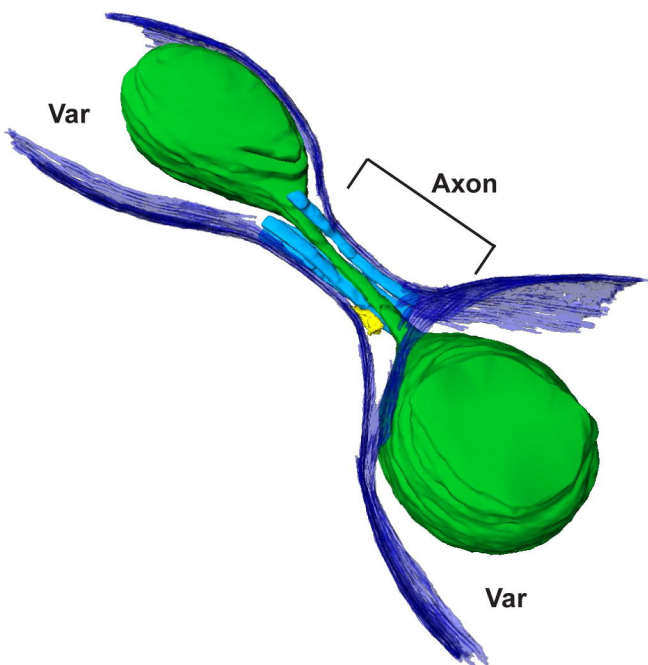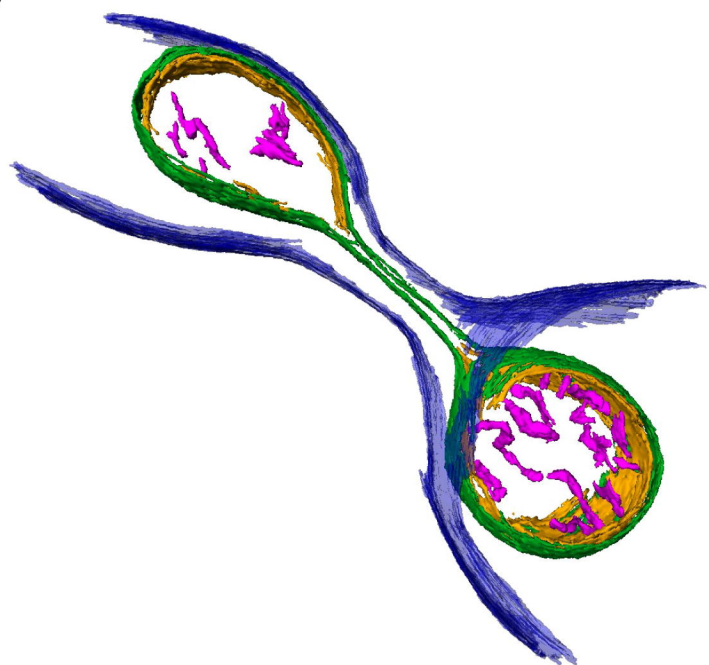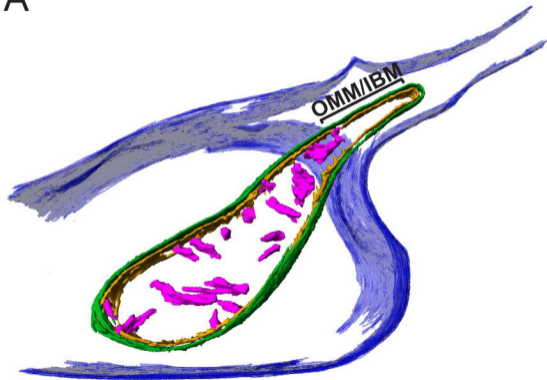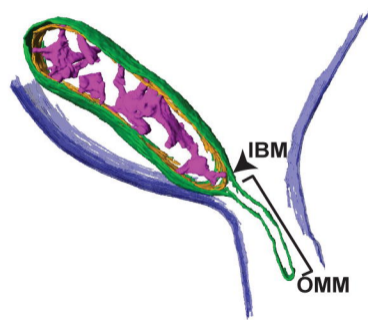824   26217710; PubMed Central PMCID: PMCPMC4509992.
825

A — Axon, Var, Mito

B — Mito, Var, Axon

C — Var, Mito1, Mito2, Mito3, Axon

A

B

Var

Axon

Var

C