Figure 1. Workflow for preparation of fresh brain tissue for cryo-EM imaging. (I) 100-200 μm-thick sample of fresh mouse brain tissue is painted into a carrier. **(II)** Carrier is loaded into a high-pressure freezer (HPF) for immediate cryo-preservation. **(III)** The HPF sample is trimmed in a cryo-ultramicrotome to 20-30 μm thickness. **(IV)** The sample is thinned via cryo-FIB milling to a final 100-300 nm thickness, followed by imaging of the lamellae in a cryo-TEM.

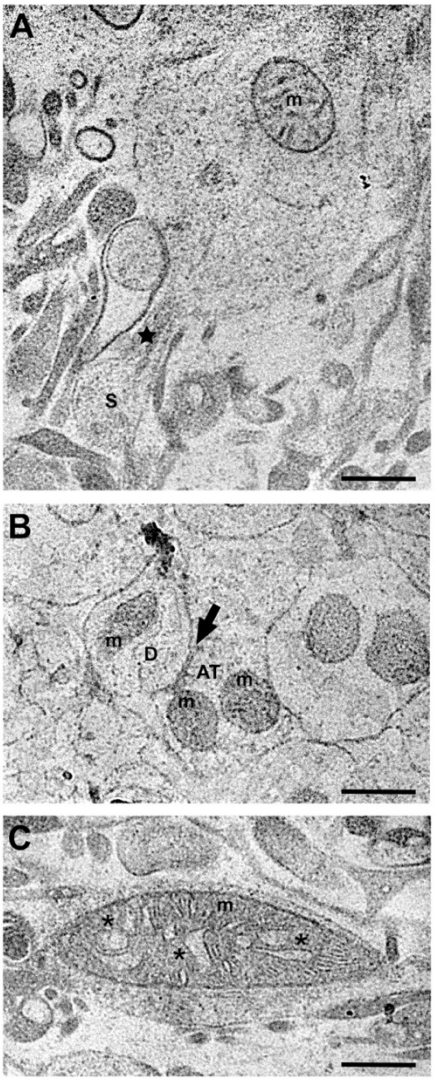

Figure 2. Ultrastructural preservation of fresh, unfixed mouse brain tissue via HPF/freeze substitution. (A) A dendritic spine (S) protruding from the parent pyramidal neuron dendritic shaft (D). The dendritic shaft contains a mitochondrion (m). A putative spine apparatus (star) is apparent in the spine neck. **(B)** A neuronal axon terminal (AT) containing two mitochondria (m) and forming an apposition (arrow) onto a dendritic shaft (D) containing a mitochondrion (m). **(C)** A large mitochondrion located within a dendritic shaft, displaying characteristics of a condensed state, which include an electron-dense matrix and multiple open cristae (*). Scale bars = 500 nm.

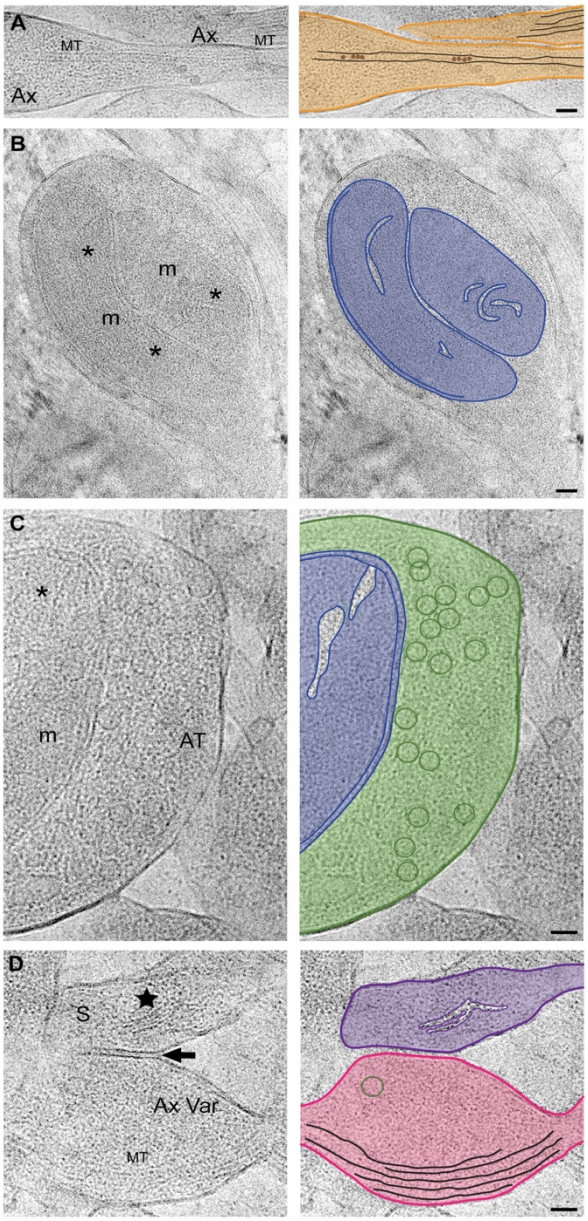

Figure 3. Representative micrographs from cryo-FIB-milled lamellae of fresh, unfixed mouse cortical neuropil imaged via cryo-EM. Left panels are annotated original cryo-EM images. Right panels feature segmentation of salient neuronal features. **(A)** Neuronal axons (Ax, orange) containing microtubule tracks (MT, black). Putative intraluminal proteins (brown) are visualized inside the central microtubule lumen. **(B)** Two nested mitochondria (m, blue) with visible cristae (asterisk) within a neuronal process. **(C)** Axon terminal (AT, green) containing clusters of synaptic vesicles (green circles) alongside a mitochondrion (m, blue) featuring open cristae (asterisk). **(D)** Apposition (arrow) between an axonal varicosity (Ax Var, pink) and a dendritic spine (S, purple), featuring a spine apparatus (star). The axonal varicosity contains a synaptic vesicle (green circle) and microtubule tracks (MT, black). Scale bars = 50 nm.