Cell

Docking of Axonal Mitochondria by Syntaphilin Controls Their Mobility and Affects Short-Term Facilitation

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DOI 10.1016/j.cell.2007.11.024

SUMMARY

Proper distribution of mitochondria within axons and at synapses is critical for neuronal function. While one-third of axonal mitochondria are mobile, a large proportion remains in a stationary phase. However, the mechanisms controlling mitochondrial docking within axons remain elusive. Here, we report a role for axon-targeted syntaphilin (SNPH) in mitochondrial docking through its interaction with microtubules. Axonal mitochondria that contain exogenously or endogenously expressed SNPH lose mobility. Deletion of the mouse *snph* gene results in a substantially higher proportion of axonal mitochondria in the mobile state and reduces the density of mitochondria in axons. The *snph* mutant neurons exhibit enhanced short-term facilitation during prolonged stimulation, probably by affecting calcium signaling at presynaptic boutons. This phenotype is fully rescued by reintroducing the *snph* gene into the mutant neurons. These findings demonstrate a molecular mechanism for controlling mitochondrial docking in axons that has a physiological impact on synaptic function.

INTRODUCTION

The proper transport and intracellular distribution of mitochondria are critical for the normal physiology of neurons. Mitochondria accumulate in the vicinity of active growth cones of developing neurons (Morris and Hollenbeck, 1993) and are present at some synaptic terminals (Shepherd and Harris, 1998; Rowland et al., 2000). In addition to the aerobic production of ATP, mitochondria regulate Ca^{2+} concentrations (Werth and Thayer, 1994) and have been implicated in certain forms of short-term synaptic plasticity by buffering Ca^{2+} at synapses (Tang and Zucker, 1997; Billups and Forsythe, 2002; Levy et al., 2003; Yang et al., 2003). Loss of mitochondria from axon terminals in *Drosophila* results in defective synaptic transmission (Stowers et al., 2002; Guo et al., 2005; Verstreken et al., 2005). Mitochondria in the cell bodies of neurons are transported down neuronal processes in response to changes in the local energy state and metabolic demand. Because of their extreme polarity, neurons require specialized mechanisms to regulate the transport and retention of mitochondria at specific subcellular locations. While cytoplasmic dynein is the driving force behind retrograde movement, the kinesin family of motors and their adaptors Milton and syntabulin are responsible for anterograde transport of axonal mitochondria (Stowers et al., 2002; Górska-Andrzejak et al., 2003; Hollenbeck and Saxton, 2005; Cai et al., 2005; Glater et al., 2006). Defective transport of axonal mitochondria is implicated in human neurological disorders and neurodegenerative diseases (see reviews by Hirokawa and Takemura, 2004; Chan, 2006; Stokin and Goldstein, 2006).

Mitochondria in axons display distinct motility patterns and undergo saltatory bidirectional movements where they stop and start moving, frequently changing direction. While approximately one-third of axonal mitochondria are mobile at instantaneous velocities of 0–2.0 μm/s in mature neurons, a large proportion remains in stationary phase. Their net movement is significantly influenced by recruitment between stationary and motile states (Hollenbeck, 1996; Ligon and Steward, 2000). Such complex mobility patterns suggest that axonal mitochondria might be coupled to two opposing motors and docking machinery. Efficient control of mitochondrial docking at particular sites of axons in response to cellular processes and synaptic stimuli is likely essential for neuronal development and synaptic function (Chada and Hollenbeck, 2004; Reynolds and Rintoul, 2004). However, the proteins mediating mitochondrial docking and retention within axons have not yet been identified.

Syntaphilin (SNPH) is a neuron-specific protein initially identified as a candidate inhibitor of presynaptic function (Lao et al., 2000). We recently generated mouse mutants with a homozygous deletion for the *snph* gene, leading to the discovery of a novel role for SNPH as a docking receptor of axonal mitochondria. Our findings indicate that SNPH is targeted to and required for maintaining a large portion of axonal mitochondria in

stationary state through an interaction with the microtubule-based cytoskeleton. First, we show that axonal mitochondria containing exogenously expressed GFP-SNPH are nearly immobilized. Second, endogenous SNPH-tagged mitochondria are strongly correlated with stationary mitochondria. Third, deletion of the *snph* gene in mice dramatically increases mitochondrial motility, reduces their density in axons, and consequently influences short-term facilitation during prolonged high-frequency stimulation, probably by affecting calcium dynamics at presynaptic boutons. The observed phenotype can be fully rescued with the reintroduction of the *snph* gene into the mutant neurons. Furthermore, the *snph* mutant mice show impaired motor coordination. These combined molecular, cellular, and genetic studies elucidate a mechanism underlying the docking of axonal mitochondria and provide evidence that the increased motility and/or reduced density of axonal mitochondria have a significant impact on presynaptic function.

RESULTS

SNPH Is an Axon-Targeted Protein Associated with Mitochondria

Previous studies using recombinant proteins suggested that SNPH is a neuron-specific protein that likely plays an inhibitory role for presynaptic function (Lao et al., 2000). To further evaluate its role, we examined the subcellular localization of SNPH in neurons. First, transfection of GFP-SNPH into cultured hippocampal neurons revealed punctate or vesicular-tubular structures preferentially enriched in axonal processes (see Figure S1A available online). Second, coimmunostaining for SNPH with an antibody against the SNPH residues 225–428 and the dendritic marker MAP2 in mouse brain slices demonstrated that the majority of SNPH staining is not colocalized with the MAP-2-positive dendritic processes. In contrast, most axonal tracts in the CNS, including the internal capsule, cerebral and cerebellar peduncles, fimbria of the hippocampus, and almost all cranial nerves, showed relatively enriched expression of SNPH (Figure S1B). Third, immunocytochemical analysis of cultured hippocampal neurons demonstrates that endogenous SNPH is predominantly distributed in the MAP2-negative and Tau-positive axonal processes (Figure 1A) and clustered alongside but not within dendritic profiles (Figure S2A), thus indicating its axonal localization. Furthermore, 65 ± 14% (mean ± SD, 54 images) of axonal mitochondria are colocalized with SNPH (Figure 1B), whereas little SNPH is colocalized with the synaptic vesicle marker synaptophysin (Figure S2B).

Identification of the molecular determinants for its axonal localization and mitochondrial targeting is critical to understand the role of SNPH in neurons. Localization analysis of GFP-SNPH truncated mutants reveals that its carboxyl terminal tail (TM1 and TM2) is necessary for mitochondrial association (Figures 1C and S3). TM1 and TM2 have similar signal structures specific for mitochondrial outer-membrane-targeting (Rapaport, 2003), which are moderately hydrophobic and relatively short (16–20 residues) with net positive charges flanking both sides (Figure S3A). The mitochondrial targeting of SNPH is consistent with the immunogold EM observations that showed its localization to the outer mitochondrial membrane (Das et al., 2003).

We next sought to determine the sequence responsible for axonal sorting. The majority of SNPH staining is localized at axonal mitochondria (Figures 1 and S3B). In support of these observations, we identified an axon-sorting domain of SNPH (residues 381–469)(Figure 1C). Expression of the truncated mutant of SNPH lacking this sequence results in its distribution into all mitochondria including those in the soma and dendrites (Figure S3B), suggesting that SNPH may use this axon-sorting sequence for axonal localization independent of its mitochondrial targeting.

SNPH Immobilizes Axonal Mitochondria

Proper axonal transport and distribution of mitochondria are critical for neuronal activity and synaptic transmission. To characterize the role of SNPH in axonal mitochondrial trafficking, we conducted live cell time-lapse imaging using confocal microscopy in cultured hippocampal neurons coexpressing DsRed-mito and GFP-SNPH. Axonal motile and stationary mitochondria were identified using kymographs, as previously described (Miller and Sheetz, 2004). Those mitochondria not labeled with GFP-SNPH migrate dynamically and bidirectionally along the axonal process, whereas GFP-SNPH-labeled mitochondria remain stationary (Movie S1). Consistent with previous reports (Morris and Hollenbeck, 1993; Ligon and Steward, 2000; Miller and Sheetz, 2004), 38 ± 16% (mean ± SD) of axonal mitochondria are motile in control neurons expressing DsRed-mito alone. Strikingly, almost no mitochondrion (0.3 ± 1%, mean ± SD) labeled with GFP-SNPH is mobile (Figure 2). This phenomenon is consistent across all axons (n = 39) examined, indicating that GFP-SNPH-tagged mitochondria are stationary. In contrast, 35 ± 9% (mean ± SD) of axonal mitochondria labeled with SNPH-ΔMTB, a SNPH mutant lacking the microtubule-binding domain (130–203), are mobile (Figure 2B, Movie S2, and Figure S4) with no significant difference from the DsRed-mito control group ($p = 0.34$, *U* test). These data suggest that exogenously expressed SNPH inhibits the motility of axonal mitochondria and its microtubule-binding domain is required for the SNPH-mediated immobilization.

To exclude an artificial effect due to overexpression of SNPH, we next examined whether there are distinct motion patterns of axonal mitochondria in relation to the association with endogenous SNPH. Mitochondrial movement in living hippocampal neurons was recorded, followed by retrospective immunostaining for endogenous SNPH. Statistical analysis reveals a strong correlation ($r = 0.98$, 6 paired experiments) between SNPH-positive mitochondria (green and yellow puncta shown in Figures 3C and 3D) and stationary mitochondria (vertical red lines shown in Figure 3B). Axonal mitochondria appear as two populations: one associated with SNPH and the other not, demonstrating a binominal $B(n, p)$ distribution (Figure 3E). The proportion (ρ) of SNPH-tagged mitochondria is approximately 62% based on quantitative analysis (Supplemental Data). The mean percentage of endogenous SNPH-tagged mitochondria (65 ± 14%, mean ± SD) is consistent with the mean percentage of stationary mitochondria (62 ± 15%, mean ± SD), and both fit well with 90% confidence intervals based on binomial statistics (Figures 3F and 3G). These results suggest a complementary relationship between endogenous SNPH-tagged mitochondria and motile