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Coordinated bi-directional trafficking of synaptic vesicle and active zone proteins in peripheral nerves

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ABSTRACT

Synaptic transmission is mediated by neurotransmitters that are stored in synaptic vesicles (SV) and released at the synaptic active zone (AZ). While in recent years major progress has been made in unraveling the molecular machinery responsible for SV docking, fusion and exocytosis, the mechanisms governing AZ protein and SV trafficking through axons still remain unclear. Here, we performed stop-flow nerve ligation to examine axonal trafficking of endogenous AZ and SV proteins. Rat sciatic nerves were collected 1 h, 3 h and 8 h post ligation and processed for immunohistochemistry and electron microscopy. First, we followed the transport of an integral synaptic vesicle protein, SV2A and a SV-associated protein involved in SV trafficking, Rab3a, and observed that while SV2A accumulated on both sides of ligation, Rab3a was only noticeable in the proximal segment of the ligated nerve indicating that only SV *trans*-membrane protein SV2A displayed a bi-directional axonal transport. We then demonstrate that multiple AZ proteins accumulate rapidly on either side of the ligation with a timescale similar to that of SV2A. Overall, our data uncovers an unexpected robust bi-directional, coordinated -trafficking of SV and AZ proteins in peripheral nerves. This implies that pathological disruption of axonal trafficking will not only impair trafficking of newly synthesized proteins to the synapse but will also affect retrograde transport, leading to neuronal dysfunction and likely neurodegeneration.

1. Introduction

Neurotransmitters are stored in synaptic vesicles (SV) and released in a temporally and spatially regulated manner. SV mediated neurotransmitter release mostly occurs at highly specialized membrane domain, the synaptic active zone (AZ) [1]. AZs are visualized as electron dense structures with transmission electron microscopy [2,3] and are composed of large multi-domain protein molecules including Piccolo, Bassoon, Rims, Liprins-α, Munc-13 and Elks/CAST [4]. The proteins within the active zones perform valuable function in SV recycling process, including SV clustering, docking, priming for fusion, anchoring of calcium channels close to vesicles as well as short and long-term presynaptic plasticity. Many of the AZ proteins during synaptogenesis may traffic to the presynapse on specialized transport vesicles with a dense core, dubbed as Piccolo transport vesicles (PTVs) [5]. However, post-synaptogenesis the number of putative PTVs diminishes indicating that other mechanisms of AZ protein transport may be operational to maintain AZ after synaptogenesis [6]. Most of the studies on AZ protein axonal transport were performed in primary neuronal cultures. The studies demonstrated that, in vivo, synaptogenesis is supported by a coordinated trafficking of AZ and SV proteins [7]. All of the AZ proteins mentioned earlier, however, are also present in the presynapse of the neuromuscular junction (NMJ) [8]. Skeletal muscle motor neurons have very long axons requiring fast axonal trafficking of both SV proteins

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and AZ proteins not only during development, but also to maintain a post-synaptogenic steady state level of recycling AZ and SVs. In this study we describe axonal trafficking of three of the AZ proteins, namely Piccolo, Bassoon and Rim1, in rat sciatic nerve and compare it to trafficking of integral SV proteins, SV2A and a SV associated protein Rab3a. To study the trafficking pattern, we have performed a stop-flow sciatic nerve ligation and examined accumulation of AZ and SV protein on either side of ligation using immunolabeling. Our study indicates that surprisingly both AZ and SV protein undergo bi-directional, antero- and retrograde fast coordinated trafficking.

2. Materials and methods

Animals: Sixteen house-bred adult male Sprague Dawley rats, weighting 200–250 g, were used in the study. In order to study rates of antero- and retrograde fast axonal transport, rats were deeply anesthetized and underwent peripheral nerve stop-flow ligation surgery as described previously [9,10]. Following surgery, at 1 h, 3 h and 8 h time point rats were transcardially perfused with 4% paraformaldehyde in 0.02 M phosphate buffered saline (PBS, pH 7.4) and sciatic nerves were collected for immunofluorescent studies. All animal procedures were approved by the Lund University Local Ethical Committee and were performed in accordance with the National Institutes of Health Animal Care Guidelines.

Immunofluorescence: Nerve samples were postfixed for 3 h in 4% paraformaldehyde solution, as described earlier and transferred to 20% sucrose solution for cryopreservation. After cryopreservation, sections were embedded in OCT Tissue Tek (Sakura Finetek), frozen at −20 °C, cut longitudinally at 10 μm thickness and collected on polylysine coated slides (SuperFrost Plus, Fisher Scientific). Immunofluorescent staining was performed according to optimized laboratory protocols. Following primary and secondary antibodies were used: anti-Bassoon (1:400), anti-Piccolo (1:600), anti-Rim1 (1:200), anti-SV2 (1:400), anti-Rab3a (1:500) and anti-Synaptophysin (1:500) – all purchased from Synaptic Systems (Goettingen, Germany); and anti-mouse FITC (1:2000) and anti-rabbit Cy5 (1:2000), both from Thermo Fisher Scientific (Waltham, MA, USA). All sections from each time-point series were immunostained in parallel as described previously [11,12]. In order to control for antibody specificity and to minimize risk of false positive results, standard immunostaining procedures with primary antibody omission or replacement were carried out on sections from each tissue sample parallel to the experimental staining.

Electron microscopy: Following previously described procedures [10,11], 6 h after the stop-flow surgery, rats were deeply anesthetized, transcardially perfused with a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde in 0.05 M phosphate buffer (PB, pH 7.4) and sciatic nerve were collected. Proximal nerve segments close to the ligation site were teased into thin bundles, transferred to eppendorf tubes and postfixed in the same fixative at 4 °C for 1 h. In order to disassociate myelin sheaths and increase antibody penetration, samples were homogenized mechanically using small grinders. After homogenization, samples were rinsed with PB, preincubated in 1% bovine serum albumin in PBS for 1 h and overnight incubated with primary antibodies. Following day, sections were rinsed in PBS, incubated with 6 and 12 nm gold particle conjugates and embedded in Epon 812. After embedding, sections were cut ultrathin and viewed under JEOL transmission electron microscope.

Fiber quantification and statistical analysis: The number of immunopositive fibers was examined at a length of 0.5–3 mm proximally to and distally from the ligation site with a confocal microscope (Leica SP5, Goettingen, Germany) at 20x objective (Leica 20x Plan-Apochromat air) at one focal plane. Each microscope frame counted as one region of interest. Single and double positive fibers were counted using Image J open source software (http://rsbweb.nih.gov/ij/) with cell counter plugin.

Statistical analysis was performed by non-parametric ANOVA (Kruskal-Wallis test corrected for ties) with Dunn multiple comparison post-test (GraphPad Instat, La Jolla, CA, USA).

3. Results

Nerve ligation experiment is a well-established technique used for over five decades to study axoplasmic transport [13,14]. Here we use stop-flow nerve ligation in sciatic nerves of rats to examine axoplasmic transport of SV and AZ proteins. We first tested accumulation of a membrane anchored SV protein, SV2A accumulation 1, 3 and 8 h after ligation. As expected of a rapidly transported membrane protein, SV2A accumulated quickly at the proximal side of ligation. There was also a parallel and similar accumulation on the distal side, suggesting that SV2A undergoes a bi-directional trafficking, both antero- and retrograde (Fig. 1A). Over the period of 8 h the number of SV2A-labeled fibers nearly tripled on either side of the ligation (Fig. 1A). We next examined Rab3, a SV associated protein, in the proximal and distal segments of the ligated nerve. Rab3a does not have a transmembrane domain and anchors to membrane lipid bilayer via prenylation by a Rab geranylgeranyl transferase. Rab3a is thought to play a role in delivering SV proteins to the presynapse [15]. We saw rapid accumulation of Rab3a in the proximal segment with the increasing number of positively labeled fibers over the 8 h period, however no Rab3a signal was present on the distal side (Fig. 1B). Our findings here are consistent with previous studies that demonstrated that Rab3a did not undergo a fast retrograde trafficking in axon [10,11]. Overall this data validates our ligation experiment to further study AZ protein transport.

AZ proteins do not have transmembrane domains or signal peptides and are therefore most likely synthesized in cytoplasmic ribosomes rather than in rough endoplasmic reticulum. However multiple studies have suggested that AZ proteins are trafficked to the presynapse on specific transport vesicles such as PTV [5,16–18]. Here we examined trafficking of several AZ proteins including Piccolo, Bassoon and Rim1 in the axons of sciatic nerve. Our data indicate that all studied AZ proteins accumulate rapidly on both sides of ligation with a timescale similar to that of SV2A, suggesting that in long axons of the sciatic nerve, AZ proteins much like SV proteins, undergo a bi-directional fast axonal transport (Fig. 2A,B,C). We then sought to determine if SV proteins and AZ proteins would exhibit a co-localized distribution on both proximal and distal segments of the ligated nerve. Our data indicates that both classes of presynaptic proteins (SV and AZ proteins) exhibit a high degree of co-localization on either side of the ligation, with as many as 80% of the fibers positively labeled for SV2A and Piccolo and SV2A and Bassoon on either side of ligation. Similarly, we found 80% of fibers in the proximal segment also positive for both Piccolo and Rab3 (Supplemental Figs. 1–2). It has been shown that in cultured neurons SV protein and AZ proteins are trafficked in a very coordinated fashion [7]. Furthermore, it has been suggested that AZ proteins may travel to the presynapse in golgi-derived vesicles. A high coordination of SV and AZ protein trafficking within axons in our ligation experiment however raises the possibility that AZ and SV proteins may co-migrate on a shared vesicle. To test this idea, we first imaged the fibers with high magnification and examined co-localization of AZ proteins with synaptophysin, a classical marker of synaptic vesicles. Surprisingly, we found that AZ proteins and Synaptophysin in these fibers do not show a high degree of co-localization (Fig. 3A). However, since the diameters of SV (30–50 nm) and PTV ~80 nm are both well below resolution power of a confocal microscope we performed immuno-electron microscopy on teased proximal segment of the ligated sciatic nerve. We

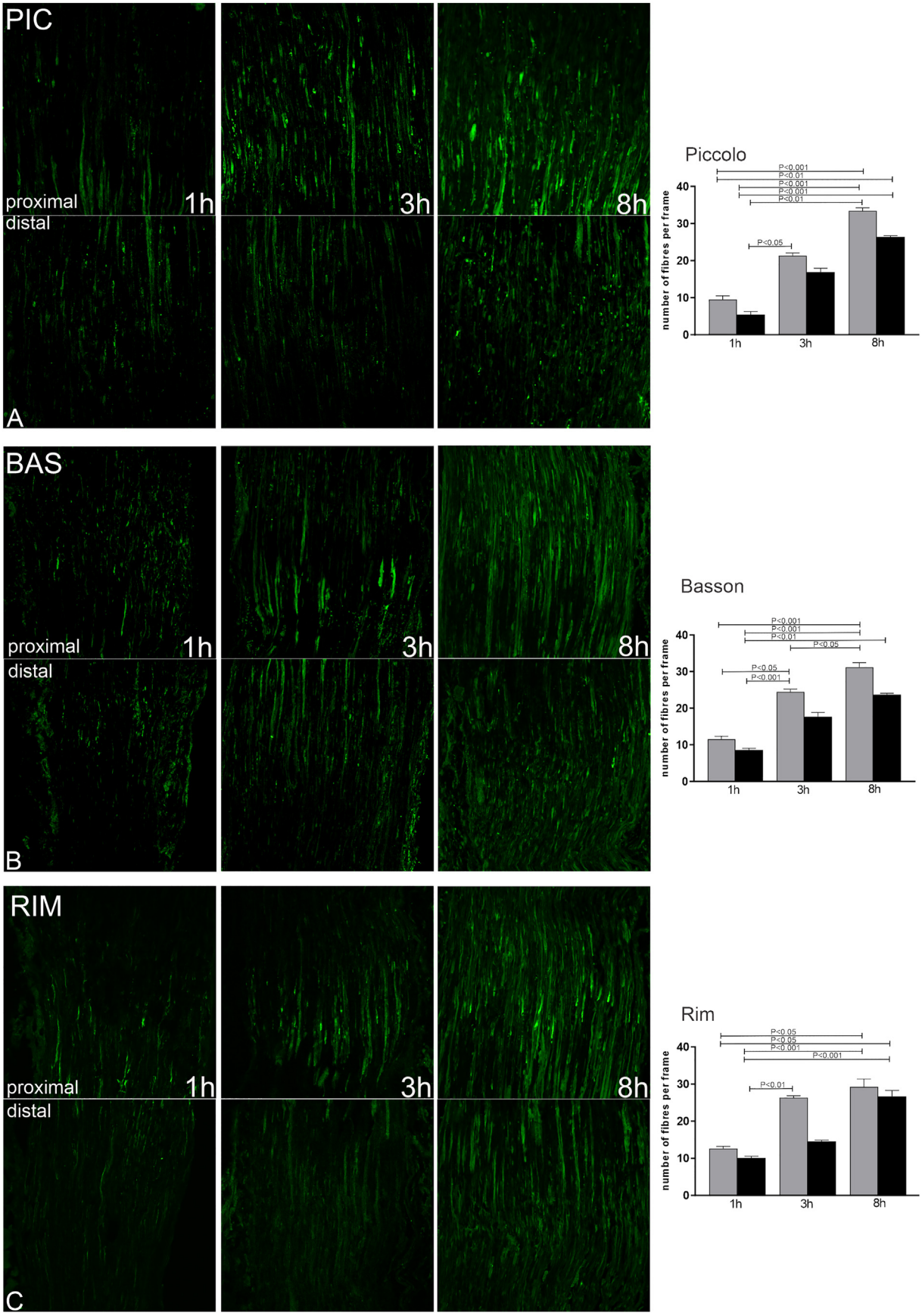

Fig. 1. Time dependent accumulation of synaptic vesicle proteins at the ligation sites, 200x magnification.
A) the number if immunostained fibers positive for SV2 increased over time, reflecting the rates of fast axonal transport at 1 h, 3 h and 8 h after the procedure. SV2 was present at both proximal and distal site, demonstrating both antero- and retrograde trafficking patterns. B) Similarly to SV2, the number Rab3a immunostained fibers increased over time, but only at the proximal site. No staining was observed at the distal site, reflecting a lack of retrograde transport of this protein.

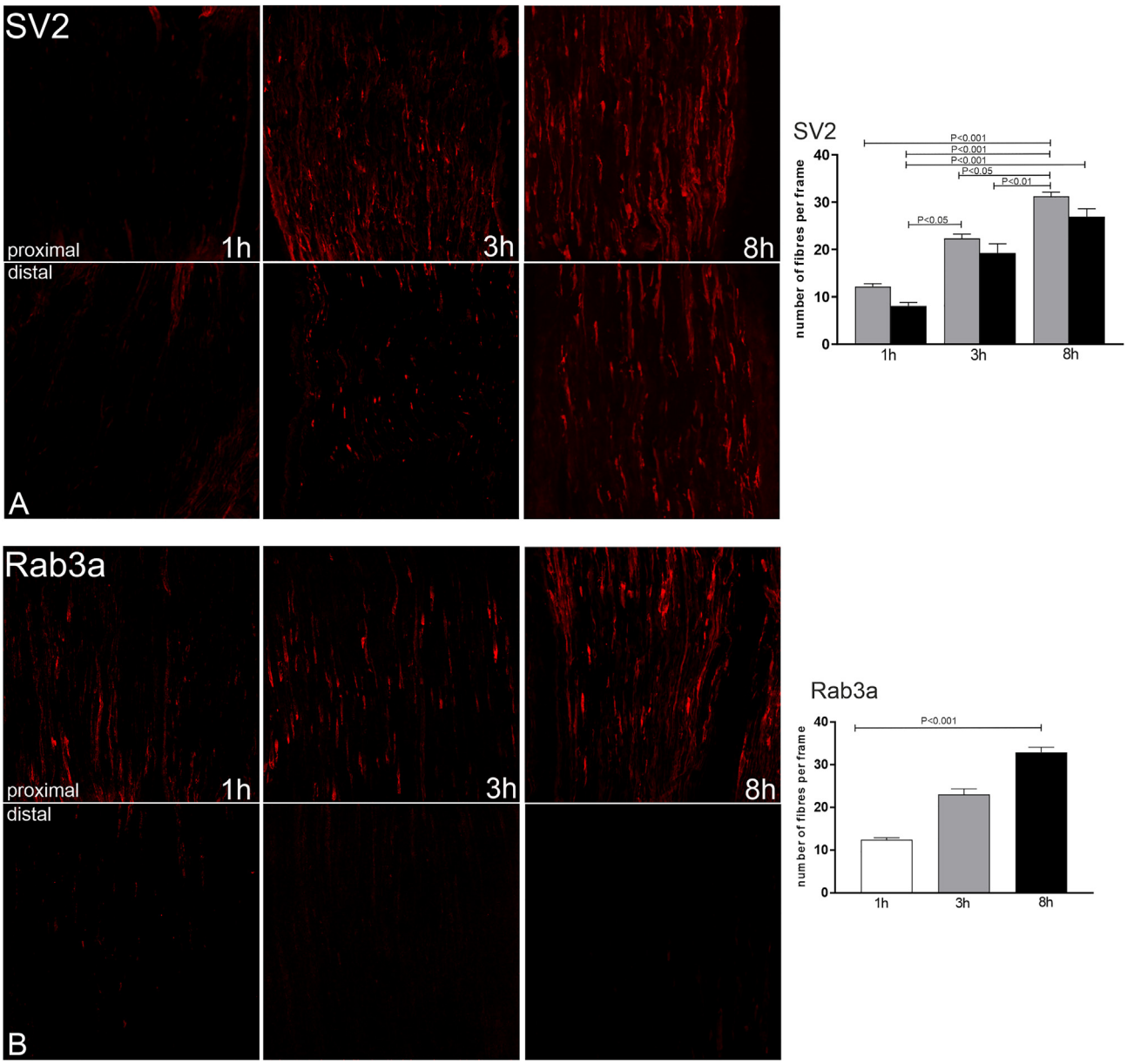

Fig. 2. Time dependent accumulation of active zone proteins, 200x magnification.
Piccolo (Pic, A), Bassoon (Bas, B) and Rim1 (C) immunolocalization at the ligation sites. Same as SV2, the number AZ protein-positive fibers increased over time, the highest number present 8 h post ligation. All proteins showed similar patterns of accumulation and were observed at both ligation sites. The accumulation at the proximal site reflects the rates of anterograde trafficking and the accumulation at the distal site reflecting the rate of retrograde transport, with statistical differences in number of immunopositive fibers between and within time points.

inspected co-localization of two different antigens using two differently sized gold-conjugated secondary antibodies for our experiments. Our data clearly indicates that AZ proteins mostly localizes to membranes different than those of SV proteins (Fig. 3B). Thus, over all our data indicates that in spinal cord neurons such as those whose axons form the sciatic nerve, AZ and SV proteins exhibit rapid coordinated-migration in post-synaptogenesis phase. Furthermore, our data also establishes that even in axons where the presynaptic active zone is located at the axonal terminal there is a robust retrograde fast trafficking of AZ proteins.

4. Discussion

Presynaptic AZ is the site where SVs undergo exocytosis, releasing neurotransmitters at the chemical synapse [19,20]. Within AZ, Munc13 and Rim1 are involved in docking and priming of SVs [21–23], Rim1 and Rim-bp are critical for anchoring calcium channel [21], Rim1 also participates in several forms of presynaptic plasticity [24,25], Liprins-α are involved in organization of AZ as well as exocytosis [26–28], and large molecules like Piccolo and Bassoon may be involved in SV clustering [29]. The latter notion is supported by mass spectrometry studies of SVs, where, of all AZ protein examined, only peptides belonging to Piccolo and Bassoon were identified [30]. The formation of AZ has been predominantly studied in the central nervous system. In central neurons, Piccolo during synaptogenesis is transported on dense core golgi-derived vesicles called PTVs [5,17]. Typically, a dense core within vesicles is formed when it emerges from the *trans*-golgi network due to condensation of peptides belonging to the granin family [31]. The composition of the dense core in PTV remains uncertain, it is also

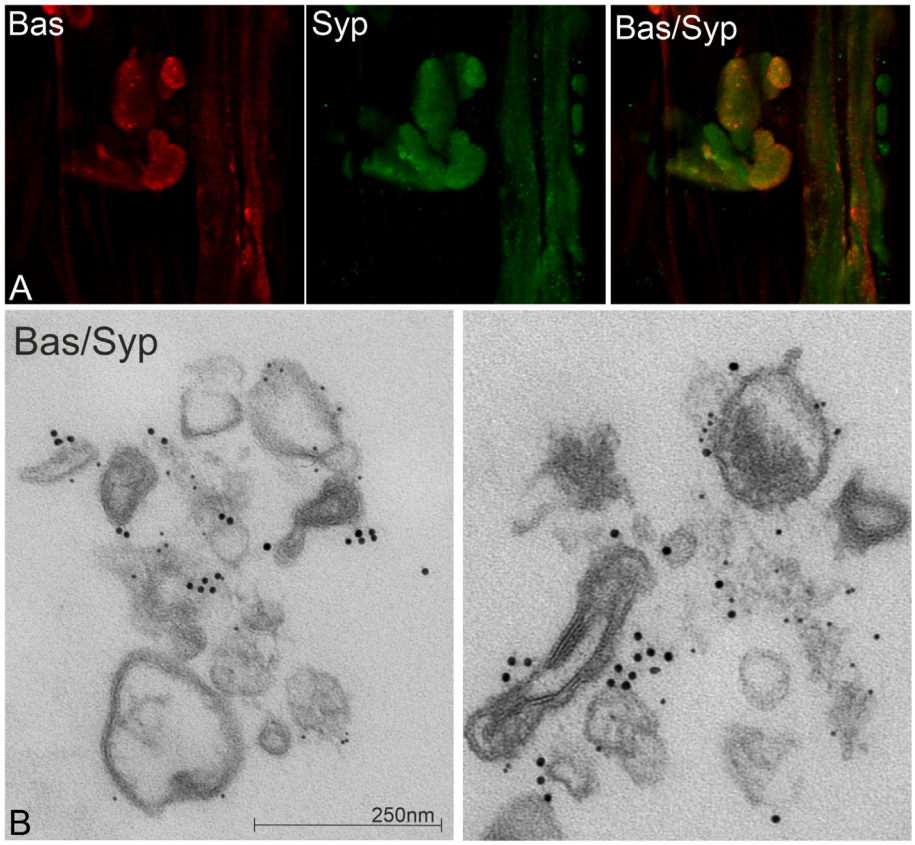

Fig. 3. High resolution localization of SV and AZ proteins. Representative high magnification (A) images of double labeled fibers, stained for Bassoon, used here as a typical marker of synaptic Active Zone and Synaptophysin, used here as a synaptic vesicle marker, 8 h post ligation, proximal side, 630x magnification. Double stained, individual dots are visible for Bassoon and Synaptophysin. Representative electron microscopy (B) images of individual synaptic vesicles double labeled for Bassoon (small black dots) and Synaptophysin (large black dots). Clustering of both proteins on membranes derived from axonal fibers are noticed.

not clear whether these vesicles are involved in secretion of any of the condensed peptides from the dense core. After the synaptogenic stage, the number of PTVs observed is greatly reduced [6], thus the mechanism by which AZ proteins are transported to synapse after synaptogenesis in central synapses is not known.

In a previous study, we demonstrated that AZ proteins were also present in nerve endings of the peripheral nervous system, specifically, at NMJs of skeletal muscles which harbor all previously described AZ proteins. A subset of AZ proteins can also be found in axonal release sites within smooth muscles [8]. The skeletal muscle NMJs are highly stable synapses [32] and thus require continuous maintenance of AZ by trafficking of component proteins over the lifetime [33]. Since axons of spinal cord neurons are anatomically separated from neuropil and clearly identifiable in peripheral nerve, it provides unique advantages for studying axonal trafficking. In fact, axonal trafficking has been mostly studied using nerve ligation method [13,14,34]. The biggest advantage of this method is that the observations are made by accumulation of native endogenous protein and are not prone to artifacts as a result of overexpression of protein or fusion with non-physiological fluorescent proteins. Here, we have examined AZ protein versus SV protein trafficking in the sciatic nerve axons. Similar to central neurons, AZ proteins in the axons of sciatic nerve also exhibit fast trafficking. We did not find any dense core vesicles in our electron microscopy analysis of sciatic nerve. It is likely that clear core vesicles are involved in trafficking of AZ proteins such as Piccolo in sciatic nerve. It may be possible that PTVs may be involved only during synaptogenesis in both central and peripheral synapses. AZ proteins most likely undergo synthesis in cytosolic ribosomes, and thus need to be recruited on the outer surface of *trans*-golgi network in order to be transported on a vesicle. The central region of Bassoon protein may bind to the golgi membrane [17], but little is known about such mechanism in other AZ proteins. Many AZ proteins have C2 domains which may or may not bind to calcium. C2 domain can bind to molecules like PIP2 (phosphoinositol biphosphate) within the lipid bilayer thus recruiting the protein to membrane [35]. Similarly, several AZ proteins have PDZ domains that binds to cytosolic tails of membrane anchored proteins. Such interactions may also be important for membrane binding of AZ proteins. PDZ domain themselves may also bind directly to lipid moieties [36]. Thus it is possible that AZ proteins may bind to the membrane of golgi-derived vesicles by many different possible molecular interactions yet to be examined. Proteins like Liprins-α also may indirectly bind to membrane anchored protein like Neurexins [37]. AZ molecules like Piccolo and Bassoon are selectively sorted to the axon and then to the presynaptic terminal. Thus, mere anchoring of these proteins to golgi membrane itself is not sufficient as the anchoring must take place at specific membrane domains or on specific vesicles with cargo that are destined to be sorted to the axons. How AZ proteins containing vesicles are sorted at the golgi yet remains to be addressed.

Biochemically, many AZ proteins exhibit a low solubility even in presence of non-denaturing detergents [20]. It is likely that even during trafficking they form large protein complexes that potentially also interact with cytoskeletal proteins. Indeed, it has been proposed that unitary AZs travel down the axons as preassembled units on vesicles that fuse to nascent presynaptic terminals to form functional AZ [38]. However, we and others have noted that

different AZ proteins may also have different distribution and trafficking patterns during development which argues against unitary assembled trafficking [17,39]. Overall, the axonal trafficking of non-membrane AZ proteins seems to be very different from that of other soluble (non-membrane anchored) proteins [40] which may allow for rapid transportation, formation and maintenance of this important presynaptic subdomain. Finally, a robust retrograde transport of AZ protein suggest that AZ proteins marked for degradation are most likely targeted retrogradely to the cell soma for destruction via the proteasome pathway. In fact, AZ may be a highly dynamic structure, with proteins getting exchanged between synapses in central neurons [41]. In NMJ, where synapses are at the terminal far from the cell soma, such local exchanges may be possible but the retrograde trafficking is intriguing and may indicate a low proteasomal activity at NMJ presynaptic compartment. In fact, it has been noted that stable synapses in general may have a low proteasome activity [42]. In many neurodegenerative disorders as well as axonopathies, axonal trafficking is affected, this likely leads to accumulation of aged AZ proteins at the NMJ and contributes to stress and degeneration of the junction.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.04.041.

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