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Finding Order in Slow Axonal Transport

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Slow axonal transport; cytosolic proteins; synapsin; actin; clathrin; cytoskeleton

ORDER IN FAST AND SLOW AXONAL TRANSPORT

Order is everywhere in neurons. Starting from the orderly Golgi-stacks that allow sequential processing of proteins as they exit the soma, to the exquisite uniformly-sized synaptic vesicles that control quantal release of neurotransmitters – all remind us of the order and method that seem to dictate cellular processes in neurons, and more broadly, the natural world. Understanding *order* is key in gaining mechanistic insight and designing the right experiments, and this brief review will discuss the logic and order underlying slow axonal transport – a vital but poorly understood phenomenon in neurobiology.

Axonal transport a constitutive phenomenon by which macromolecules synthesized in the neuronal cell-bodies are conveyed into axons and synapses. Numerous studies have shown that axonal transport is essential for neuronal survival, including observations dating back to Ramon y Cajal in the nineteenth century, that axons disconnected from their cell bodies degenerate [1]. The first glimpse of an orderly arrangement in axonal transport came from pulse-chase radiolabeling studies that characterized the movement in vivo. In these experiments, pulse-incorporation of radiolabeled amino acids into newly-synthesized proteins in neuronal cell-bodies of living animals, and subsequent egress of the labeled proteins into axons, generated an overall map of cargo-movement (Fig. 1A).

Radiolabeling experiments revealed two overall rate-classes called fast and slow axonal transport. While the fast component consisted of membranous organelles moving at ~ 50–200 mm/day, the slow component conveyed an entirely different set of cargoes, namely cytoskeletal and cytosolic (or “soluble”) proteins moving at ~ 0.2–10 mm/day; overall rates

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Conflict og interest
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that were several orders of magnitude slower [2,3]. While there were some rate-variations in different neuronal types, the basic distinction between slow and fast transport has been seen in essentially every species studied to date, including mice, rats, guinea pigs, rabbits, *Aplysia*, *Xenopus*, and others [4–14]. The sustained slow transit over long axons precluded diffusion as the sole mechanism, which decays exponentially over time and cannot deliver diffusible molecules (such as free radiolabel) to axon-tips [8].

Detailed studies in many species showed that the cytoskeletal and cytosolic proteins in the slow component largely moved as distinct radiolabeled "peaks". While the slowest group – termed 'Slow Component a' or SCa – was composed of the main cytoskeletal proteins neurofilaments and microtubules; a slightly faster group within slow component carried cytosolic proteins and actin, termed 'Slow Component b' or SCb (Fig. 1A–B). Interestingly, studies in long axons that allowed good spatial and temporal separation of the transport components showed that there was very little overlap between SCa and SCb proteins, as seen in 2-D gels that can resolve individual transported proteins (Fig. 1C). The co-transport of cargoes within a given rate-component – and the distinct cargo-composition between the three components – was a striking feature of the radiolabeling data and immediately suggested an underlying order in axonal transport; a concept that has intrigued researchers ever since.

What is the basis for this co-transport? Regarding fast transport, several decades of studies have revealed many details behind the orderly movement of vesicles. For instance, a striking polarization of microtubules in the axon maintains unidirectional vesicle-transport in and out of the axon, ensuring homeostasis. A general theme has emerged, where vesicles bind to motor proteins Kinesin and dynein, allowing vectorial movement on microtubules, and various regulators and modulators act upon this transport machinery to fine-tune the movement [15–18]. Though there are variations and improvisations on this overall theme, it seems reasonable to say that the underlying *order* of fast axonal transport – and the basis for the rapid exit of pulse-labeling seen in the earlier radiolabeling studies – is understood.

Amongst the models that were put forth to explain the seemingly cohesive movement of clusters of proteins in slow axonal transport, the "structural hypothesis" was particularly influential. The central idea was that proteins are transported in the axon as "component parts of intact cytological structures", where "each rate component represents a discrete macromolecular assembly that moves as a unit" [6,19]. For instance, regarding cytoskeletal transport in SCa, a prediction was that cytoskeletal monomers would be transported in association with mobile polymers, and that other molecules associated with the cytoskeleton (microtubule-associated proteins for instance) would move in association with the moving polymers. The movement of vesicles in fast axonal transport seemed to validate the hypothesis because radiolabel associated with a discrete structure – i.e. a vesicle or membranous organelle – was clearly responsible for all fast transport.

The idea of structural hypothesis was also extrapolated to cytosolic proteins moving in SCb. Individual radiolabeled "peaks" of selected SCb proteins suggested a remarkable cotransport that lasted over days (see Fig. 2, from [7]). Such coherence in slow transport was proposed as evidence of a common structure or scaffold that was moving these cytosolic proteins. One

idea was that proteins at the very front of the moving SCb peaks might be the anchor on which other cytosolic molecules might assemble as multiprotein complexes [20]. Alternatively, since the cytoskeletal protein actin moved in SCb, it was also proposed that “actin-cages” might corral various cytosolic proteins during slow transport [21].

VISUALIZATION OF SLOW AXONAL TRANSPORT

While pulse-chase radiolabeling characterized overall axonal transport, there were several limitations of this technique. First, the transport was not directly visualized, but could only be inferred by the changing pattern of radioactivity in sequential axonal segments over time. Second, the in vivo system could not be easily manipulated to evaluate mechanistic details. For instance, it was not feasible to inhibit the movement of a single cargo and determine effects on other cargoes using pulse-chase radiolabeling. A third conundrum was that axonal transport in these experiments could only be evaluated on the time scales of hours to days, and events that happened on shorter timeframes remained unknown; limiting the mechanistic insight that could be derived from such experiments. In the absence of a method to visualize slow transport, the structural hypothesis remained controversial, with many arguing for a transport paradigm involving movement of monomeric proteins [22].

Direct visualization of slow axonal transport in cultured neurons have overcome these limitations and offered new insights. Observation of neurofilament transport in axons of cultured neurons showed that neurofilament proteins were transported as assembled polymers [23,24]. Moreover, when neurofilament transport was observed at shorter time scales, polymers were found to move rapidly at rates similar to fast axonal transport. However, unlike fast transport, neurofilaments paused for prolonged periods during transit, and the overall movement was infrequent. Correlative live and EM studies have firmly established that single neurofilaments are transported [25]. Known as the “stop and go” model [26,27], this concept cleared up a number of confusing aspects of slow axonal transport, including the absence of a “slow” kinesin motor. Visualization of tubulin transport has been more difficult because of the dense packing of microtubules in the axon shaft. However, the current thinking is that short microtubules are intermittently transported in axons [28,29]. The mechanistic basis of the intermittent movement in slow axonal transport is unclear.

VISUALIZATION OF CYTOSOLIC SLOW AXONAL TRANSPORT (SCb)

Unlike punctate vesicles, many cytosolic cargoes are diffusely distributed along the axon and tagging by conventional fluorophores only gives a diffuse glow. Thus, photoactivatable probes have been used to visualize SCb transport. In these experiments, a small pool of cytosolic molecules was photoactivated in the axon-shaft, and the overall anterogradely biased flow of the activated fluorophores was visualized over time (Fig. 3A - left; also see [30,31] for details). Quantification of this biased flow revealed a slow, directed motion towards the axon-tip, at rates consistent with slow axonal transport. Such anterogradely biased flow in cultured neurons has been demonstrated for several cytosolic SCb proteins – synapsin, CamKII, dynein, α-synuclein, clathrin [31–34] – and also for actin, a cytoskeletal protein moving in SCb [35]. Using this paradigm in cultured hippocampal neurons, the slow

axonal transport of synapsin – an SCb protein enriched at synapses – has been studied the most, revealing some insights. As a technical point, though diffusion can rapidly move soluble molecules in the relatively short axons of cultured neurons – for instance soluble GFP will fill up the axon over time – the time course of synapsin enrichment in axons in distal synapses is clearly distinct from that of soluble GFP (see Fig. 2 of [32]). Moreover, in axons, the fast, exponentially decaying mobility of purely soluble molecules is very different from the energy-dependent, anterogradely-biased motion of SCb proteins [31]; making these neurons a good model-system for slow transport studies. Application of pulse-chase radiolabeling to cultured neurons growing in chambers that segregate soma from terminal axons also confirm the existence of slow transport in cultured neurons [36].

An early idea was that soluble molecules were assembling into multiprotein complexes, followed by axonal transport of those complexes [7]. Visualization of slow synapsin transport in cultured axons suggested transient recruitment of soluble molecules onto a moving structure – the ‘dynamic recruitment’ model [31,32]. The idea here is that various cytosolic molecules (or complexes) can transiently associate on the surface of a persistently mobile unit moving in fast axonal transport. Consequently, the overall displacement of the slow transport cargo will be inefficient and sluggish, compared to its faster counterpart. Interestingly, blocking vesicle transport also inhibited the slow anterograde bias of synapsin in axons, suggesting that ultimately, slow transport of synapsin was dependent on fast vesicle transport [32]. Whether this is a general theme for slow transport remains to be seen. Using a combination of live imaging and proteomics, more recent studies also suggest that the dynamic recruitment of synapsin on a persistently moving unit – presumably vesicles – is regulated by Hsc70 chaperone activity, offering clues into the regulation of SCb [37]. Visualization of synapsin-Hsc70 complexes in axons by super-resolution microscopy also support the idea of SCb complexes [37].

AN UNEXPECTED DIVERSITY IN CYTOSOLIC SLOW AXONAL TRANSPORT

An overarching theme from radiolabeling experiments was the mechanistic commonality in slow axonal transport in general, and SCb in particular. However, visualization of individual cargoes in slow transport indicate that multiple mechanisms operate in SCb, perhaps masquerading as overlapping waveforms in the pulse-chase experiments. Steady-state distribution along the axon can also offer clues into the transported cargo-structure. For instance, the steady-state distribution of membrane-anchored cargoes in the axon – like synaptophysin or VAMP2 – is punctate, and indeed, punctate motile vesicles are seen by live imaging, which is the basis for fast transport. If all cytosolic proteins in slow axonal transport were moving coherently, it would make sense that their steady-state distribution along the axon-shaft would also be similar. However, that is not the case. For instance, the distribution of the three SCb proteins synapsin, clathrin, and actin in the axon-shaft is very different. While synapsin is uniformly distributed, clathrin has a punctate appearance [31,34]. Axonal actin is distributed along the shaft as puncta and filaments, and live imaging showed focal “hotspots” where actin continuously polymerizes and depolymerizes, as well as rapidly-elongating actin filaments that extended bidirectionally along the axon-shaft (“actin trails”, see Fig. 3A and [38–40]).

The three cargoes are also transported very differently in axons. Kymographs from live imaging of three SCb proteins – synapsin, clathrin and actin – in axons of cultured hippocampal neurons is shown in Figure 3A. Pulse photoactivation of synapsin tagged to photoactivatable GFP (PAGFP) revealed an anterogradely-biased 'flow' of synapsin in axons (Fig. 3A – left; also see [37]). On the other hand, axonal clathrin particles move intermittently and frequently, but with an overall anterograde bias – distinct from vesicle transport, but reminiscent of moving neurofilaments (Fig. 3A – middle and 3B; also see [34]). Slow anterograde transport of actin appears to be generated by an unconventional mechanism involving local assembly and biased polymerization (Fig. 3A – right; see [35] for details). Importantly, manipulations that block slow transport of one cargo have no effect on another, suggesting multiple mechanisms in SCb. For example, a formin-inhibitor that blocks the anterogradely-biased transit of actin in axons has no effect on axonal synapsin dynamics (Fig. 3C; also see [35]). Similarly, while disruption of microtubules inhibited synapsin and clathrin mobility in the axon-shaft, it has no effect on axonal actin dynamics [34,38]. Thus, the emerging data suggest that multiple mechanisms are at play in SCb transport.

EMERGING MECHANISTIC THEMES IN SLOW AXONAL TRANSPORT

Recent studies using live imaging and other contemporary tools have revealed a complexity in slow axonal transport that was not fully appreciated by earlier studies. However, long-term pulse-chase and live imaging experiments offer complementary information across temporal and spatial scales, and some general concepts have emerged from synthesizing information from these two experimental paradigms. First, the main difference between fast and slow transport seems to be related to "duty-ratio"; the proportion of time that the cargo-structures spend moving [26]. While vesicles in fast transport have a high duty-ratio – continuously engaging with moving motors – slow transport cargoes associate infrequently, thus making the overall movement slow. These associations can be extremely transient, with "dynamic recruitment" of cytosolic molecules (like the biased flow of synapsin), or pre-assembled structural units can be intermittently transported (like neurofilaments or clathrin). What is the nature of the rapidly-moving cargo? Available evidence suggest that slow cargoes may directly bind to motors [41–43], or associate with mobile vesicles [32]; however, these two scenarios are difficult to distinguish because methods blocking one invariably block the other. A previous study applying pulse-chase radiolabeling to cultured sympathetic neurons found that Golgi disruption by Brefeldin-A inhibited the transport of tubulin (and apparently, also other SCb proteins) in axons [36]. Though these studies suggest that slow transport might be ultimately dependent on fast transport, the data are far from conclusive and further studies with better tools are needed.

Though the concept that individual rate components represent co-transport of macromolecular assemblies, conveyed by a common mechanism, is probably incorrect – at least for slow axonal transport – a caveat is that amongst the hundreds of cytosolic SCb proteins, live imaging experiments have only looked at a handful. Thus, it is possible that the imaging studies are looking at a few slow-component cargoes that are not co-transported, biasing our view. Moreover, proteomics-data do indicate that SCb proteins can organize into multi-protein complexes [34,37], so it is likely that there is some order to this movement.

One possibility is that cytosolic and cytoskeletal proteins organize into complexes of functionally related proteins that are assembled in cell bodies before transit, and then "shipped" to their destinations within axons and synapses. Recent studies suggest important axonal and synaptic roles for GAPDH [44–46], a cytosolic protein conveyed in slow axonal transport [13]; but studies combining visualization of slow transport, axonal/synaptic targeting, and function are lacking. Moreover, studies characterizing transport kinetics of more slow-component proteins are needed to make progress. In fact, even the identity of most SCb proteins are unknown to this day. Newer model-systems that bridge temporal and spatial scales – offering both a big-picture as well as molecular view of slow axonal transport – may also bring more clarity. Finally, fast transport has been visualized in vivo [47], and in iPSC-neurons [48], and application of these systems to slow transport is warranted.

Since its initial characterization over 50 years ago, the sluggish nature of slow axonal transport has intrigued researchers; but much of it is still a mystery. Despite the undisputable importance of this rate-class – that conveys three times as much material as its faster counterpart – progress has been slow because of difficulties in visualizing the movement. However, recent studies have begun to offer concrete answers, and one remains optimistic that mechanistic details underlying the *order* behind this final remaining puzzle in axonal transport will soon emerge.

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Slow axonal transport conveys cytosolic and cytoskeletal proteins into axons and synapses at overall velocities that are several orders of magnitude slower than the fast transport of membranous organelles such as vesicles and mitochondria. The phenomenon of slow transport was characterized by in vivo pulse-chase radiolabeling studies done decades ago, and proposed models emphasized an orderly cargo-movement, with apparent cohesive transport of multiple proteins and subcellular structures along axons over weeks to months. However, visualization of cytosolic and cytoskeletal cargoes in cultured neurons at much higher temporal and spatial resolution have revealed an unexpected diversity in movement - ranging from a diffusion-like biased motion, to intermittent cargo dynamics and unusual polymerization-based transport paradigms. This review provides an updated view of slow axonal transport and explores emergent mechanistic themes in this enigmatic rate-class.

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Highlights

- Pulse-chase radiolabeling studies characterized fast and slow axonal transport
- Fast and slow axonal transport convey distinct, non-overlapping cargoes
- Apparent coherence is seen in transport of slow component cargoes
- Live imaging indicates surprising diversity amongst slow transport cargoes
- Slow component may convey diverse multiprotein complexes with distinct mechanisms
- Slow component may depend on vesicle transport

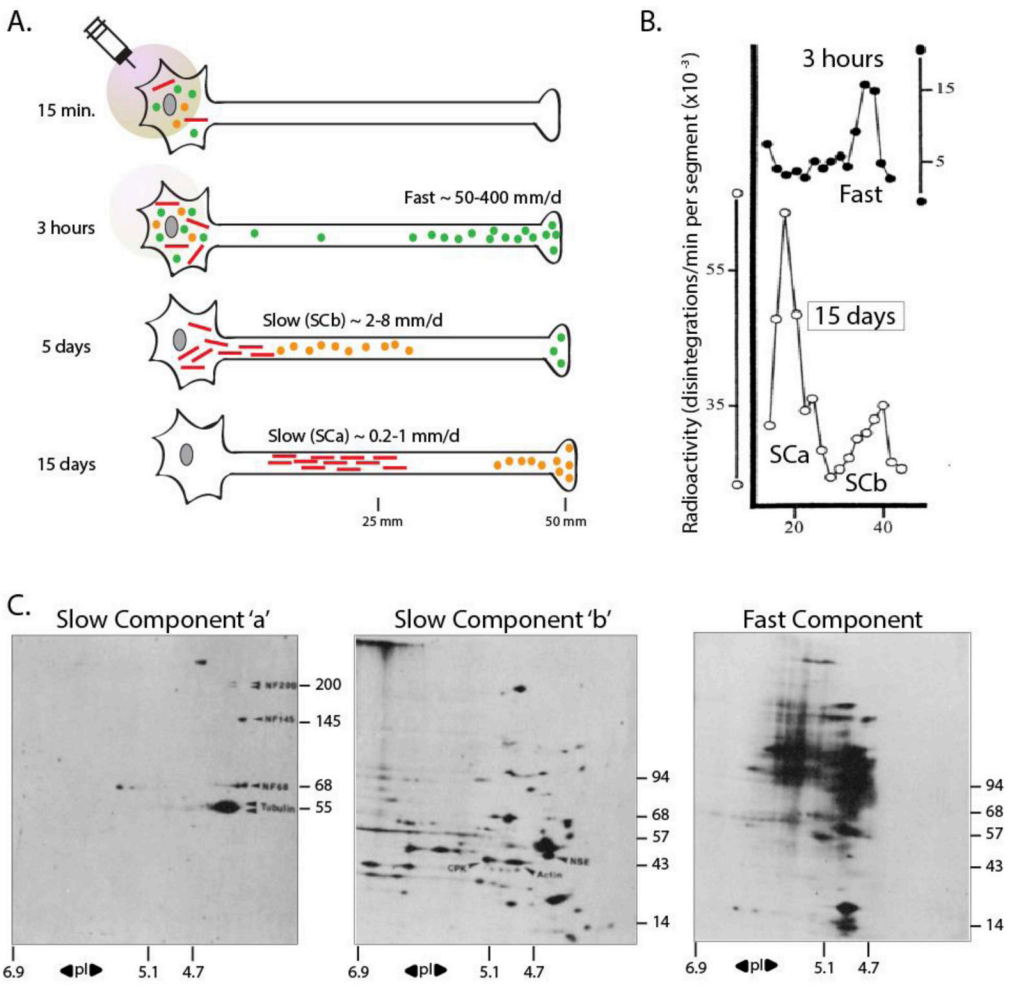

Figure 1: Radiolabeling studies to evaluate axonal transport.

A. Schematic showing radiolabeled amino-acids (purple halo) injected in the vicinity of neuronal cell bodies; subsequently incorporated into new synthesized proteins (green, orange and red shapes) and transported along axons. Note egress of fast and slow components into the axon. Figure reproduced with permission from [2].

B. Axonal transport data from hypoglossal axons of guinea pigs, after injection of radiolabeled amino-acids into the hypoglossal nucleus (site of neuronal cell-bodies). X axis shows distance from the hypoglossal nucleus (in mm), and radioactivity-counts are shown in Y-axis. Note that at 3 hours after labeling (top), a well-defined wave is seen, representing the fast component. After 15 days, two distinct radiolabeled waves are seen, representing the two rate-classes within the slow component, SCa and SCb (see text for details). Figure reproduced with permission from [6].

C. Comparing cargo-composition of fast and slow components. Axonally transported proteins from guinea pig optic nerves were radiolabeled as noted above and analyzed by two-dimensional electrophoresis and fluorography at 6 hours, 6 days, or 40–60 days – to analyze the fast component, SCb and SCa respectively (pH gradient of gel at bottom and apparent molecular weights on right). The resolution of this technique allows separation of individual proteins and highlights the distinct cargo composition of each rate-class. Some

individual proteins are identified by arrowheads on the gel: neurofilaments (NF) and tubulin (SCa, left); creatine phosphokinase (CPK), actin and nonspecific enolase (NSE, SCb, middle). Figure reproduced with permission from [14].

Fig. 1A, 1B and 1C reproduced with permission from [2], [6] and [14] respectively.

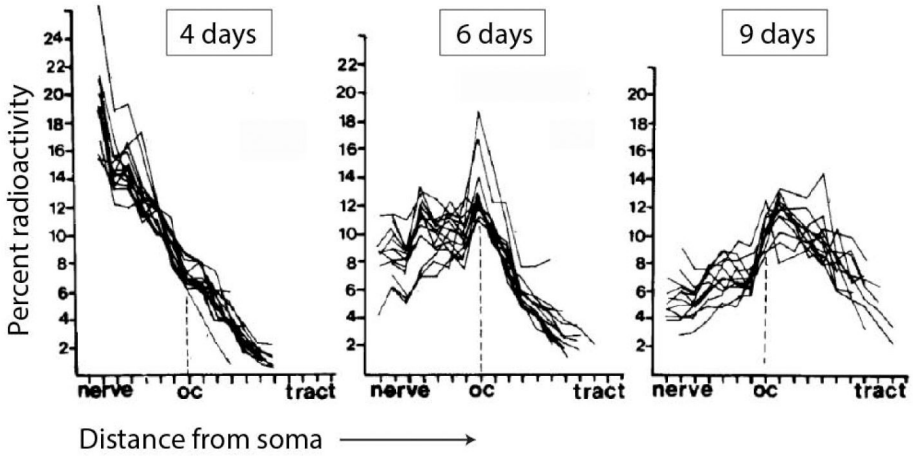

Figure 2:

Overlaid radiolabeled “wave profiles” of 20 selected SCb proteins in guinea pig optic axons; four, six, and nine days after intraocular injection of radiolabeled amino acids. Note the overall coherence in transport-profiles of diverse cytosolic proteins, even after days of transport. Optic chiasm (oc). Distance in x-axis (mm units). Relative radioactivity units on y-axis. Reproduced with minor modifications from [7], with permission.

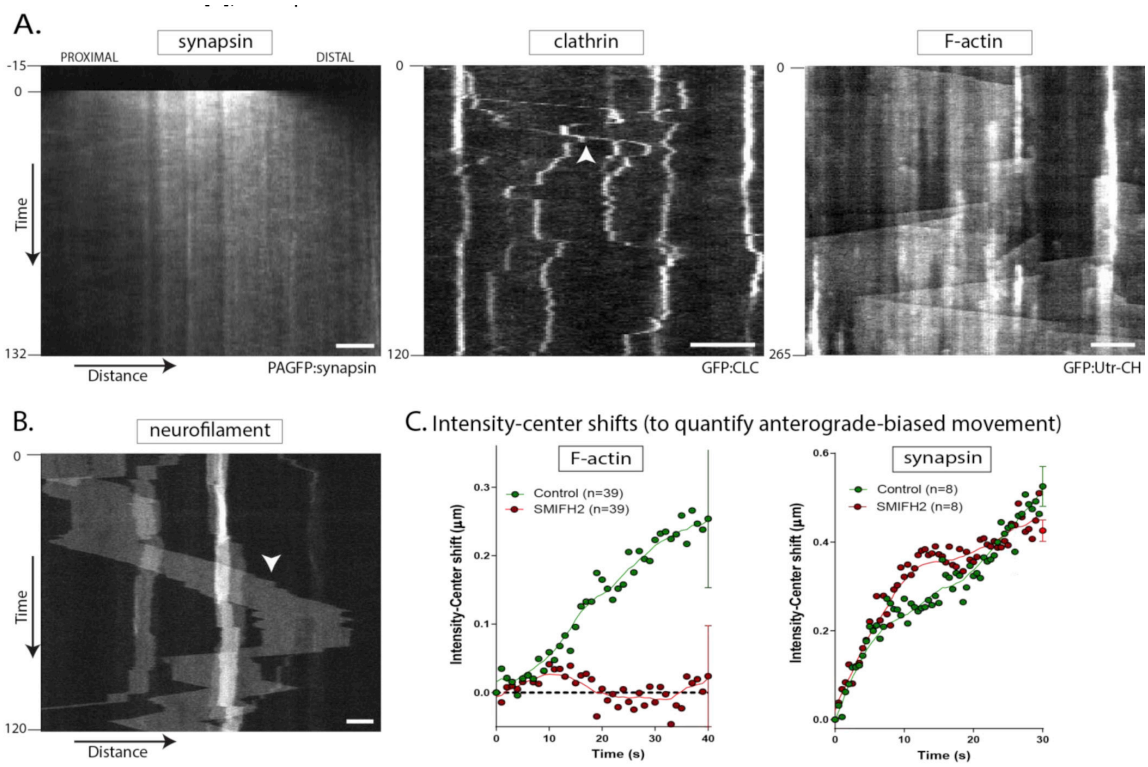

Figure 3: Distinct dynamics of slow component proteins in axons.

A. Kymographs showing axonal dynamics of three cytosolic proteins moving in SCb. Distance in microns on x-axis and time in seconds on y-axis, and movements from upper-left to lower-right reflect anterograde transport in these kymographs. Cultured hippocampal neurons were transfected with PAGFP tagged to synapsin (left); GFP tagged to clathrin light chain (GFP:CLC, middle); or GFP tagged to the calponin homology domain of utrophin (UtrCH) – a marker of filamentous actin (F-actin). Note that pulse-activated synapsin at t=0 appears to diffuse bidirectionally in the axon, but with an anterograde bias (relatively more PAGFP:synapsin fluorescence is seen on the distal, i.e. right, half of kymograph as time passes). However, also note that the intermittent movement of clathrin particles (one marked by arrowhead) is very different from synapsin. F-actin imaging shows focal hotspots (vertical interrupted lines) where actin continuously polymerizes and depolymerizes; as well as elongating polymers extending bidirectionally (diagonal plumes). Kymograph on left is reproduced from [33], with permission. Kymographs in middle and right panels are unpublished, but are similar to those published in [34, 35]. Scale bar is 5µm.

B. Kymographs from live imaging of GFP tagged neurofilament-M protein in cultured sympathetic neurons. Note translocation of a single filament (arrowhead), with frequent pauses in transport. Figure reproduced from [27], with permission. Scale bar is 5µm.

C. Quantification of the anterogradely biased transit of F-actin (GFP:Utr-CH) in axons, using an “intensity-center shift” assay that quantifies shifts in the centroid of a photoactivated axonal-pool over time (see [35] for details). Anterogradely-biased transport is depicted by positive intensity-center shifts. Note that while the formin inhibitor SMIFH2 blocks anterograde shift of F-actin in axons, it has no effect on the anterogradely biased movement of synapsin (fig. reproduced from [35], with permission).

Figure 3A – left, 3B and 3C adapted from [33], [27], and [35] respectively. Figure 1A – middle and right are unpublished kymographs, but similar to previously published data [34, 38].