Differential roles of kinesin and dynein in translocation of neurofilaments into axonal neurites

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Summary

Neurofilament (NF) subunits translocate within axons as short NFs, non-filamentous punctate structures (‘puncta’) and diffuse material that might comprise individual subunits and/or oligomers. Transport of NFs into and along axons is mediated by the microtubule (MT) motor proteins kinesin and dynein. Despite being characterized as a retrograde motor, dynein nevertheless participates in anterograde NF transport through associating with long MTs or the actin cortex through its cargo domain; relatively shorter MTs associated with the motor domain are then propelled in an anterograde direction, along with any linked NFs. Here, we show that inhibition of dynein function, through dynamitin overexpression or intracellular delivery of anti-dynein antibody, selectively reduced delivery of GFP-tagged short NFs into the axonal hillock, with a corresponding increase in the delivery of puncta, suggesting that dynein selectively delivered short NFs into axonal neurites. Nocodazole-mediated depletion of short MTs had the same effect. By contrast, intracellular delivery of anti-kinesin antibody inhibited anterograde transport of short NFs and puncta to an equal extent. These findings suggest that anterograde axonal transport of linear NFs is more dependent upon association with translocating MTs (which are themselves translocated by dynein) than is transport of NF puncta or oligomers.

Key words: Dynein, Kinesin, Axonal transport, Neurofilament, Nocodazole, Microtubule

Introduction

Neurofilaments (NFs) are among the most abundant constituents of the axonal cytoskeleton. In conjunction with microtubules (MTs) and the submembrane actin skeleton, they establish a longitudinally oriented network that provides stability to axons (Pant and Veeranna, 1995; Shea and Chan, 2008). Like all axonal components, NFs must be transported into axons (Galbraith and Gallant, 2000; Shea and Yabe, 2000). Introduction of haptenized exogenous NF fusion proteins into neurons has facilitated analyses of NF axonal transport. NF subunits translocate within axons in multiple forms, including as short NFs, non-filamentous punctate structures (‘puncta’) and diffuse material that might represent unassembled subunits or hetero-oligomeric subunit assemblies (Jung et al., 1998; Motil et al., 2006; Roy et al., 2000; Prahlad et al., 2000; Theiss et al., 2005; Wang et al., 2000; Yabe et al., 1999; Yabe et al., 2001a; Yabe et al., 2001b; Yuan et al., 2009). The interplay, if any, among these various forms remains unresolved. Punctate structures have been shown to participate in remodeling of the intermediate filament network in non-neuronal systems (Helfand et al., 2004; Prahlad et al., 1998). Whether punctate forms of NF subunits carry out a similar role, and/or whether any such role is confined to perikarya or the initial elaboration of neurites, has not been clarified. However, punctate structures and short NFs transport along axons at overlapping rates, and the predominant form of transporting NF subunits varies according to the neuronal differentiation state, leaving open the possibility that short NFs and punctate structures each contribute in distinct manners to the establishment and/or regional remodeling of the NF network (Prahlad et al., 2000; Yabe et al., 1999; Yabe et al., 2001a; Yabe et al., 2001b). Turnover of haptenized NFs has been observed in the absence of any participating macrostructure (e.g. NF or punctuate structures), leaving open the additional possibility that turnover might also be mediated, at least in part, by subunits or small oligomers (Yuan et al., 2009).

Transport of NFs into and along axons requires an intact MT system (Francis et al., 2005; Jung et al., 1998; Jung et al., 2004; Yabe et al., 2000) and the action of the MT motor proteins kinesin and dynein (He et al., 2005; Motil et al., 2006; Prahlad et al., 2000; Shah et al., 2000; Theiss et al., 2005; Uchida et al., 2009; Xia et al., 2003; Yabe et al., 1999; Yabe et al., 2000). As with other axonal constituents, kinesin mediates anterograde NF transport (Prahlad et al., 2000; Theiss et al., 2005; Xia et al., 2003; Yabe et al., 1999; Yabe et al., 2000), whereas dynein mediates retrograde transport (He et al., 2005; Motil et al., 2006; Shah et al., 2000; Theiss et al., 2005). However, the dynamics of assembly and transport of NFs are complex; these MT-based motor proteins have multiple interactions with actin and the actin-based motor myosin (Francis et al., 2005; Jung et al., 2004; Rao et al., 2002). Unexpectedly, the retrograde-directed motor dynein also participates in the anterograde delivery of NF forms into axonal neurites (Motil et al., 2006). The probable mechanism underlying this initially counterintuitive role of dynein is the unique role of this motor protein in delivery of MTs into axons; as dynein can associate with MTs through its cargo domain, as well as its motor domain, it is capable of translocating shorter MTs in an anterograde direction through cargo-based interactions with relatively larger MTs and/or actin filaments. This process maintains a uniform orientation of axonal MTs, with their plus-end directed towards the synapse (Ahmad et al., 1998; Baas, 1998; Dillman et al., 1996). Those NFs that undergo dynein-mediated delivery into axons presumably do so through ‘piggybacking’ on MTs that are themselves undergoing dynein-mediated translocation (Motil et al., 2006). Such NFs might
associate directly with translocating MTs (Hisanaga et al., 1993) and/or might be crosslinked to translocating MTs through kinesin (e.g. Liao and Gundersen, 1998; Kushkuley et al., 2009).

Although previous studies have confirmed a role for both kinesin and dynein in the delivery of NFs into axonal neurites, they did not investigate whether kinesin or dynein preferentially translocated short NFs or punctate structures into axonal neurites. Here, we present evidence that either motor protein can translocate punctate structures, but that translocation of short NFs is more crucially dependent upon the activity of dynein rather than kinesin.

Results
Axonal neurites of differentiated NB2a/d1 cells display a prominent longitudinal NF bundle, along with short individual NFs and puncta (Fig. 1). Both unstable and stable MTs extend from the perikaryon into the axonal shaft (Fig. 1). We set out to quantify the relative impact of inhibition of kinesin and dynein on the delivery of GFP-tagged puncta and short NFs into the axonal hillock by intracellular delivery of antibodies against these motor proteins and by transfection with a construct expressing Myc-tagged dynamitin. Intracellular delivery of antibodies against kinesin or dynein did not disrupt the overall cellular architecture within the 2-hour experimental window utilized; no differences were detected in the neurite length or the distribution of steady-state levels of MTs or filamentous actin (Fig. 2). However, intracellular delivery of antibodies against either kinesin or dynein disrupted the normal perikaryal localization of the Golgi complex within 2 hours (Fig. 2), consistent with previous studies demonstrating a requirement for a MT motor function in the maintenance of Golgi distribution (Corthesy-Theulaz et al., 1992; Johnson et al., 1996; Rogalski and Singer, 1984; Thyberg and Moskalewski, 1999). Consistent with previous studies (Ahmad et al., 1998; He et al., 2005; Motil et al., 2006; Yabe et al., 1999), these analyses confirmed that there was an acute inhibition of motor protein function following intracellular delivery of the antibodies but a lack of overall cytoskeletal trauma.

Intracellular delivery of anti-dynein antibody and overexpression of Myc–dynamitin each reduced the percentage of short NFs within the axonal hillock by ~50%, with a corresponding increase in the percentage of puncta (Fig. 3). Similar levels of diffuse GFP signals remained dispersed throughout the perikarya and axonal hillock, which might correspond to less-organized oligomers or individual subunits (Shea et al., 1990; Yabe et al., 2001a; Yuan et al., 2009). By contrast, inhibition of kinesin activity did not alter the percentage of short NFs or puncta within the axonal hillock (Fig. 3).

The reduction in the percentage of short NFs within the axonal hillock was not caused by inhibition of NF assembly as the number of short NFs or puncta within the soma was not reduced by intracellular delivery of the anti-dynein antibody, overexpression of dynamitin or intracellular delivery of the anti-kinesin antibody (Fig. 3). Moreover, interference with dynein activity did not significantly reduce the total number of puncta within the axonal hillock region; similar numbers of puncta were observed within the hillock before and after intracellular delivery of anti-dynein antibody (30±2 and 33±3, respectively; P<0.23), as well as before and after overexpression of Myc–dynamitin (28±5 and 21±3, respectively; P<0.84). It should be noted that the substantially larger volume of the perikarya compared with that of the axonal hillock precluded detection of a corresponding increase in perikaryal short NFs following their decrease within the axonal hillock; as sections of perikarya occupied an area 11.3±(±2)-fold larger than their corresponding hillock (n=5 cells), a 50% decrease in short NFs within a given hillock could only generate a ≤5% increase within the corresponding perikarya, which is smaller than the >10% variance observed when quantifying short NFs within perikarya (see Fig. 3). The lack of short NFs within the shaft following intracellular delivery of anti-dynein antibody means that the inhibition of dynein did not accelerate NF translocation out of the hillock and into the shaft [Fig. 4; see also Motil et al. (Motil et al., 2006)]. However, it remained possible that intracellular delivery of anti-dynein antibody provoked retrograde transport of NFs from the shaft into the hillock. Although this was unlikely to occur as a result of dynein inhibition, we addressed this possibility through real-time analyses as follows: we transfected cells with GFP–NF, waited until the GFP was prominent throughout perikarya and neurites (~18–24 hours), introduced antibody against dynein or kinesin, through ProVektin, and immediately captured images of live cells at 1-second intervals (Fig. 5). We then subtracted the fluorescence from the images from those captured 1 second previously (which depletes colocalized pixels and highlights differences between sequential images) and quantified the pixel distribution along the axonal hillock and proximal axon as described previously (Yabe et al., 2001c) (Fig. 5A). As anticipated, net retrograde transport was not detected in mock-treated cells; it has been demonstrated previously that 40–50% of NFs and NF subunits normally undergo anterograde transport, whereas only 10% undergo retrograde transport (Chan et al., 2004; Motil et al., 2006). Cells receiving ProVektin alone demonstrated a progressive sequential increase in pixels appearing in more distal segments. Cells receiving anti-dynein antibody also had an increase in distal segments, but this increase was reduced by ~30%, consistent with the role of...
dynein in anterograde delivery of NFs and NF subunits into axonal neurites (Motil et al., 2006). By contrast, cells receiving anti-kinesin antibody displayed an ~10% reduction in both distal and proximal segments, consistent with the prior demonstration that treatment with anti-kinesin antibody prevented all anterograde NF transport but permitted retrograde transport (Chan et al., 2004; Yabe et al., 1999; Motil et al., 2006). We also false-colored every other image in the sequence red, merged them with the prior (green) GFP image and quantified the percentage of colocalized (yellow) pixels within the axonal hillock (Fig. 5B). Treatment with anti-dynein antibody increased the percentage of colocalized pixels by ~40% compared with that upon treatment with ProVectin alone; this correlates with the 50% inhibition of anterograde delivery of NFs and NF subunits into axonal neurites when using this antibody (Motil et al., 2006). Anti-kinesin antibody caused a >100% increase in colocalized pixels, consistent with the impact of this antibody on anterograde transport (Yabe et al., 1999). We also compared the number of short NFs and puncta within the hillock at the start and end of the 10–15-second sequence of captured images (Fig. 5B). Treatment with ProVectin alone or with anti-dynein antibody did not alter the number of short NFs or puncta within the hillock in this short time interval. Anti-kinesin antibody, by contrast, induced 40% and 50% increases in puncta and NFs, respectively, within the axonal hillock; these values serve as a positive control for the lack of any such observation following intracellular delivery of anti-dynein antibody. These real-time analyses argue against the unlikely possibility that dynein inhibition resulted in accumulation of NFs within the hillock through retrograde transport out of axonal neurites.

These findings collectively indicate that dynein plays a more substantial role in translocation of short NFs, compared with that of puncta, out of the perikaryon and into axonal neurites. We reason that those NFs undergoing dynein-mediated delivery into axonal neurites do so by ‘piggybacking’ on short MTs that were themselves undergoing dynein-mediated transport into axonal neurites, as occurs when dynein associates with longer MTs and/or the actin network through its cargo domain (Ahmad et al., 1998; Baas, 1998; Dillman et al., 1996). This possibility is consistent with prior observations that both short NFs and puncta associate with, and translocate along, MTs and actin (Jung et al., 2004; Motil et al., 2006). If this were the case, we reasoned that elimination of short MTs would also impair the delivery of short NFs in the axonal hillock. We therefore examined the consequences of a short-term (2 hour) treatment with nocodazole on the distribution of NFs and puncta. Nocodazole treatment under these conditions reduced the MT content of the hillock (Fig. 6), eliminating non-stabilized

Fig. 2. Impact of intracellular delivery of antibodies against kinesin and dynein on NB2a/d1 homeostasis. (A) Antibodies against kinesin or dynein were delivered into cultures as described in the Materials and Methods section and, 2 hours later, were processed, along with mock-loaded controls, for immunofluorescence analysis, to confirm intracellular delivery of antibodies, by incubation with the appropriate secondary antibody (Internal antibody), or to monitor MTs or filamentous actin, as indicated. Representative images are presented. The accompanying graphs present densitometric quantification (means ± s.d.; through ImageJ) of the relative amount of MT and actin within axonal neurites compared with that in the total cell as the integrated density of the neurite (integrated density of neurite plus soma) (n=50–70). The cells presented for MT distribution are the identical cells to those presented for internalized antibodies. The distribution of MTs and filamentous actin was not statistically different under all conditions. (B) Relative lengths of neurites at 2 hours following antibody loading, as described in A, quantified in phase-contrast images with Image J (means ± s.e.); neurite lengths were not statistically different under all conditions. (C) Representative images of the perikarya of cells processed with rhodamine-conjugated wheat germ agglutinin to visualize the Golgi complex 2 hours after antibody delivery. Note the disruption, by both antibodies, of the normal perikaryal localization of the Golgi complex compared with that in mock-loaded cells (arrows) following intracellular delivery of either antibody. The accompanying graph presents quantification of the percentage of the soma occupied by the Golgi complex; both antibodies significantly increased this percentage. *P<0.05.
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Fig. 3. Differential role of dynein and kinesin in NF translocation into axonal neurites. Upper panels: representative fluorescent images of the axonal hillocks of cells transfected with the GFP–M construct alone (no treatment) or co-transfected with a construct expressing Myc–dynamitin, as indicated. Images are also presented in reverse-contrast. Note the presence of puncta (arrowheads) and short NFs (arrows) in the absence of dynamitin and the selective depletion of short NFs following dynamitin overexpression (not all NFs are labeled). The accompanying graphs present a quantification (means + s.e.) of the percentage of total GFP-tagged puncta and short (linear) NFs within hillocks or perikarya in cells expressing GFP–M alone (–, n=36), co-transfected with Myc–dynamitin (n=4) or subjected to intracellular delivery of antibodies against dynein (n=8) or kinesin (n=22) (+ indicates the presence of the respective additional agent) during the final 2 hours before observation. Note that Myc–dynamitin and the anti-dynein antibody selectively reduced the percentage of short NFs within hillocks, with a corresponding increase in puncta; neither altered these percentages within perikarya. Intracellular delivery of the anti-kinesin antibody did not alter these percentages in hillocks or perikarya. Anti-dynein antibody and Myc–dynamitin each reduced the percentage of short NFs within the axonal hillock by ~50%, with a corresponding increase in the percentage of puncta. By contrast, anti-kinesin antibody did not alter the percentage of short NFs or puncta within the hillock (see Fig. 2). *P<0.05, between – and + conditions. Values without asterisks are not statistically different (P>0.05).

Discussion

Previous studies have demonstrated that, although kinesin mediates anterograde axonal NF transport, and dynein mediates retrograde axonal NF transport, both kinesin and dynein mediate translocation of NFs from perikarya into axonal neurites [inhibition of either motor reduced delivery of NFs into the hillock by ~50% (Motil et al., 2006; Yabe et al., 1999)]. The findings presented here extend these prior studies by suggesting that kinesin and dynein play distinct roles in delivery of NFs into axonal neurites. Although inhibition of kinesin reduced the overall delivery of NF subunits into axonal neurites (Yabe et al., 1999), it did not alter the percentage of NFs relative to puncta within the axonal hillock, indicating that it is involved in translocation of both of these NF forms into axonal neurites. However, inhibition of dynein only reduced the percentage of NFs within the axonal hillock, with a corresponding increase in puncta. This suggested that dynein plays a unique role in translocation of short NFs into axonal neurites. The influence of dynein on the relative percentage of NFs and puncta within the axonal hillock was not due to an artifactual inhibition of NF assembly as the relative percentages of NFs and puncta were not altered within the soma during dynein inhibition. Selective reduction of short NFs within the axonal hillock is therefore caused either by inhibiting their entry or increasing their rate of exit. As previous studies have demonstrated that inhibition of dynein accelerated anterograde NF transport within axonal neurites [presumably owing to elimination of an opposing retrograde ‘drag’ (Motil et al., 2006)], the possibility existed that dynein inhibition similarly fostered an increased transport of short NFs out of the hillock. This possibility was eliminated, however, because there was a reduction in the translocation of NFs into the axonal shaft, as well as the hillock, following microinjection of dynamitin. Although the remaining possibility, i.e. that dynein mediates anterograde delivery of linear NFs into axonal neurites, might seem counterintuitive, previous studies on the role of dynein in delivery of MTs into axons have suggested potential mechanisms. Dynein is known to associate with MTs through its cargo domain and a...
...MTs, and any associated short NFs, in an anterograde direction. Although dynein could continue retrograde NF transport along MTs and actin on NF distribution. Nocodozole treatment selectively depleted translocation of short NFs and not puncta, whereas cytochalasin D did not display any such selectivity. Nocodozole treatment, as utilized here, depletes unstabilized (tyrosinated) MTs but preserves longer stabilized (acetylated) MTs (Motil et al., 2006). This increase in dynein-mediated retrograde NF transport could also be mediated in part by the increased density of relatively long MTs within the axonal shaft. Any contribution of dynein to anterograde transport of MTs (Reed et al., 2006), which are prominent within the axonal shaft. Any contribution of dynein to anterograde transport of MTs could be obscured by the increased density of relatively long MTs within the axonal shaft. These long MTs might facilitate coordinated kinesin processivity and, therefore, accentuate the role of kinesin in direct MTs, as previously demonstrated for vimentin and MTs (Liao and Gundersen, 1998; Kreitzer et al., 1999). Thus, even if kinesin more readily and/or directly translocates puncta and hetero-oligomers as opposed to NFs, it is also likely to play a crucial role in the dynein-mediated translocation of linear NFs.

A similar level of inhibition of translocation for both linear and punctate NFs following cytochalasin treatment is consistent with the above interpretations, given that actin is known to play a role in translocation of NFs by both dynein and kinesin. In the case of dynein, the actin cortex, like longer MTs, functions as a substrate against which dynein can translocate MTs (and any associated NFs) into and along axonal neurites (Ahmad et al., 1998; Hasaka et al., 2004; Myers et al., 2006; Pfister, 1999; Susalka et al., 2000). The actin cortex also participates in kinesin-mediated anterograde translocation of NFs, both by providing anchorage (Alami et al., 2009; Francis et al., 2005; Jung et al., 2004; Rao et al., 2002) and perhaps through interactions between kinesin, and/or NFs, and one or more forms of the actin-based motor myosin (Ali et al., 2008; Huang et al., 1999; Jung et al., 2004). Depletion with cytochalasin D can, therefore, equally affect translocation of punctate and linear NFs into axonal neurites by inhibiting the anterograde translocation of both MT motor proteins.

The dynamics of NF transport are likely to differ dramatically within the axonal shaft, as compared with those of the hillock, owing to the increased density of relatively long MTs within the shaft. These long MTs might facilitate coordinated kinesin processivity and, therefore, accentuate the role of kinesin in direct anterograde transport of NFs, as well as puncta. This possibility is supported by increased kinesin-mediated transport along acetylated MTs (Reed et al., 2006), which are prominent within the axonal shaft. Any contribution of dynein to anterograde transport of ‘piggybacking’ NFs within the shaft might then be obscured by increased kinesin-mediated anterograde NF transport, as well as the more substantial role for dynein in retrograde NF transport (Motil et al., 2006). This increase in dynein-mediated retrograde NF transport could also be mediated in part by the increased density of long MTs within the shaft compared with that in the axonal hillock.

Establishment and maintenance of the axonal NF array remains the subject of debate. Studies collectively suggest that those NFs that transport at a rate consistent with that displayed by the fast-transport MT motors kinesin and dynein (Roy et al., 2000; Trivedi et al., 2007; Wang et al., 2000; Yuan et al., 2009) can undergo exchange with axonal NFs that undergo substantially slower transport or do not translocate at all (Yabe et al., 2001b; Yan and Brown, 2005; Yuan et al., 2009). The role of puncta in axonal NF establishment and/or maintenance is less clear. Notably, as...
endogenous subunits are also observed within puncta in non-transfected cells, puncta are not simply derived from overexpression of exogenous subunits (Chan et al., 2003; Yabe et al., 2001a). Puncta are also not necessarily exclusively derived from the most recently expressed NF subunits, as they can contain late-appearing C-terminally phosphorylated epitopes (Yabe et al., 1999); this leaves open the possibility that some puncta might derive from exit of NF subunits from filamentous forms and/or can undergo phosphorylation-dependent dissociation from kinesin and phosphorylation-dependent crosslinking with other NFs. Short NFs and puncta might play distinct roles in the initial establishment of the axonal cytoskeleton compared with its remodeling and/or maintenance. In this regard, the predominant form of transporting NF subunits vary in accordance with the neuronal differentiation state, leaving open the possibility that both short NFs and puncta contribute to regional remodeling of the NF network and that these roles might be temporally, as well as spatially, segregated (Yabe et al., 1999; Yabe et al., 2001a; Yabe et al., 2001b). Whether or not puncta need to convert into linear structures before incorporation into the network is also unclear. Non-filamentous assemblies of vimentin and keratin, including puncta, undergo MT-motor-based translocation and contribute to the establishment of the intermediate filament network (Ho et al., 1998; Martys et al., 1999; Prahlad et al., 1998; Windorffer and Leube, 1999). In non-neuronal systems, incorporation of puncta into the filament network at the edges of newly plated cells has been observed in confocal microscopic

Fig. 5. Real-time analyses of the impact of antibodies against dynein and kinesin on NF distribution. (A) Representative gray-scale images of the hillock (top left-hand images) and the proximal axonal region of cells treated with ProVectin with or without antibodies against dynein or kinesin, as indicated. Cells were transfected with GFP–M and the following day treated with ProVectin with or without antibodies against dynein or kinesin, and images were immediately captured at 1-second intervals. The “Image” panels present the difference between sequential images, obtained by subtracting images from the one captured 1 second previously using the Photoshop subtraction function. Images were divided into three equivalent segments and the accompanying graph (right-hand panel) presents a quantification (means + s.e.) of the distribution of pixels along neurites in three sequential images from three cells under each condition, as described in the Materials and Methods section. Note an increase in pixels in more distal compared with that in proximal segments at each interval in cells receiving ProVectin alone, and the attenuation of this increase in cells receiving anti-dynein antibody. Note also the ~10% reduction in pixels in distal compared with that in proximal segments in images from cells receiving anti-kinesin antibody. (B) Images of axonal hillocks, each captured 1 second apart. The second image (Image 2) is false-colored in red. The merged image has colocalized pixels in yellow or orange, with pixels that are not colocalized retaining their green or red color. Arrows denote representative NFs, and arrowheads denote representative puncta in the merged image; not all NFs or puncta are indicated. To quantify differences between the sequential images, green and red pixels were depleted from the merged image with the Photoshop levels tool, the persisting yellow or orange pixels are false-colored in blue (Co-localized pixels). The graph in the upper right-hand panel presents a quantification (means + s.e.) of the total increase in colocalized pixels within the hillock cytosol for cells receiving the antibodies against dynein or kinesin compared with those receiving ProVectin alone. The graph in the lower right-hand panel presents a quantification (means + s.e.) of NFs and puncta within the hillock in the first compared with that in the last image of the 10–15-second sequence of captured images. Note that ProVectin alone and anti-dynein antibody did not alter the number of NFs or puncta within the hillock during this interval, whereas anti-kinesin antibody induced a 40% increase in puncta and a 50% increase in NFs within the hillock (P<0.17 and <0.18, respectively).
analyses. The robust level of NFs within axons has precluded routine observation of the interconversion of punctate and filamentous NF assemblies, but apparent conversion of GFP-tagged puncta into NFs and association of puncta with axonal NF bundles, respectively, during the final 2 hours of transfection, as indicated. Images are also presented in reverse-contrast. Note the presence of puncta (arrowheads) and short NFs (arrows) in transfected cells receiving no additional treatment and following treatment with cytochalasin D but the selective depletion of short NFs following nocodazole treatment; not all NFs are labeled. The accompanying graph presents a quantification (means ± s.e.) of the percentage of GFP-tagged puncta or short (linear) NFs within hillocks or perikarya without or following treatment with nocodazole or cytochalasin D during the final 2 hours before observation (n=18 for all conditions). Note that nocodazole treatment, but not cytochalasin D treatment, selectively reduced the percentage of short NFs within hillocks, with a corresponding increase in puncta. *P<0.05. Values without asterisks are not statistically different (P>0.05).

Kinesin and dynein might also play different roles in the establishment of the axonal NF array, as compared with their maintenance role in more mature axons. Dynein-mediated anterograde delivery of MTs is responsible for the formation of MT bundles within developing axonal neurites (Dehmelt et al., 2006). This observation has led to speculation regarding the potential role of dyenin-mediated MT transport in the development of the resident axonal NF population (Fig. 8). A MT undergoing dyenin-mediated anterograde transport could fuse with, or bind to, the resident MT bundle (Rothwell et al., 1987). Those NFs (or puncta) that are associated with such MTs through an intervening kinesin might simply continue anterograde transport, through kinesin processivity, along the resident MTs. By contrast, any directly bound NFs might instead cease transport and remain bound to the MT bundle. Multiple such MT-associated NFs, perhaps aligned along a developing MT bundle, could undergo end-to-end annealing (Colakoglu and Brown, 2009). Furthermore, additional transporting NFs that undergo temporal dissociation from their anterograde motor (Yabe et al., 1999; Yabe et al., 2000) could form phosphorylation-dependent crosslinks with these non-transporting NFs (Kushkuley et al., 2009). Such events could initiate the development of a resident NF population, which could lead to NF bundling and establishment of stationary NFs along the developing axonal cytoskeleton (Nixon and Logvinenko, 1986; Yabe et al., 2001b; Yuan et al., 2009).

These speculations put forth the notion that transporting NFs might play a more substantial role in elaboration and maintenance of the axonal cytoskeleton, whereas puncta might be more involved in local remodeling and/or repair. Determining whether this is the case, and whether either motor protein plays a more substantial role in the initial establishment of the NF network during early axonal outgrowth compared with the continued maintenance of the axonal cytoskeleton, is of considerable interest.

Materials and Methods

Cell culture and differentiation

Mouse NB2a/d1 neuroblastoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; high-glucose formulation) containing 10% fetal bovine serum, and the formation of axonal neurites was induced by addition of 1 mM dibutyryl cyclic AMP (dbcAMP), as previously described (Yabe et al., 1999), for 72 hours. For simplicity and clarity of writing only, dbcAMP-induced neurites are at some
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Transfection with GFP-conjugated NF subunit constructs

Transfection with our NF-M–eGFP construct has been previously described (Yabe et al., 1999). Briefly, cultured cells were transfected following 2 days of dbcAMP treatment with 0.62 μg/ml NF-M–eGFP (GFP–M) using Lipofectamine (Invitrogen) for 4 hours in 1 ml of serum-free medium, after which time cultures received an additional 1 ml of serum-supplemented medium containing dbcAMP. Additional cultures were also transfected with a construct expressing Myc-conjugated dynamitin (Myc–dynamin; a generous gift from Ron Valee (University of Massachusetts, Amherst, MA) (Helfand et al., 2002)). Cells were incubated for an additional 18–24 hours to allow routine visualization of GFP-tagged NF subunits, after which time the medium was replaced with fresh medium containing serum and dbcAMP; the cells were therefore viewed on the third day of dbcAMP treatment. Over 90% of the cells expressed GFP–M at this time, and, consistent with previous studies (Helfand et al., 2002), immunofluorescence analyses with an anti-Myc antibody confirmed that 90–100% of cells in cultures transfected with this Myc–dynamin construct expressed Myc–dynamin (data not shown) (Motil et al., 2006).

An anti-dynein antibody (clone 70.1; Sigma-Aldrich) and a pan-specific anti-kinesin polyclonal antibody (Cytoskeleton, Denver, CO) were delivered into transfected cells, during the final 2 hours of the above incubation, or into non-transfected cells, along with biontinylated NF-L (Jung et al., 1998), that had been treated for 5 days with dbcAMP using ProVector, as described previously (Chan et al., 2004). These antibodies had been utilized previously to visualize the association of NFs with their respective motor protein and to perturb the respective motor protein function (Motil et al., 2006; Shah et al., 2000; Yabe et al., 1999; Yabe et al., 2000). Successful intracellular delivery of antibodies was confirmed by processing of cultures for immunofluorescence analyses with the corresponding secondary antibody only.

Perturbation of MTs

Additional cultures were treated with 300 μM nocodazole, which eliminates nonstabilized MTs but preserves stabilized MTs (Jung et al., 1998; Shea, 1999; Yabe et al., 1999), or 5 μM cytochalasin D (Jung et al., 2004; Motil et al., 2006), which perturbs the actin cytoskeleton, for the final 2 hours following transfection.

Immunofluorescence

To visualize biotin-conjugated NFs, cultures were fixed 1 hour after microinjection with 4% paraformaldehyde in Tris-buffed saline (TBS; pH 7.4) for 5 minutes at room temperature. The cultures were then rinsed twice in TBS (5 minutes per rinse), three times (5 minutes per rinse) with 10 mg/ml sodium borohydride in TBS (to reduce autofluorescence) and then once with TBS. Cultures were blocked for 30 minutes in TBS containing 1% BSA and 2% normal goat serum. Cultures were then incubated overnight at 4°C in TBS containing 1% BSA and a 1:100 dilution of rabbit anti-biotin antibody. The following morning, cultures were rinsed three times with TBS, re-blocked for 30 minutes, rinsed once with TB and then incubated for 30 minutes at 37°C in TBS containing 1% BSA and a 1:150 dilution of Texas-Red-conjugated goat anti-rabbit-IgG.

To visualize MTs, cells were extracted with a MT-stabilizing solution, which eliminates unassembled tubulin subunits while preserving existing MTs (Brown et al., 1992), with minor modifications, as follows. Cultures containing 1 ml of medium received an additional 1 ml of 60 mM PIPES (pH 6.9) containing 10 mM EGTA, 2 mM MgCl₂, 1% saponin and 10 μM taxol for 1 minute. Cultures were then rinsed with PBS, fixed for 10 minutes with 4% paraformaldehyde and rinsed again with PBS. Fixed cultures were incubated for 36 hours with gentle rocking at 4°C in PBS containing 1% BSA and a 1:500 dilution of a monoclonal antibody directed against total (DM1A) or acetylated (6-11B-1) or tyrosinated (TUB-1A2) tubulin (all obtained from Sigma-Aldrich). Cultures were then rinsed with PBS and incubated at 37°C in PBS containing 1% BSA and a 1:500 dilution of Texas-Red-conjugated goat anti-mouse-IgG. Cultures were rinsed in PBS and stored at 4°C in TBS until the time of visualization (≤5 hours). To visualize actin, cultures were incubated with rhodamine-conjugated phalloidin as described previously (Jung et al., 2004). To visualize the Golgi complex, fixed cultures were incubated for 1 hour at 37°C in 75 μg/ml rhodamine-conjugated wheat germ agglutinin (Sigma-Aldrich) as described previously (Ahmad et al., 1998).

Image acquisition and densitometric analysis

Epifluorescent, immunofluorescent and corresponding phase-contrast images were captured with a Hamamatsu Orca camera operated by Open Lab software (Improvement, Waltham, MA) on a Macintosh G5 using a Zeiss Axiosvert epifluorescence microscope. Images were stored as TIF or PICT files and densitometric analyses were carried out using the NIH ImageJ software (available at http://rsbweb.nih.gov/ij/), as described previously (Jung et al., 1998). Briefly, regions of cells were encircled with the program’s freehand selection tool. Background was subtracted using the program’s automated function. Perikarya, axonal hillocks or respective axonal neurites were recorded individually and the densitometric value points referred to as ‘axons’, and the translocation of material into and along the dbcAMP-induced axonal neurites of these cells is referred to as ‘axonal transport’. It is recognized that these neuroblastoma cultures might lack crucial features characteristic of neurons in culture or in situ.

Fig. 8. Putative roles of dynein and kinesin in translocation of NFs into and along axonal neurites. The upper panel (Axonal NF transport) depicts how NFs might undergo translocation along microtubules as ‘classical’ cargo of either motor. The middle panel (Delivery of NFs to Axons) depicts various non-exclusive models by which kinesin and dynein could translocate NFs from perikarya into neurites. Anterograde transport is to the right, as indicated. NFs can undergo anterograde transport of NFs as a cargo of kinesin. Dynein might mediate anterograde translocation of NFs that have linked to a short MT that is itself undergoing anterograde transport. This could either be through a direct association of the NFs with these translocating MTs, or through kinesin, which might facilitate this association by acting as a linker. The bottom panel (If a short MT integrates with MT bundle) presents the potential consequences should a short MT, undergoing kinesin-mediated anterograde transport (1), associate with resident axonal MTs (2; represented as crosslinked). Such MTs are represented here if they underwent end-to-end annealing with a resident MT for simplicity. NFs associated directly with such an MT might cease transport, and could represent a nucleation site for the development of axonal NF bundles (Yabe et al., 2001a) that correspond to the NF stationary phase (Nixon and Logvinenko, 1986; Yuan et al., 2009). NFs associated with such MTs through kinesin could simply continue transport, through kinesin processivity, along the resident MTs. NFs are depicted as being solely filamentous for simplicity; punctate forms could be substituted in these diagrams. Adapted from Motil et al. (Motil et al., 2006).
obtained for each was divided by the total value for all regions. The percentage of NFs and punctate structures in each region was determined by dividing each respective value by the total number of particles. For simplicity of writing, NFs that display a clear profile are referred to as ‘short NFs’ or simply ‘NFs’ and those that display a round or elliptical profile as ‘puncta’, with the caveat that there might be a continuum between these forms.

For real-time analyses of the immediate impact of the intracellular delivery of the antibodies against dynein or kinesin, cells were transfected with GFP-M and treated with ProVecin with or without the antibodies against dynein or kinesin the following day (when GFP was prominent throughout cells). Images were immediately captured at 1-second intervals for a total of 10–15 seconds; this limited capture interval was utilized to avoid significant photobleaching. Images were analyzed for transllocation of NFs or NF subunits by two methods. First, the difference between sequential images was obtained by ‘subtracting’ images from the one captured 1 second previously with the Photoshop subtraction function. Resultant images were then divided into three equivalent segments, and the pixel density was quantified in each segment with ImageJ. The difference in pixel density between adjacent segments was quantified in three sequential images from three cells under each condition (e.g. Yabe et al., 2001c). The mean (±SEM) difference between segments (e.g. segment 2 minus segment 1, and segment 3 minus segment 2) was calculated for all images under each condition. Second, the extent of pixel colocalization between sequential images was calculated as the index of transport, with the reasoning that an increase in colocalization indicated less net transport. To accomplish this, the second of two sequential images of GFP-M-transfected cells was false-colored in red. This image was then merged with the previously captured (green) image and all red and green pixels were depleted from the merged image with the Photoshop levels tool; the resultant image was then merged with the previous captured (green) image and all red and green pixels were depleted from the merged image with the Photoshop levels tool; the resultant images were then false-colored in red. This image was then quantified using ImageJ. The relative number of NFs and puncta within hillocks was then compared in the first and last image of each captured sequence for 9–15 individual cells under each condition. The percentage of the soma occupied by the Golgi complex before and after intracellular delivery of antibodies was quantified as described previously (Temple et al., 2005).

Values obtained from multiple cells were exported into Microsoft Excel for calculation of ratios and statistical analyses. All ratios of relative distribution were individually calculated and then compiled.

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References


