Regulation of Cytoplasmic Dynein via Local Synthesis of its Cofactors, Lis1 and p150^{Glued}

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ABSTRACT

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Within the past thirty years, the discovery and characterization of the microtubule-associated motor proteins, kinesins and cytoplasmic dynein, has radically expanded our understanding of intracellular trafficking and motile phenomena. Nevertheless, the mechanisms by which eukaryotic cells integrate motor functionality and cargo interactions over multiple subcellular domains in a spatiotemporally controlled way remain largely mysterious.

During transport within the neuronal axon, dynein and the kinesins run in opposite directions along uniformly polarized microtubule tracks, so that each motor must switch between active transport and being, itself, a cargo in order to be properly positioned and carry out its function. The axon thus represents a model system in which to study the regulatory mechanisms governing intracellular transport, especially under conditions when it must be modulated in response to changing environmental cues, such as during axon outgrowth and development.

Recently, the localization of certain messenger RNAs and their local translation to yield protein has emerged as a critical process for the development of axons and other neuronal compartments. I observed that transcripts encoding the dynein cofactors Lis1 and dynactin are among those localized to axons, so I hypothesized that stimulus-dependent changes in axonal transport may occur via local synthesis of dynein cofactors. In these studies, I have shown that different conditions of nerve growth factor signaling on developing axons trigger acute changes in the transport of various axonal cargoes, contemporaneous with rapid translational activation and production of Lis1 and dynactin's main subunit, p150^{Glued}, within the axons themselves.

Differential synthesis of these cofactors in axons was confirmed to be required for the observed stimulus-dependent transport changes, which were completely prevented by axon-specific pharmacologic inhibition of protein synthesis or RNA interference targeted against Lis1 and $p150^{Glued}$. In fact, Lis1 was, in an apparent paradox, locally synthesized in response to both nerve growth factor stimulation and withdrawal. I demonstrated that this is due to the fact that Lis1 is produced from a heterogeneous population of localized transcripts, differentiated chiefly by whether they interact with the RNA-binding protein APC. Preventing the binding of APC to Lis1 transcripts thus inhibited axonal synthesis of Lis1 and its resultant transport effects under conditions of nerve growth factor stimulation, while having no bearing on the similar phenomena seen during nerve growth factor withdrawal. This demonstrates that association with RNA-binding proteins can functionally distinguish sub-populations of localized messenger RNAs, which, in turn, provides a foundation for mechanistically understanding how localized protein synthesis is coupled to specific stimuli.

Axonally synthesized Lis1 also was shown to have a particular role in mediating transport of a retrograde death signal originating in nerve growth factor-deprived axons, as neurons exhibited greatly reduced cell death when axonal synthesis of Lis1 was blocked. Through the application of pharmacologic agents inhibiting different steps in the propagation of this pro-apoptotic signal, I established that the signal depends upon effective endocytosis and the activity of glycogen synthase kinase 3β. It is therefore likely that the retrogradely transported signaling cargo in question is a glycogen synthase kinase 3β-containing endosome or multivesicular body—a type of large cargo consistent with Lis1's known role in adapting the dynein motor for high-load transport. Preliminary results further indicate that axons exposed to

another type of degenerative stress, in the form of toxic amyloid-β oligomers, may also employ local synthesis of Lis1 as a means of regulating transport and survival signaling.

These findings establish a previously undescribed mechanism of regulating dynein activity and cargo interactions through local synthesis of its cofactors, allowing for rapid responses to environmental cues and stimuli that are especially relevant during the development of the nervous system. In addition to illustrating a regulatory principle that may be generally applicable to subcellular compartments throughout polarized cells, these studies provide new insights into intracellular transport disruptions that occur in lissencephaly, neurodegeneration, and other human disease states.

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ABBREVIATIONS

5-FdU	5-fluorodeoxyuridine
Aβ	amyloid-β
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APC	adenomatous polyposis coli
ATF	activating transcription factor
ATP	adenosine triphosphate
BACE	beta-site amyloid precursor protein cleaving enzyme
BDNF	brain-derived neurotrophic factor
BME	β-mercaptoethanol
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CLIP	CAP-GLY domain containing linker protein
Da	dalton (unified atomic mass unit)
DIV	day(s) *in vitro*
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DRG	dorsal root ganglion
eIF	eukaryotic initiation factor
Em	emission maximum

EtOH	ethanol
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
HMN	hereditary motor neuropathy
HPLC	high performance liquid chromatography
LiCl	lithium chloride
LNA	locked nucleic acid
MLK	mixed lineage kinase
MAPK	mitogen-activated protein kinase
mTOR	mechanistic target of rapamycin
MVB	multivesicular body
NGF	nerve growth factor
NMDA	*N*-Methyl-D-aspartate
NT	neurotrophin
PAFAH	platelet-activating factor acetylhydrolase
PBS	phosphate-buffered saline
PBS-T	PBS with 0.1% Tween-20
PCR	polymerase chain reaction
RT-PCR	reverse transcription PCR

PFA	paraformaldehyde
PDL/PLL	poly-D-lysine/poly-L-lysine
PNA	peptide nucleic acid
QD	quantum dot
RNA	ribonucleic acid
RNAi	RNA interference
mRNA	messenger RNA
miRNA	microRNA
siRNA	short interfering RNA
tRNA	transfer RNA
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
RNP	ribonucleoprotein
SSC	saline sodium citrate
SEM	standard error of the mean
SUMO	small ubiquitin-like modifier
TBS	Tris-buffered saline
TdT	terminal deoxynucleotidyl transferase
Trk	tropomyosin receptor kinase
UTP/dUTP	uridine triphosphate/deoxyuridine triphosphate
UTR	untranslated region
TUNEL	TdT dUTP nick end labeling
ZW10	Zeste White 10

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Dedicated to my wife and family

CHAPTER I. GENERAL INTRODUCTION

The earliest report of motile phenomena in living cells dates to the pioneering microscopic studies of van Leeuwenhoek, but the first mechanistic explanations for the observed movements within cells came only in the 19th century. Wilhelm Kühne, coiner of the term "enzyme," broke new ground by linking the contraction of muscle cells to the function of a protein, identified as myosin (Kühne 1859, Kühne 1864). That myosins are themselves enzymes, utilizing ATP to power their movement relative to an actin scaffold, would gradually become clear a century later (Engelhardt and Ljubimowa 1939, Banga and Szent-Györgyi 1942, Huxley and Niedergerke 1954, Huxley and Hanson 1954). With these discoveries, the notion of a molecular motor was born.

By the mid-1950s, George O. Gey and his colleagues could leverage the resources of ultrastructural and time-lapse microscopy to assemble the most vivid picture to-date of the living, dynamic cell, including its remarkable "endocellular traffic" (Gey, Shapras et al. 1954). Palade would go on to show the importance of vesicular trafficking for the secretory pathway of protein synthesis, tying cell biology into the advancement of the molecular biology revolution (Caro and Palade 1964). However, the nature of this intracellular transport would remain very poorly understood until several studies established the existence of the cytoskeleton, an intracellular network with actin, tubulin, and other polymeric components, common to many types of eukaryotic cell (Inoue and Sato 1967, Ishikawa, Bischoff et al. 1969).

A veritable explosion of new data followed on the heels of this seminal work: the characterization of new motor proteins, pharmacological inhibitors, and regulatory pathways acting upon the cytoskeleton. Yet, despite our greater insights into the molecular basis of

transport, many questions remain unresolved at the point of convergence between the cellular motors, their cargoes, and the cytoskeletal substrate. For instance, how does the cell coordinate the participation of all these players into an intracellular transport regime that is, at once, locally adaptable and coherent as a whole? Recent advances in RNA and protein biology, beyond the ken of Crick, Palade, or their contemporaries, challenge much of our received wisdom and furnish new paradigms to explain these nuanced aspects of cellular physiology.

In this introductory chapter, a focused overview will be provided on three topics of interest to contemporary biological research: the intracellular transport system, localization of messenger RNA and its translation, and positive and negative factors in the neurochemical milieu. Individually, these subjects could each brook exhaustive analysis that might fill, at the very least, the space allotted for this entire chapter. However, the most relevant aspects for comprehension will necessarily be emphasized, as well as those areas where these topics potentially intersect or inform one another. The later chapters, hopefully, will succeed in demonstrating how these facets of cellular life are, in fact, intimately linked, taking into account new evidence presented here for the first time.

I.1. Molecular Mechanisms of Intracellular Transport

In evolving beyond the size and relative simplicity of prokaryotes, eukaryotic cells had to overcome a fundamental biophysical problem: how to maintain the rate of certain vital chemical reactions when the enzymes and reactants involved had ever-greater space in which to diffuse away from each other. One solution was the origin of internal membranes, which enclose smaller spaces, within vesicles and organelles, where the necessary elements for a given reaction can be brought into close proximity and kept away from other cellular contents. Another, associated endeavor was the further development of the prokaryotic cell's rudimentary polymeric scaffolds and force-generating enzymes into a system of active transport that would facilitate the spatial orientation and interaction of macromolecules as well as the new membranous organelles. Research continues into the structural basis of this transport system and the host of proteins that enable its work, but its centrality to eukaryotic cellular functioning is already evident.

a. Microtubule-based motors: dyneins and kinesins

Besides the aforementioned myosins, which were eventually shown to be a family of ubiquitous actin-based motors not restricted only to muscle cells (Pollard and Korn 1973, Pollard and Korn 1973, Hammer, Jung et al. 1986), the other motor proteins responsible for transport in eukaryotic cells—dyneins and kinesins—associate with microtubules. Structurally, a microtubule is a hollow cylinder measuring 24 nanometers in its outer diameter, whose wall is formed by a sheet of protofilaments assembled from heterodimeric complexes of α- and β-tubulin as building blocks (Gibbons 1961, Shelanski and Taylor 1968, Feit, Slusarek et al. 1971). The consistent orientation of heterodimers within protofilaments gives microtubules an inherent polarity (Amos and Klug 1974). For cytosolic microtubules, minus-ends are generally associated with a

microtubule organizing center near the nucleus, which, in animal cells, is an organelle called the centrosome (Gould and Borisy 1977). Plus-ends, meanwhile, are characterized by "dynamic instability," meaning that they tend to exist in either a shrinking phase or a growing phase protected from disassembly by a cap of GTP-bound tubulin (Mitchison and Kirschner 1984).

In addition to their differing structures and genetic lineages, the two superfamilies of microtubule motors are functionally distinguished by which microtubule end they tend to be directed toward. Dyneins are the principal group of minus-end directed motors. The first representative of this motor family to be discovered was axonemal dynein, which is not involved in transport but, instead, produces the beating of cilia and flagella (Gibbons and Rowe 1965). Two cytoplasmic dyneins were subsequently identified: cytoplasmic dynein 1 is the general motor for transporting minus-end directed cargo in cells (Paschal, Shpetner et al. 1987, Schnapp and Reese 1989), whereas cytoplasmic dynein 2 is specific to intraflagellar transport (Gibbons, Asai et al. 1994, Pazour, Dickert et al. 1999). The cytoplasmic dynein holoenzyme is a dimerized, multisubunit complex, with each monomer containing one heavy chain bearing the ATPase force-generating and microtubule-binding domains, one intermediate chain (~74 kDa), two light intermediate chains (~53-59 kDa), and several distinct light chains [**FIGURE 1-A**] (Neely, Erickson et al. 1990, Holzbaur and Vallee 1994, Hughes, Vaughan et al. 1995). The intermediate chains form a focal platform for integrating dynein's binding with the various light chains and other regulatory cofactors (Susalka, Nikulina et al. 2002), which, interestingly, appear to distinguish functional subfractions of cytoplasmic dynein through combinatorial complexity (Tai, Chuang et al. 2001). Reductively, however, the processive movement of cytoplasmic dynein along microtubules requires only dimerization of the two heavy chains, whose

microtubule-binding stalks shuffle past each other in rather uneven steps that appear to be significantly influenced by diffusion (Reck-Peterson, Yildiz et al. 2006).

The kinesins, which facilitate plus-end directed transport, stand in contradistinction to many of the described qualities of the dynein motor. To begin with, it is worth noting that, while there is only one cytoplasmic dynein in non-ciliated cells, myriad different kinesin-related proteins have been found in eukaryotes (Endow and Hatsumi 1991), some of which are even minus-end directed (Walker, Salmon et al. 1990). Conventional kinesins in metazoans form dimers of two identical heavy chains each bound to a light chain (Vale, Reese et al. 1985), but other kinesin family members function as heterotrimers (Cole, Chinn et al. 1993), homodimers without light chains (Seiler, Kirchner et al. 2000, Setou, Nakagawa et al. 2000), or monomers (Nangaku, Sato-Yoshitake et al. 1994, Okada, Yamazaki et al. 1995). Association with different kinesin light chain isoforms is thought to mediate selective interactions with different types of cargo or even modulate the motility characteristics of individual motors (Rahman, Friedman et al. 1998). Mechanistically, kinesins are consistent motors that hydrolyze one ATP molecule to take one step (Schnitzer and Block 1997) of discrete, 8 nanometer size (Svoboda, Schmidt et al. 1993), without attempts at lateral stepping (Gelles, Schnapp et al. 1988).

Before continuing to discuss dynein and kinesins more specifically in the context of transporting macromolecules and membrane-bound cellular structures, it must be mentioned that these motors have a variety of other roles in the life of the cell. Aside from well-characterized involvement in assembling the mitotic spindle (Hagan and Yanagida 1990, Merdes, Ramyar et al. 1996), cytoplasmic dynein and kinesins have also emerged as organizers of the cytoskeleton in non-dividing cells. Both microtubule motors are essential for the assembly and maintenance of intermediate filament networks containing vimentin (Gyoeva and Gelfand 1991, Helfand,

Mikami et al. 2002) or neurofilaments (Yabe, Pimenta et al. 1999, Shah, Flanagan et al. 2000). Combined with evidence that these motors interact with actin (Kuriyama, Gustus et al. 2002, Rehberg, Kleylein-Sohn et al. 2005, Meiri, Marshall et al. 2012) and can even regulate nucleation of their own microtubule tracks at the centrosome (Young, Dictenberg et al. 2000), it becomes clear that dynein and kinesins help to coordinate the actin, tubulin, and intermediate filament components of the cytoskeleton in a manner which should not be overlooked.

b. Motor cofactors and regulatory interactions

At this point, the question arises as to how a single type of cytoplasmic dynein can effectively perform for minus-end directed transport the plenitude of functions associated with all the various kinesins in plus-end directed transport. In point of fact, cytoplasmic dynein is not truly alone, as it is subject to regulation by signal transduction pathways as well as a multitude of cofactors that adapt the motor's functionality to its specific task at hand. Of these cofactors, two of the most essential and best-characterized are dynactin and Lis1.

Dynactin is a large complex of 11 protein subunits with a combined molecular mass rivaling that of dynein itself [**FIGURE 1-A**] (Schroer 2004). *In vitro*, dynactin was shown to be necessary for dynein-mediated vesicle translocation (Gill, Schroer et al. 1991), and it is believed to be required, in one way or another, for virtually all of dynein's functions *in vivo* (Karki and Holzbaur 1999). The largest and primary functional subunit of dynactin is the *DCTN1* gene product $p150^{Glued}$ (Holzbaur, Hammarback et al. 1991), which has domains that bind dynein intermediate chain (Karki and Holzbaur 1995), microtubules, and another dynactin subunit, Arp1 (Waterman-Storer, Karki et al. 1995). Within each dynactin complex, an octameric polymer of Arp1 forms a short rod that provides a scaffold to which all but one of the other subunits bind:

p150^{Glued} and p50/dynamitin (Echeverri, Paschal et al. 1996); a pointed-end complex consisting of Arp11, p62, p27, and p25 (Eckley, Gill et al. 1999); the actin-capping protein CapZ and a conventional β-actin monomer (Schafer, Gill et al. 1994). The Arp1 rod also seems to mediate dynactin's interaction with many membranous cargoes by binding directly to spectrin-family proteins found on the surface of certain vesicles and organelles (Holleran, Ligon et al. 2001, Muresan, Stankewich et al. 2001). Dynactin's smallest subunit, p24/p22, is closely associated with the flexible, extensible "arm," formed by p150^{Glued} and p50/dynamitin, which projects out of the Arp1 rod (Karki, LaMonte et al. 1998). Given its interactions with both dynein and microtubules, the dynactin complex is generally understood to improve the processivity of the motor by enhancing its attachment to microtubule tracks, thereby increasing average run length (King and Schroer 2000, Ross, Wallace et al. 2006). On the other hand, it has been argued that dynactin's microtubule binding is important principally in the initiation step of dynein-based transport, a function which is separable from its enhancement of motor processivity. While this explains the observation that mutations in the microtubule-binding domain of p150^{Glued} reduce minus-end directed vesicular flux without altering the transport characteristics of moving cargoes (Kardon, Reck-Peterson et al. 2009, Moughamian and Holzbaur 2012), it only makes the mechanism behind dynactin's influence on motor processivity that much more mysterious.

Encoded in humans by the gene *PAFAH1B1*, platelet-activating factor acetylhydrolase IB subunit alpha—also known as Lis1—is a ubiquitously expressed protein, albeit most prominent in the brain, heart, and skeletal muscle (Reiner, Carrozzo et al. 1993). Disruption of Lis1 function is the etiology of classic lissencephaly and Miller-Dieker syndrome (Dobyns, Reiner et al. 1993, Lo Nigro, Chong et al. 1997), due to the failure of neuronal precursors to migrate along radial glia and populate the layers of the cerebral cortex (Feng, Olson et al. 2000). It was first

identified through genetic screens of mutants with perturbed dynein-dependent positioning of the nucleus (Xiang, Beckwith et al. 1994), also characteristic of its binding partners NudE (Minke, Lee et al. 1999, Efimov and Morris 2000) and NudEL (Niethammer, Smith et al. 2000, Sweeney, Prokscha et al. 2001). The NudE homologues are considered to be enhancers of Lis1's interaction with dynein (Li, Lee et al. 2005), as overexpression of Lis1 has been seen to rescue the effects of NudE and NudEL loss of function (Markus, Punch et al. 2009). Curiously, in addition to Lis1-NudE/NudEL binding to dynein intermediate chain and LC8, one of the light chain subunits (Stehman, Chen et al. 2007), Lis1 is also the only cofactor currently known to interact directly with the dynein heavy chain (Sasaki, Shionoya et al. 2000, Mesngon, Tarricone et al. 2006). Due to apparent contacts at both the ATPase and microtubule-binding domains, Lis1 has been proposed to act as a molecular "clutch" (Huang, Roberts et al. 2012), inducing moving dynein to remain in a force-producing, microtubule-attached state (McKenney, Vershinin et al. 2010). At the ensemble level, Lis1 thus appears to help dynein crews withstand high-load transport, such as during nuclear migration (Tanaka, Serneo et al. 2004) or when moving other large structures through a constrained environment with high drag forces (Yi, Ori-McKenney et al. 2011). Nevertheless, as with dynactin, alternative models of Lis1 function have been put forward stressing its role as a potential "initiation factor" for dynein-mediated transport. According to evidence from lower eukaryotes, without Lis1 dynein accumulates at microtubule plus-ends (Zhang, Li et al. 2003), even though Lis1 is absent from and has no effect on the motility of dynein-cargo complexes that are already in motion (Egan, Tan et al. 2012). Given the seeming conflict between these findings and those from metazoans supporting the idea of Lis1 as a high-load motor adaptor, Lis1's part in dynein transport initiation remains controversial.

Additional dynein regulators include CLIP-170, whose principal function does, in fact, seem to lie in facilitating dynein transport initiation at microtubule plus-ends (Carvalho, Gupta Jr et al. 2004, Lomakin, Semenova et al. 2009), and the Bicaudal D homologues, which are metazoan-specific cargo adaptors especially important for linking dynein to mRNA (Swan, Nguyen et al. 1999, Bullock and Ish-Horowicz 2001) and Golgi vesicles (Hoogenraad, Akhmanova et al. 2001, Matanis, Akhmanova et al. 2002). Another set of proteins only found in metazoans, Spindly and the Rod-Zwilch-ZW10 complex, work together in recruiting dynein and its cofactors to the kinetochore (Starr, Williams et al. 1998, Williams, Li et al. 2003, Griffis, Stuurman et al. 2007) in order to remove mitotic checkpoint inhibition (Howell, McEwen et al. 2001) and effect chromosome separation (Sharp, Rogers et al. 2000).

Many of the dynein regulatory proteins have notable interactions with each other, which can be cooperative or competitive. Direct binding between Lis1 and CLIP-170 has been detected, and the two appear to jointly regulate dynein motor complex function at kinetochores and other microtubule plus-ends (Coquelle, Caspi et al. 2002, Tai, Dujardin et al. 2002). Outside of mitosis, ZW10's participation in dynein-mediated Golgi membrane trafficking is thought to involve its interaction with dynactin's p50/dynamitin subunit (Hirose, Arasaki et al. 2004, Varma, Dujardin et al. 2006). Bicaudal D2, dynactin, and Lis1 have all been reported to cooperate in recruiting dynein to certain vesicular cargoes for transport (Splinter, Razafsky et al. 2012), although other studies indicate that dynactin and Lis1-NudE/NudEL antagonize each other by competing for binding sites on CLIP-170 (Lansbergen, Komarova et al. 2004) and cytoplasmic dynein's own intermediate chain [**FIGURE 1-A**] (McKenney, Weil et al. 2011). By excluding the possibility of dynein's simultaneous regulation by both dynactin and Lis1, it is

proposed that the dynein motor can be shifted between different modes of transport specialized for diverse cellular tasks—*e.g.* processive, long-distance *versus* high-load, multi-motor transport.

Like cytoplasmic dynein, some kinesins have also been shown to undergo regulation by motor cofactors. According to a recent study, the microtubule-associated protein ensconsin helps to relieve autoinhibition of kinesin-1 and is thus an essential cofactor enabling the motor to undertake its primary function of organelle transport (Barlan, Lu et al. 2013). Furthermore, several dynein cofactors, including Lis1 and dynactin, are known to interact with kinesins. During plus-end targeting of dynein, Lis1 serves as an adaptor linking dynein, as a cargo, to the active kinesin motor (Li, Lee et al. 2005, Yamada, Toba et al. 2008). Meanwhile, dynactin's p150^{Glued} subunit associates with kinesin-5 (Blangy, Arnaud et al. 1997) and kinesin-2, the latter of which it assists by linking it to some types of organelles and enhancing its processivity (Deacon, Serpinskaya et al. 2003, Berezuk and Schroer 2007). Indeed, interfering with kinesin function has long been known to disrupt transport in either direction along microtubules (Brady, Pfister et al. 1990), and the near-identical effects of dynactin inhibition suggest that it supplies a critical regulatory link between kinesin and dynein motors (Waterman-Storer, Karki et al. 1997).

As in many physiological systems, another major regulatory approach to controlling intracellular transport is through post-translational modification, particularly by protein kinases. Phosphorylated kinesins bind vesicular cargoes less efficiently (Sato-Yoshitake, Yorifuji et al. 1992) and may be impaired at transporting higher loads (DeBerg, Blehm et al. 2013). Direct phosphorylation of dynein heavy chain correlates with increased motor activity in metazoan cells (Dillman and Pfister 1994, Lin, Ferro et al. 1994), though *in vitro* data and studies in lower eukaryotes have suggested that phosphorylation of various dynein subunits generally diminishes its ATPase activity (Runnegar, Wei et al. 1999, Kumar, Lee et al. 2000). Such an effect might be

attributable to suppression of the complete assembly of the dynein motor complex, since phosphorylation of dynein light chains promotes their dissociation from the holoenzyme (Song, Benison et al. 2007) and phosphorylation of the intermediate chain interferes with its binding of dynactin (Vaughan, Leszyk et al. 2001) or Lis1-NudE/NudEL (Gao, Hebbar et al. 2015). During mitosis, intermediate chain phosphorylation has even been proposed as a switch determining whether dynein associates with ZW10 or dynactin, thereby regulating dynein's initial targeting to kinetochores and subsequent detachment (Whyte, Bader et al. 2008). Dynein cofactors, too, are themselves subject to phosphoregulation, as has been demonstrated with NudEL (Sasaki, Shionoya et al. 2000) and dynactin's p150^{Glued} subunit (Farshori and Holzbaur 1997).

The fact that various characteristics of the microtubule motors can be adjusted via covalent modification and association with different cofactors offers a glimmer of insight into how the cell might coordinate motor activity, both systematically and within its subcellular compartments. In cell types with many such differentiated compartments, such as neurons, the regulation of intracellular transport is beset by a number of unique challenges. Yet despite—or, really, because of—their complexity, neurons have proven to be an exemplary model system in which to study intracellular transport, enabling us to better understand how the regulatory principles already discussed impact the lives of cells and entire organisms.

c. Axoplasmic transport and other microtubule motor functions in neurons

Essentially all types of eukaryotic cells feature morphological polarity, *i.e.* the differentiation of structurally and functionally distinct regions within the cell. In neurons, the fundamental distinction is between the soma, or cell body, and neurites, which are cytoplasm-containing projections arising from the soma. Neurites are subdivided into dendrites and axons.

Dendrites are highly branched, generally tapering extensions possessing a cytoplasm continuous with that of the soma (Deiters 1865, Luse 1956), through which, as Ramón y Cajal understood, neurons often receive many of their synaptic inputs (Ramón y Cajal 1899). A single nerve cell can have a ramification of many dendrites, although some kinds have none at all. In contrast, only one neurite per cell is distinguished as the axon, usually forming the longest process, of more or less uniform diameter over its entire course, without extensive branching. Action potentials, the electrochemical units of information output in the nervous system, propagate along axons after being initiated in the axon initial segment (Edwards and Ottoson 1958), which also comprises a barrier separating the somatic membranes and cytoplasm from those of the axon [**FIGURE 1-A**] (Kobayashi, Storrie et al. 1992, Winckler, Forscher et al. 1999, Song, Wang et al. 2009).

In terms of their internal structure, one of the most striking features of axons is that virtually all of their microtubules are oriented in a uniform direction, with their minus-ends proximal and plus-ends distal to the soma (Heidemann, Landers et al. 1981). Dendrites, meanwhile, possess mixed-polarity microtubules in roughly equal proportions for much of their length, although their most distal segments do display uniform polarity similar to axons (Baas, Deitch et al. 1988, Burton 1988). Curiously, when a neuron's projections are just beginning to grow and develop, before one is differentiated to become the axon, they all, in fact, contain uniformly polarized microtubules (Baas, Black et al. 1989). Another way of framing the difference between dendrites and the axon, therefore, is to say that dendrites acquire mixed microtubule polarity as they mature, whereas the axon is basically the one projection that maintains its initial uniform orientation of microtubules. Moreover, consistent with what has been mentioned previously regarding the motor proteins as regulators of cytoskeletal structure, it

is known that these processes of neurite development and differentiation require the activity of kinesins and cytoplasmic dynein. Several of these motors localize specifically to the neurite that will become the axon; if their localization or function is inhibited, this neurite loses uniform microtubule polarity like all the others, and no axon develops (Nishimura, Kato et al. 2004, Jacobson, Schnapp et al. 2006, Zheng, Wildonger et al. 2008). At the migrating, amoeboid tip of a developing axon, the growth cone, dynein is also required to power microtubule advance in the face of an opposing flow of actin. This process, during which dynein must partner specifically with Lis1, is essential for the directional turning and stimulated outgrowth of axons, thus enabling them to reach their target fields and establish the connectivity of whole nervous system (Myers, Tint et al. 2006, Grabham, Seale et al. 2007).

The unidirectional activity of each family of microtubule motors, combined with the uniform orientation of axonal microtubules, immediately suggests how they might be applied to axoplasmic transport. Indeed, as would be expected, the transport of membrane-bound structures in the anterograde direction—that is, away from the cell body, or towards plus-ends—is undertaken by kinesins (Vale, Reese et al. 1985, Vale, Schnapp et al. 1985), while retrograde transport utilizes cytoplasmic dynein (Schnapp and Reese 1989). Anterograde and retrograde transport of membrane-bound structures take place at roughly the same rate, on the order of hundreds of millimeters per day (Ochs, Sabri et al. 1969, Forman, Padjen et al. 1977). Though there is some overlap between the kinds of membranous cargo moving in either direction, the anterograde and retrograde transport pools are by no means identical. Retrogradely moving cargoes tend to be endocytic in nature, ranging from pinocytic vesicles to large lysosomes and multivesicular bodies, while anterograde transport principally involves smooth endoplasmic reticulum and other small vesicular and tubular structures, such as synaptic and plasma

membrane precursor vesicles (Smith 1980, Tsukita and Ishikawa 1980, Hollenbeck 1993). Mitochondria, on the other hand, are transported in both directions (Zelena 1968), as are some non-membranous cellular components, such as proteins (Bray and Austin 1968, Fink and Gainer 1980) and mRNA granules (Li, Volknandt et al. 1999).

Not all cellular components transported into the axon, however, move at the same pace. The axon's own cytoskeletal polymers, including microtubules, neurofilaments, and actin filaments (Grafstein, McEwen et al. 1970, Hoffman and Lasek 1975, Black and Lasek 1979), as well as some apparently soluble metabolic enzymes (Erickson and Moore 1980, Brady and Lasek 1981) move much more slowly in the anterograde direction than membrane-bound structures, reaching maximum velocities of less than 20 millimeters per day (Willard, Cowan et al. 1974, Willard and Hulebak 1977). The trafficking of tRNAs into axons appears to proceed at a similarly slow rate (Ingoglia, Grafstein et al. 1973, Black and Lasek 1977). Juxtaposed with the "fast anterograde transport" of vesicles and organelles, this enigmatic mechanism has been dubbed, simply, "slow transport," and it remains the subject of considerable debate and speculation even after many years of research. Only recently has a rough consensus emerged affirming the participation of the microtubule motors in slow transport, with what has been termed the "stop-and-go" model being the predominant explanation at present. According to this model, slow transport components are actually moved by microtubule motors, but their coupling to the transport machinery is intermittent and highly inefficient in comparison to that of membranous cargoes (Miller and Heidemann 2008). This hypothesis is bolstered by experiments showing that cytoskeletal polymers in axons undergo rare but rapid bidirectional movements, consistent with the velocities of kinesins and dynein, interspersed between long pauses (Roy, Coffee et al. 2000).

As described previously, the transport behaviors of the microtubule motors can be regulated by signal transduction cascades, and mounting evidence suggests that one particular phosphorylation pathway is of critical importance to the coordination of axonal transport. CDK5 is a protein kinase that is strongly localized to axons (Tsai, Takahashi et al. 1993). One of its phosphorylation targets is the dynein cofactor and Lis1 binding partner NudEL. Since overexpression of dominant-negative CDK5 or an unphosphorylatable mutant of NudEL strongly inhibits retrograde transport of organelles, it has been argued that CDK5 phosphorylation of NudEL stimulates Lis1-NudEL's interaction with dynein (Pandey and Smith 2011). On the other hand, CDK5 also phosphorylates GSK3 in axons, inactivating it. Because active GSK3's phosphorylation of kinesin light chains causes kinesin to dissociate from cargo, CDK5 therefore enhances kinesin-based transport as well (Morfini, Szebenyi et al. 2004), establishing CDK5 and its downstream effectors as major regulators of both anterograde and retrograde transport.

d. Transport dysfunction in human disease

Experiments of nature provide us with copious examples of the consequences that follow from failure of the intracellular transport machinery or its regulatory mechanisms. Besides Lis1 haploinsufficiency resulting in the devastating neurodevelopmental disorder of lissencephaly, genetic abnormalities in the kinesins, cytoplasmic dynein, and their cofactors produce several other neurodegenerative diseases. Defects in the kinesin family members KIF5A and KIF1A cause hereditary spastic paraplegias (Reid, Kloos et al. 2002, Erlich, Edvardson et al. 2011), and KIF1B mutation is responsible for Charcot-Marie-Tooth disease type 2A (Zhao, Takita et al. 2001). Each of these motors is involved in axoplasmic transport of various essential cargoes, including synaptic vesicle precursors (Okada, Yamazaki et al. 1995), mitochondria (Nangaku,

Sato-Yoshitake et al. 1994, Karle, Mockel et al. 2012), and neurofilaments (Xia, Roberts et al. 2003). Meanwhile, mutations at the *DCTN1* locus, encoding p150^{Glued}, cause distal spinal and bulbar muscular atrophy HMN7B (Puls, Jonnakuty et al. 2003), the early-onset parkinsonism of Perry syndrome (Farrer, Hulihan et al. 2009), as well as some cases of familial ALS (Munch, Sedlmeier et al. 2004). Recent work has also linked mutations in *DYNC1H1*, a dynein heavy chain gene, to a number of familial neurological disorders: Charcot-Marie-Tooth disease type 2O (Weedon, Hastings et al. 2011), distal spinal muscular atrophy with lower extremity predominance (Harms, Ori-McKenney et al. 2012), and a form of hereditary mental retardation (Vissers, de Ligt et al. 2010).

Even neurological disorders that do not have simple, monogenic causes directly affecting the motor proteins often feature obvious dysfunctions in intracellular transport, which may contribute to disease progression. In models of Huntington's disease, mutant huntingtin protein has been seen to aggregate in axons, interrupting transport and causing axonal degeneration that conspicuously precedes the disease's hallmark neuronal loss (Li, Li et al. 2001, Szebenyi, Morfini et al. 2003). Likewise, axoplasmic transport disruption is considered to be one of the earliest abnormalities detectable in transgenic models of ALS (Williamson and Cleveland 1999) and Alzheimer's disease (Ishihara, Hong et al. 1999, Stokin, Lillo et al. 2005). Regarding the latter disease, in particular, one of its most prominent pathologic characteristics is aggregation of the microtubule-associated protein tau, which might directly inhibit transport (Ebneth, Godemann et al. 1998) or lead to transport dysfunction by destabilizing the cytoskeleton (Patrick, Zukerberg et al. 1999). In any case, the outsized impact that defects in the molecular motors have on the health of the nervous system clearly demonstrates that the complex architecture of neurons demands efficient intracellular transport in order to remain viable.

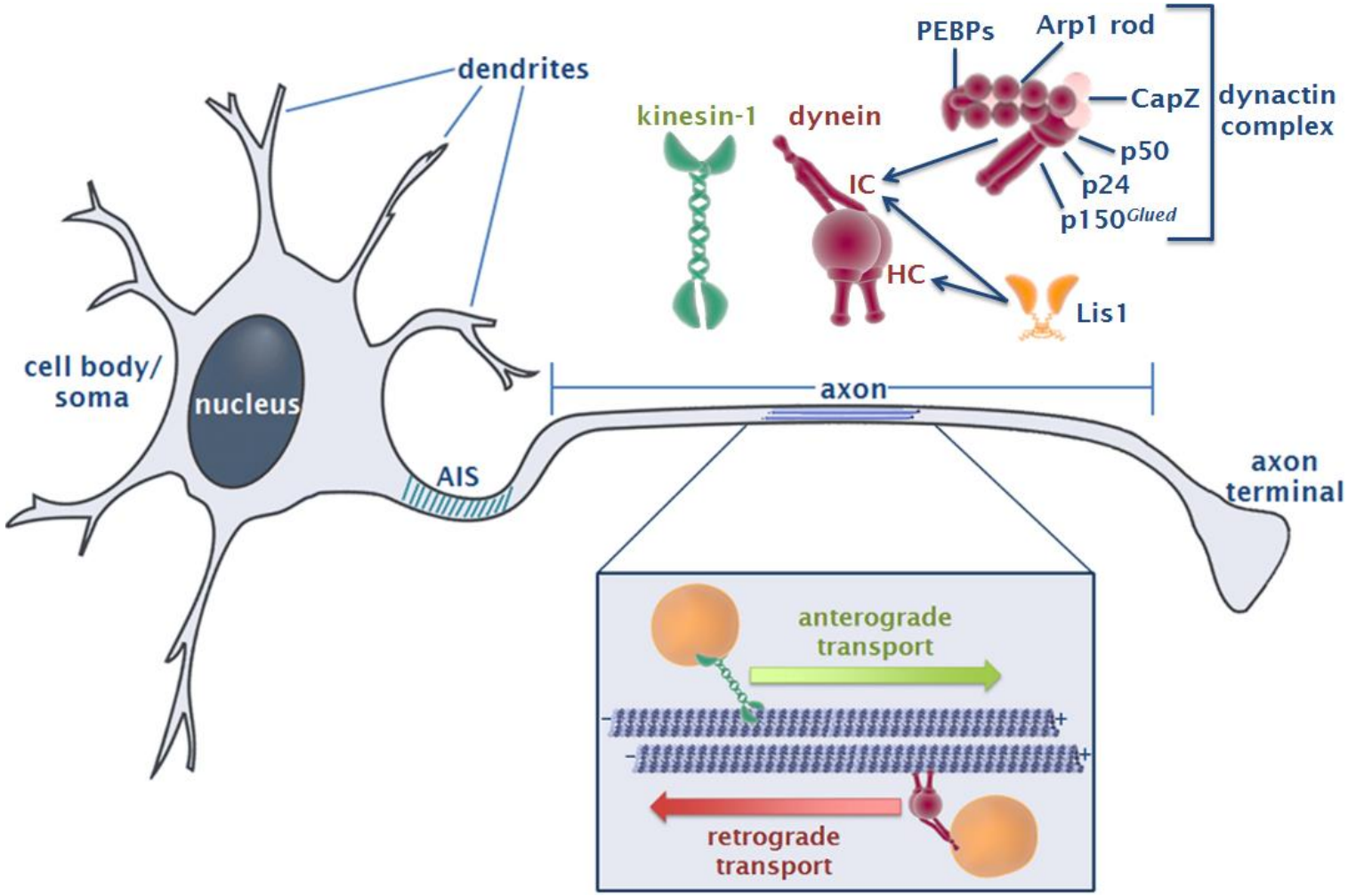

FIGURE 1-A: Diagrammatic overview of neuronal structure and the molecular motor toolbox. The axon initial segment (AIS) forms a diffusion barrier which segregates the axoplasmic contents from those of the soma. Within axons, all microtubules are uniformly polarized, and transport towards minus-ends—that is, towards the soma—is mediated by dynein. Dynein's regulatory interactions with its cofactors Lis1 and dynactin take place at either its heavy chain (HC) or intermediate chain (IC). The dynactin complex rivals dynein itself in size, consisting of many subunits. PEBPs: pointed-end binding proteins.

I.2. Localized Protein Synthesis in Neurons

From our examination of the protein machinery comprising the intracellular transport system, it should be apparent that much of cellular behavior and function can be explained in terms of what proteins a cell contains and where they are. The latter factor—that is, protein localization—is substantially determined through the very means of intracellular transport, but this is not the only process involved. In fact, recent discoveries have illustrated that, rather than being synthesized and then trafficked long distances from their point of origin, some proteins are made precisely where they are needed; their localization is thus defined from the beginning of their existence. This special mode of localized protein synthesis adds considerable opportunities for fine-tuned spatiotemporal control within cellular systems, and it is continually being recognized in new contexts and applications.

a. Post-transcriptional regulation and RNA granules

In his famous "Central Dogma of Molecular Biology," Francis Crick formulated that the flow of genetic information within cells culminates in protein synthesis (Crick 1958), and it was soon after shown that this involves, first, the transcription of DNA to mRNA and, then, the translation of mRNA by ribosomes to make protein (Brenner, Jacob et al. 1961). At the same time, groundbreaking studies on bacterial "operons" demonstrated that induction or repression of gene expression occurred at the level of transcription, through different factors that modify the rate of mRNA synthesis (Jacob and Monod 1961). For almost two decades after these initial discoveries, the conventional understanding dictated that the mRNA intermediate, being short-lived, did not really afford itself as a target for regulatory control (Darnell Jr 1979). Rather, it was expected that the mRNA transcript, once it was fully processed and exported into the

cytoplasm, would be translated to synthesize the corresponding protein more or less immediately. This limited view of genetic regulatory opportunities began to broaden with the realization that mRNAs can have widely differing half-lives, with some persisting for many hours, and that such variability in transcript stability can be attributed to differences in how the mRNAs are handled after transcription (Lubimova, Chernovskaja et al. 1975, Harpold, Wilson et al. 1981). Furthermore, it was observed that accumulation of mRNA and increased protein synthesis can occur well out of proportion to any changes in the transcription rate, highlighting the genuine importance of post-transcriptional and translational control mechanisms for regulating gene expression (Chen and Osborne 1970, Guyette, Matusik et al. 1979).

Since this shift in understanding, several molecular determinants of cytoplasmic mRNA regulation have been identified: miRNAs (Lee, Feinbaum et al. 1993) and the RNA-induced silencing complex (Hammond, Bernstein et al. 2000); the exon junction complex, which mediates nonsense-mediated mRNA decay (Peltz, Brown et al. 1993); and a multitude of sequence-specific RNA-binding proteins (Crawford and Richter 1987). The existence of endogenous pathways for mRNA silencing was, of course, an experimental boon, exploited in the form of gene expression knockdown via RNAi (Fire, Xu et al. 1998). While not having quite the same experimental impact, though, the ongoing characterization of the diverse RBP repertoire has, arguably, been the more revolutionary development in recent RNA biology. By nucleating RNA-protein complexes called RNPs, we now understand that RBPs form crucial regulatory hubs that enable cellular oversight for virtually every aspect of mRNA function, from initial processing to translation and degradation (Visa, Alzhanova-Ericsson et al. 1996, Dreyfuss, Kim et al. 2002). Notably, many RBPs appear to bind cohorts of functionally related mRNAs,

suggesting that the cell might coordinately control the expression of associated genes by packaging their transcripts together into the same RNP (Keene and Tenenbaum 2002).

Within the cytoplasm, RNPs often aggregate into large granules visible by light microscopy (Wilsch-Brauninger, Schwarz et al. 1997, Wilhelm, Mansfield et al. 2000), and several different types of these granules have been distinguished based on their composition and presumed function. The three varieties seen in non-germ cells—and, therefore, most relevant to our concerns—are stress granules, processing bodies, and transport particles. Stress granules and processing bodies appear to participate in mRNA storage and destruction, respectively (Nover, Scharf et al. 1989, Sheth and Parker 2003); transcripts accumulate in these structures particularly when global protein synthesis is shut down due to different forms of cellular stress (Kedersha, Stoecklin et al. 2005). Whereas stress granules are mainly aggregates of stalled translation preinitiation complexes (Kedersha, Chen et al. 2002), processing bodies contain exonucleases, decapping enzymes, and RISC components (Bashkirov, Scherthan et al. 1997, Ingelfinger, Arndt-Jovin et al. 2002, Sen and Blau 2005), accounting for their distinctively degradative function. Transport particles are bundles of mRNA complexed with specific RBPs that associate with the intracellular transport machinery and mediate transcript localization into subcellular compartments, especially within neurons (Knowles, Sabry et al. 1996). One characteristic that all these kinds of granules have in common is that their constituent mRNAs are translationally repressed (Richter and Smith 1984), although, in most cases, they seem to be in dynamic equilibrium with actively translated mRNAs in polyribosomes (Kedersha, Gupta et al. 1999, Barbee, Estes et al. 2006). With regard to transport particles specifically, the inhibition of mRNA translation during translocation to the intended subcellular compartment enables the cell to

accomplish spatially restricted expression of some proteins (Chartrand, Meng et al. 2002), a phenomenon to which we must now turn our attention.

b. Translational capacity of dendrites and axons

The first examples of asymmetric mRNA localization to be observed and linked to functional consequences were in cells preparing to undergo polarized divisions into non-identical daughter cells, such as in budding yeast (Long, Singer et al. 1997), *Xenopus* oocytes (Melton 1987), or the syncytial blastoderm of early *Drosophila* embryos (Hafen, Kuroiwa et al. 1984). However, extending the logic of localized gene expression to neurons—highly polarized but non-dividing cells—had to overcome the received wisdom that translational capacity is restricted to the soma (Peters, Palay et al. 1976). This limitation was at least theoretically lifted through the detection of protein synthesis machinery in the form of polyribosomes at synaptic contacts under dendritic spines (Steward and Levy 1982). Selective transport of mRNA into dendrites would be demonstrated soon after (Davis, Banker et al. 1987), no doubt abetted by the contemporaneous advances in elucidating post-transcriptional regulation. With mRNA and polyribosomes proven to be in dendrites, it was relatively straightforward to establish that even isolated dendrites, sheared off of cell bodies, could produce their own protein (Torre and Steward 1992).

What was true for dendrites, though, was not necessarily so for axons, and the idea of intra-axonal protein synthesis would prove to be much more controversial for quite some time. From the earliest cytologic studies of neurons, it was evident that mature vertebrate axons, unlike cell bodies or dendrites, are largely devoid of Nissl substance (Schaffer 1893), which later ultrastructural investigations would show to represent ribosomes (Palay and Palade 1955). Similarly, electron micrographs of axons themselves very rarely displayed ribosomes beyond the

initial segment (Palay, Sotelo et al. 1968). It was noted that axons from younger organisms, especially those gestating or newborn, tend to contain more ribosomes than are seen in adults, but this fact does not seem to have been thought of as much more than a curiosity (Zelena 1972). Axonal protein synthesis was thus ruled out, considering that the widespread interpretation of the data was that axons lacked the molecular machinery for it. Meanwhile, the existence of anterograde axoplasmic flow moving organelles and cytoskeletal elements into the axon was already well-established (Weiss and Hiscoe 1948), even if its molecular basis remained thoroughly unknown. This lent credibility to the supposition of an exclusively somatic origin for all the components of the axon, which seemed to many the only viable explanation. Throughout the mid-twentieth century, there was sporadic but significant opposition to this apparent consensus. In a remarkable series of articles during the 1960s, Edward Koenig showed that, after irreversible inhibition, acetylcholinesterase regenerates in axons through a process of local synthesis (Koenig and Koelle 1960, Koenig 1965a), and he vigorously defended the existence of axonal mRNA (Koenig 1965b, Koenig 1967a, Koenig 1967b). A few investigations followed on protein synthesis in synaptosomes, which represent isolated synaptic terminals, but these ultimately did little to alter the *status quo* (Austin and Morgan 1967, Autilio, Appel et al. 1968).

In the decade after the uncovering of dendritic protein synthesis, refinements in neuronal cell culture techniques and the availability of new methods for detecting RNA and protein encouraged the reexamination of translational activity in axons, especially during development. Beginning with Gary Bassell's demonstration that axonal growth cones in culture contain polyribosomes as well as a specifically localized mRNA, encoding β-actin (Bassell, Zhang et al. 1998), it was soon realized that a number of cytoskeletal and other proteins are locally produced within axons. Puzzlingly at the time, the portion of such proteins as actin or tubulin that was seen

to be locally produced was less than 1% of the amount synthesized in the soma and then transported into axons; moreover, even when axonal protein synthesis was blocked with pharmacologic inhibitors, axons continued to elongate (Eng, Lund et al. 1999). The first proof that this small pool of axonally synthesized protein has unique functional significance only came later, when it was shown that local translation is part of a number of developmental processes stimulated by extrinsic cues: axon repulsion after growth past intermediate targets (Brittis, Lu et al. 2002); growth cone collapse or attraction in response to guidance cues (Wu, Hengst et al. 2005, Leung, van Horck et al. 2006); and axon outgrowth stimulated by neurotrophic factors, but not basal elongation (Hengst, Deglincerti et al. 2009). In essence, then, intra-axonal protein synthesis appears to be indispensable only for local responsiveness to sensed stimuli, but not for carrying out constitutive "housekeeping" functions that are not stimulus-induced.

c. Axonal transcriptomics: injury, degeneration, and regrowth

In the course of investigating the different functions of axonal protein synthesis, a critical resource has emerged in the form of localized mRNA libraries, or transcriptomes. While some of these transcriptomes have limited themselves to defining the typical axonal mRNA composition of a particular type of neuron (Minis, Dahary et al. 2014), others have taken a more comparative approach, such as paralleling results from embryonic and adult axons to determine which transcripts change the most based on the developmental stage of the organism (Gumy, Yeo et al. 2011). In terms of what can be generalized about the axonal transcriptome, from these studies it is apparent that transcripts involved in mitochondrial function, cytoskeletal regulation, intracellular transport, and protein synthesis—including mRNAs for ribosomal subunits—tend to be the most highly enriched in axons. Another important take-away from this basic profiling of

axonal mRNA is that the vast majority of transcripts detectable in the axon are not found in the axon's growth cone (Zivraj, Tung et al. 2010), indicating that distinct mechanisms exist for mRNA localization to the axon or selectively to the growth cone.

Among the most intriguing transcriptomes generated have been those which took the stimulus dependence of local translation into account and assayed, for example, how the axonally localized mRNA repertoire responds to axonal injury (Willis, van Niekerk et al. 2007, Taylor, Berchtold et al. 2009) or the presence of a toxic stressor (Baleriola, Walker et al. 2014). These libraries are extremely useful in highlighting for further study potential translation-dependent pathways in the axon's response to various insults and challenges. A few major players in such pathways have already been identified. After axonal injury, locally produced importin β1 (Hanz, Perlson et al. 2003), vimentin (Perlson, Hanz et al. 2005), RanBP1 (Yudin, Hanz et al. 2008), and STAT3 (Ben-Yaakov, Dagan et al. 2012) assemble a retrogradely transported signal that informs the cell body about the lesion, while intra-axonal synthesis of β-actin, neurofilaments, and other factors facilitate regrowth (Zheng, Kelly et al. 2001, Donnelly, Willis et al. 2011). Recently, it was also shown that axons exposed to neurotoxic oligomers of amyloid-β respond by recruiting mRNA for the transcription factor ATF4, which is then synthesized in axons and retrogradely transported to induce changes in gene expression at the nucleus (Baleriola, Walker et al. 2014). From what we have learned through transcriptome profiling and previous characterization of locally produced proteins, there seem to be two overarching roles for axonal protein synthesis that have strong evidentiary support: first, the cell body-independent control of morphologic and physiologic changes within axons, and, second, the propagation of axon-to-soma signals concerning the dynamic state of the neuronal periphery.

I.3. Support Signals and Stressors of the Neuronal Environment

Neuronal physiology does not depend merely on the intrinsic pathways of wholly autonomous cells, whether these are directed from the soma or locally organized. Instead, as we have seen, albeit obliquely, extrinsic signals play a major role in informing the cell about its surroundings and instructing it to adapt to changing conditions. Neurons react to many factors in their environment, including small-molecule neurotransmitters, neuropeptides, growth signals, hormones, matrix material, and even dysfunctional proteins that accumulate there. Reviewing all these substances in detail is not our present concern, but two types—neurotrophins and amyloids—merit special consideration given their relevance to the development and degeneration of the nervous system.

a. Neurotrophins, their receptors, and signaling endosomes

Neurotrophins are a family of structurally related, secreted proteins that support the survival of vertebrate neurons (Leibrock, Lottspeich et al. 1989), comprising four known members: nerve growth factor, the founder and prototype of the family (Cohen and Levi-Montalcini 1956); brain-derived neurotrophic factor (Barde, Edgar et al. 1982); NT-3 (Hohn, Leibrock et al. 1990, Maisonpierre, Belluscio et al. 1990); NT-4/5 (Berkemeier, Winslow et al. 1991, Hallböök, Ibáñez et al. 1991); and two homologues only found in fish, NT-6 (Gotz, Koster et al. 1994) and NT-7 (Nilsson, Fainzilber et al. 1998). During development, NTs secreted by target cells help to direct growing axons of specific neuron types towards them (Gundersen and Barrett 1979, Phillips, Hains et al. 1990), a phenomenon known as their “tropic” effect. As their name suggests, NTs also have a distinct “trophic”—or, more accurately, pro-survival—effect, and certain kinds of neurons, especially during development, cannot survive in their absence

(Levi-Montalcini and Angeletti 1963). Embryonic DRG neurons, for example, go through a requisite period of dependence upon NGF, during which time experimentally induced autoimmunization of the organism to NGF will result in near-total destruction of these sensory ganglia (Johnson, Gorin et al. 1980). It is commonly understood that this NT dependence is a developmental mechanism to select for neurons whose axons have made the most optimal connections in their intended target field. Assuming a limiting supply of NTs, such neurons will outcompete and thus survive, while those with less suitable connections undergo apoptosis (Heumann, Korsching et al. 1984, Harper and Davies 1990). This idea is called the "neurotrophic hypothesis," and it has become the most useful model for understanding NTs' physiologic role *in vivo*.

Cellular responsiveness to NTs is mediated by two kinds of surface receptors, $p75^{NTR}$ and Trk receptors, with distinct binding characteristics and downstream effectors [**FIGURE 1-B**] (Sutter, Riopelle et al. 1979, Meakin and Shooter 1991). $p75^{NTR}$ belongs to the tumor necrosis factor receptor superfamily (Banner, D'Arcy et al. 1993) and was first identified as a low-affinity receptor for NGF (Johnson, Lanahan et al. 1986), although later research would demonstrate that it also binds other NTs with similar affinity (Rodriguez-Tebar, Dechant et al. 1990). The Trk proteins, meanwhile, are higher-affinity receptors with different NT specificities: TrkA binds NGF and NT-3 (Cordon-Cardo, Tapley et al. 1991, Klein, Jing et al. 1991); TrkB is activated by BDNF, NT-3, and NT-4/5 (Soppet, Escandon et al. 1991, Squinto, Stitt et al. 1991, Klein, Lamballe et al. 1992); and TrkC is only known to bind NT-3 (Lamballe, Klein et al. 1991). The primary means of NT survival signaling from binding at the plasma membrane is through these Trk receptors (Glass, Nye et al. 1991, Kaplan, Hempstead et al. 1991), which function by dimerizing in the presence of ligand to activate their kinase activity (Jing, Tapley et al. 1992). In

addition to this homodimerization, there is also evidence that $p75^{NTR}$ can interact with TrkA to potentiate its activation, particularly in response to low concentrations of NGF (Hempstead, Martin-Zanca et al. 1991, Verdi, Birren et al. 1994). However, on its own, $p75^{NTR}$ has actually been implicated in initiating mainly pro-apoptotic, rather than pro-survival, signaling (Rabizadeh, Oh et al. 1993, Frade, Rodriguez-Tebar et al. 1996).

There has long been evidence that NTs, upon binding at axon terminals, can be internalized by the cell and undergo retrograde axonal transport (Hendry, Stockel et al. 1974, Bernd and Greene 1984). It even appears that some of the effects of NTs, such as in regulating new transcription, might be directly mediated by their translocation to the nucleus (Johnson, Andres et al. 1978, Riccio, Pierchala et al. 1997), providing a biological justification for this long-range transport. Inquiries into this process culminated in the finding that NTs are subject to Trk-receptor mediated endocytosis and packed into small organelles, dubbed "signaling endosomes," that are retrogradely transported (Grimes, Zhou et al. 1996). Since this discovery, it has generally been assumed that signaling endosomes are integrally involved in the neurotrophic response, although other studies have delineated signaling endosome-independent consequences of Trk receptor activation, including calcium signaling and local protein kinase cascades (Tinhofer, Maly et al. 1996, Markus, Zhong et al. 2002). In fact, contrary to the "signaling endosome hypothesis," several groups have found that NTs' support of survival does not require Trk receptor internalization or retrograde trafficking of signaling endosomes (Zhang, Moheban et al. 2000, MacInnis and Campenot 2002).

Clearly, much about Trk receptor signaling, both in the presence and absence of bound ligand, remains imperfectly understood, and so different hypothetical frameworks continue to be fashioned. Recently, new studies have claimed that TrkA and TrkC, but not TrkB, function at

least in part as "dependence receptors," meaning that, far from being inert when in their NT-unbound state, these receptors constitutively induce pro-apoptotic death pathways which only cease when they bind ligand (Tauszig-Delamasure, Yu et al. 2007, Nikoletopoulou, Lickert et al. 2010). Combined with another recent report strongly attributing the effects of NGF deprivation to the activation of a pro-apoptotic signaling pathway rather than the loss of retrogradely transported survival signals (Mok, Lund et al. 2009), this emerging evidence suggests that much of what we think we know about neurotrophic signaling might warrant reexamination.

b. Paradigms of neurodegeneration: neurotrophin deprivation and amyloid toxicity

Post-developmentally, NTs and their receptors continue to be expressed and play vital roles in the maintenance of the nervous system, regulating neurite morphology (Diamond, Holmes et al. 1992), synaptic plasticity (Kang and Schuman 1995), and neuronal responses to injury (Merlio, Ernfors et al. 1993). Given its enduring importance to neuronal function and adaptability, NT signaling has repeatedly been investigated as a potential cause or even therapeutic avenue in neurodegenerative diseases. In glaucoma, strong evidence exists that ocular hypertension causes NT deprivation and Trk receptor dysregulation, which lead, in turn, to the death of retinal ganglion cells (Rudzinski, Wong et al. 2004, Dekeyster, Geeraerts et al. 2015). Downregulation of NTs and Trk receptors has also been seen to correlate with the progression of AD, especially in brain regions and cell types that tend to be the most severely affected, although these do not appear to be early or precipitating factors (Phillips, Hains et al. 1991, Salehi, Verhaagen et al. 1996, Ginsberg, Che et al. 2006).

Indeed, identifying the chief pathogenetic event in AD has proven extremely controversial, with most investigators dividing between two camps that each advocate a single

pathological protein as key—the microtubule-associated protein tau or amyloid-β (Jack Jr and Holtzman 2013). One advantage of studying Aβ is that well-established methods exist to survey its neurotoxic effects *in vitro* (Yankner, Duffy et al. 1990), although this is, admittedly, artificial and may have little bearing on resolving the dispute as to what actually happens in AD patients. Little is known about the transition of Aβ from its physiologic role in modulating synaptic plasticity (Kamenetz, Tomita et al. 2003, Puzzo, Privitera et al. 2008, Puzzo, Privitera et al. 2011) towards pathology, but the details of its production have been fleshed out extensively. Aβ is formed by proteolytic cleavage of a transmembrane glycoprotein, amyloid precursor protein (Kang, Lemaire et al. 1987), at two distinct sites, catalyzed by the beta-secretase BACE1 and a gamma-secretase complex [**FIGURE 1-C**] (Vassar, Bennett et al. 1999, Li, Lai et al. 2000). The release of the soluble Aβ fragment occurs normally in healthy individuals (Shoji, Golde et al. 1992), but one of the striking characteristics of AD is that extracellular Aβ aggregates into insoluble fibrils and then large neuritic plaques (Masters, Simms et al. 1985). Research has found, however, that the most potent toxic species of Aβ is not in fibrils or plaques but rather in diffusible oligomers (Lambert, Barlow et al. 1998).

Interestingly, as alluded to before, Aβ oligomers have recently been shown to stimulate local protein synthesis, which helps to mediate their toxicity (Baleriola, Walker et al. 2014), and NTs' effects on synaptic plasticity in the adult brain also appear to require the local production of protein (Kang and Schuman 1996). These results suggest that the use of localized protein synthesis in facilitating neuronal responses to extracellular stimuli might be a mechanism that is reactivated, even in the mature organism, when there is a need to adjust to dangerous insults or changing circumstances.

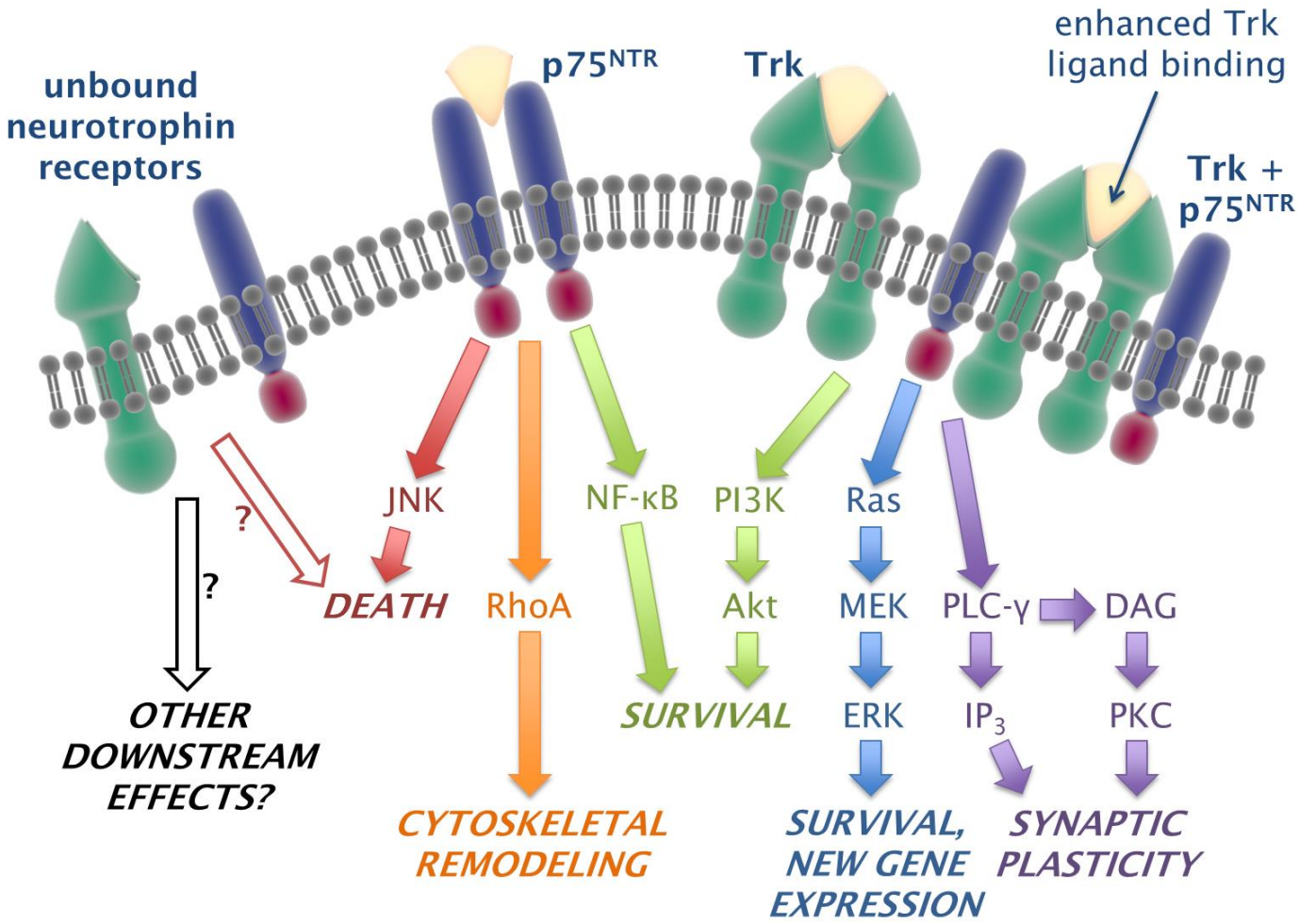

FIGURE 1-B: Summary of NT signaling through $p75^{NTR}$ and Trk receptors, including their downstream effectors and the stereotyped end results of the various signaling pathways. Although $p75^{NTR}$ and Trk receptors both can dimerize and initiate their own signal transduction cascades upon binding NTs, $p75^{NTR}$ association with Trk can also enhance Trk's affinity for ligand at low concentrations of NTs, thus enhancing Trk activity. The potential signaling pathways initiated by ligand-unbound NT receptors remain the subject of much debate.

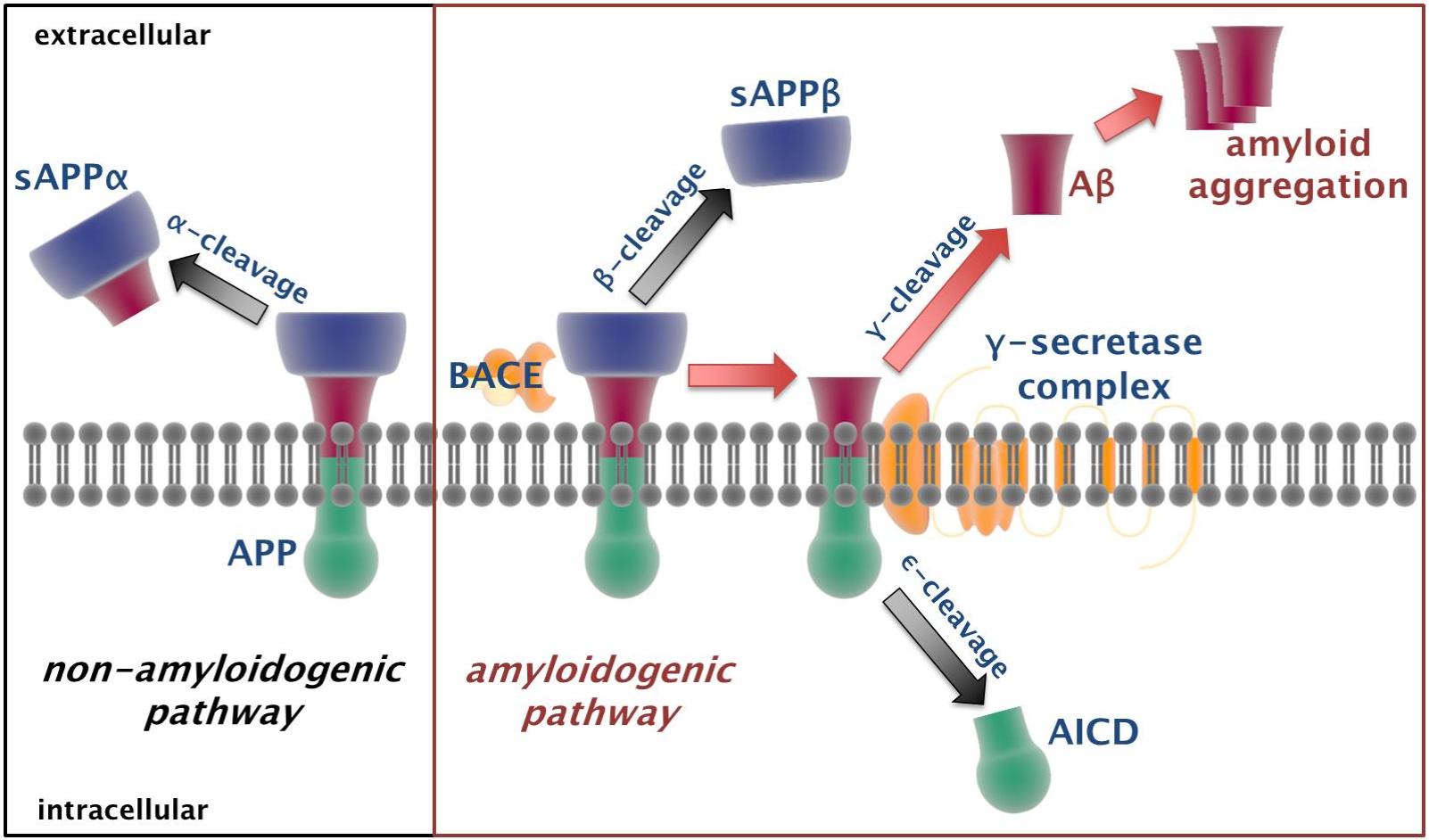

FIGURE 1-C: Summary of APP processing and Aβ generation. APP can undergo either non-amyloidogenic cleavage (left) or sequential cleavages by BACE and the γ-secretase complex in an amyloidogenic pathway (right). APP intracellular C-terminal domain (AICD) can also be released from the membrane by cleavage, freeing it for nuclear signaling and other functions.

I.4. Outline of Thesis

Thus far, we have reviewed three biological operations, all germane to the functioning of neurons, that might nevertheless seem to be largely unrelated. It is true, for instance, that NT signaling endosomes undergo active transport by molecular motors and that environmental cues can trigger the local production of certain proteins, but, as things stand, these facts do not clearly inform each other. However, these three operations could be fitted into a theoretical physiologic pathway, proceeding neatly in reverse relative to how they were introduced: *viz.* extracellular stimuli and changing environmental conditions near axons may induce local protein synthesis as a mechanism to fine-tune axonal transport for meeting new demands. What follows in this thesis is a demonstration that this proposed pathway belongs not merely to the realm of speculation but is, in fact, substantiated by new evidence as actually taking place in mammalian neurons.

In Chapter II, the materials and experimental procedures used in the described studies are detailed. Chapter III establishes that mRNAs associated with dynein-based transport are recruited to axons and undergo regulation, at the level of localization as well as translation, in response to different conditions of NT signaling. The surprisingly complex post-transcriptional regulation of axonally localized mRNA for the cofactor Lis1 is given special consideration. Chapter IV shows that NT-related changes in axoplasmic transport are mediated by localized protein synthesis, with locally produced Lis1 and $p150^{Glued}$ being important regulators of retrograde transport. Preliminary evidence for transport regulation via axonal protein synthesis in the context of pre-neurodegenerative stress is provided in Chapter V. Lastly, in Chapter VI, the present findings are situated in terms of the literature, and their implications for future research are explored. Overall, this thesis aims to broaden our conception of regulatory opportunities in polarized cells, with its presentation of a novel mechanism for regulating intracellular transport as a revealing exemplar.

CHAPTER II. MATERIALS AND METHODS

II.1. Animal Use

a. Rat ordering and husbandry

Timed-pregnant Sprague-Dawley rats (*Rattus norvegicus*) were obtained from Charles River Laboratories (Wilmington, MA) and housed in a high-level barrier facility at the Columbia University Institute of Comparative Medicine. All rodent procedures were approved by the Columbia University Institutional Animal Care and Use Committee.

b. Euthanasia

Consistent with recommendations from the Panel on Euthanasia of the American Veterinary Medical Association, rats were euthanized by gas displacement with 10% min^{-1} CO_2 for at least 5 min, followed by bilateral thoracotomy to ensure death.

II.2. Cell Culture Techniques

a. Microfluidic devices

Tripartite microfluidic devices with two sets of 200 µm-long microgroove barriers were produced according to published protocols [**FIGURE 2-A**] (Park, Vahidi et al. 2006, Desai, Freeman et al. 2009). In summary, microfluidic chambers were produced using polyurethane plastic replica molds made from silicon masters. PDMS elastomer base was combined with curing agent (Ellsworth, Germantown, WI) in a 9:1 ratio and mixed thoroughly for 5-10 min. The PDMS mixture was then poured onto the plastic mold, and the mold was placed in a vacuum desiccator for approximately 30 min to remove trapped bubbles from the PDMS. Afterward, the mold was put in a leveled oven so that the PDMS could cure for at least 4 h at 65°C. Using a razor blade, the cured PDMS was removed from the mold and cut into the individual chamber devices. For each device, a 6.0 mm biopsy punch (VWR, Radnor, PA) was used to punch out the six reservoirs. Debris was cleaned from the surface of the chambers using vinyl cleanroom tape (VWR), and the devices were sterilized by brief immersion in 70% EtOH. Lastly, the devices were dried for at least 1 h in a laminar flow hood before application.

b. Culture material preparation

Nunc cell-culture treated 6-well plates or, in the case of live-imaging experiments, glass bottom dishes (MatTek, Ashland, MA) were utilized for cell culture. 25 mm circular cover glasses (Carolina Biologicals, Burlington, NC)—which, for DRG culture only, were immersed overnight in nitric acid and then rinsed with water until neutralized—were placed in each well of a 6-well plate. Cover glasses or glass bottom dishes were coated with 100 µg ml^{-1} PDL (Sigma-Aldrich, St. Louis, MO) for hippocampal culture or PLL (Trevigen, Gaithersburg, MD) for DRG

culture; incubation with PDL or PLL was performed for 1 h, after which the coated surface was washed three times with sterile water and then dried.

c. Beginning dissection

After euthanasia, the pregnant dam's uterus was dissected out and placed in a sterile dish. Embryos were removed from the uterus within a positive pressure hood for further dissection.

d. DRG neuron culture

Primary DRG neurons were harvested from E15 rat embryos. DRGs were removed from the embryos and placed in a dish containing Leibovitz's L-15 medium. The collected DRGs were put in a 15 ml conical centrifuge tube with TrypLE Express and incubated for 20 min in a water bath at 37°C. Trypsinized DRGs were then centrifuged for 5 min at 1000 rpm in 4°C. Afterward, the TrypLE solution was carefully removed from the pelleted tissue, which was resuspended in DRG medium (Neurobasal, 1x B27, 2 mM glutamate, 20 µM 5-FdU, 50 ng ml^{-1} NGF) and dissociated by ten passes through a 1 ml pipette followed by ten more passes through a 100 µl pipette (VWR). Subsequently, approximately 60,000 cells were seeded per chamber.

e. Hippocampal neuron culture

After applying the microfluidic chambers, the cover glasses or glass bottom dishes for hippocampal cultures were coated with 2 µg ml^{-1} laminin (Trevigen, Gaithersburg, MD).

Primary hippocampal neurons were prepared from E18 rat embryos based on a well-established protocol (Banker and Goslin 1998). The embryos' brains were dissected out and placed in a dish containing HBSS. Once all of the hippocampi were isolated, they were put

together in a 15 ml conical centrifuge tube with TrypLE Express and incubated for 20 min in a water bath at 37°C. Trypsinized hippocampi were then centrifuged for 5 min at 1000 rpm in 4°C. Afterward, the TrypLE solution was carefully removed from the pelleted tissue, which was resuspended in hippocampal plating medium (Neurobasal, 10% FBS, 100 mM glutamine) and dissociated by ten passes through a regular glass Pasteur pipette followed by ten more passes through a flame-polished pipette. Cell density in the resulting suspension was measured with a hemacytometer and adjusted, with 60,000 cells ultimately being seeded per chamber. On DIV 1, the medium was changed to a serum-free growth medium (Neurobasal, 1x B27, 100 mM glutamine), half of which was replaced on DIV 5 with fresh growth medium.

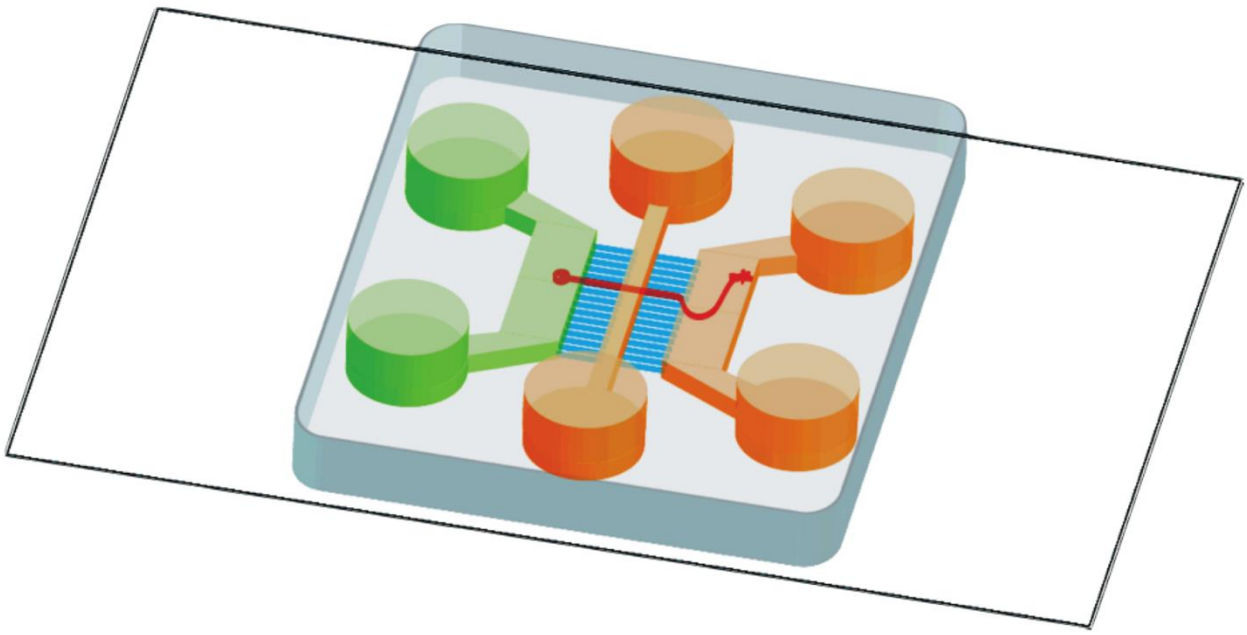

FIGURE 2-A: Schematic representation of a microfluidic chamber used to isolated axons. Embryonic DRG or hippocampal neurons (red) were seeded in the cell body compartment (green), and their axons extended through two microgroove barriers (blue) into the axonal compartments (orange). All axon-specific treatments were applied to both axonal compartments, and analyses were performed in the most distal compartment.

II.3. Experimental Interventions

a. RNA interference with siRNAs

Axon-specific silencing of *Pafah1b1* and *Dctn1* mRNAs was achieved by transfecting siRNAs into axons, concomitant with a medium exchange, using NeuroPORTER (Genlantis, San Diego, CA) as the transfection reagent. In brief, a mixture of 100 nM siRNA and 10% NeuroPORTER in serum- and antibiotic-free medium was added to axonal compartments. For hippocampal neurons only, 2 h after transfection the axonal medium was supplemented with an equal volume of Neurobasal containing 2x B27 and 100 nM glutamine; thus, in hippocampal neuron experiments, the final siRNA concentration was 50 nM. The following siRNAs were used to target rat *Pafah1b1* (NM_031763.3) and *Dctn1* (NM_024130.1):

Pafah1b1 siRNA.1- 5′CCUUUGACCACAGUGGCAAACUCUU3′

Pafah1b1 siRNA.2- 5′GGAUUUCCAUAAGACGGCACCCUAU3′;

Dctn1 siRNA.1- 5′GAGCGCUCCUUAGAUUUCCUCAUCG3′

Dctn1 siRNA.2- 5′GACAUCCGUCAGUUCUGCAAGAAGA3′.

Stealth RNAi siRNA Negative Control Med GC Duplex #3 was used as a negative control.

b. LNA transfection

Custom-made LNAs, designed to have fewer than five unmodified bases in a row so as to prevent RNase H recruitment and activation (Kurreck, Wyszko et al. 2002), were ordered from Exiqon (Woburn, MA) with HPLC purification. By a similar procedure to that described above for transfection of DRG axons with siRNA, 100 nM LNAs were transfected into the cell body compartment using NeuroPORTER. One LNA (5’-A+TTTA+CAGTA+TACAA+TT-3’)

targeted the CUGU-motif APC binding site (bases 1905-1921) of rat *Pafah1b1* mRNA, while a control LNA (5'-CA+TGAA+TACTT+TGT-3') was made to target an unrelated, upstream sequence (bases 1878-1891) [**FIGURE 2-B**]. +A/+C/+G/+T signify modified LNA bases.

c. Pharmacological inhibitors

When specified, the following inhibitor compounds were applied specifically to axons in microfluidic cultures: protein synthesis inhibitors anisomycin (1 µM, Sigma-Aldrich) or emetine (2 µM, EMD Millipore, Billerica, MA); mTOR inhibitor rapamycin (10 nM); MLK inhibitor CEP-1347 (0.5 µM); p38 MAPK inhibitor SB239063 (1 µM); GSK3 inhibitors LiCl (15 mM) or SB216763 (10 µM, Sigma-Aldrich); or the dynamin inhibitor dynasore (80 µM, Sigma-Aldrich).

d. Nerve growth factor and amyloid-β

2.5S NGF (Harlan, Indianapolis, IN) was dissolved in buffer consisting of 0.1 M sodium acetate and 0.5 M sodium chloride at pH 5.0, aliquoted, and stored at -80°C until use.

Soluble oligomeric Aβ was prepared according to an established protocol (Stine, Dahlgren et al. 2003). Synthetic $A\beta_{1-42}$ peptides (purchased from Dr. David Teplow, UCLA) were dissolved in hexafluoroisopropanol to a concentration of 1 mM, aliquoted, and dried. The peptides were resuspended to 1 mM in DMSO through bath sonication for 10 min and stored at -20°C. For oligomer formation, the peptides were diluted to 100 µM in PBS, incubated overnight at 4°C, and then used on the same day.

e. *In vivo* retrograde tracing experiments

Stereotaxic injections were performed according to established procedures (Sotthibundhu, Sykes et al. 2008) with minor modifications (Jean, Ribe et al. 2013). 9-12 month-old C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with ketamine (95 mg kg^{-1}) and xylazine (7 mg kg^{-1}), and then placed in a stereotaxic frame (Stoelting, Wood Dale, IL). Stereotaxic injections were conducted using convection-enhanced delivery at a rate of 0.5 µl min^{-1} using the Quintessential Stereotaxic Injector (Stoelting). Mice were injected with 4 µl DMSO into the left dentate gyrus and 4 µl oligomeric $A\beta_{1\text{-}42}$ (100 µM in PBS) into the right dentate gyrus [**FIGURE 2-C**]. Based on the observed spread of $A\beta 1_{\text{-}42}$, the estimated final $A\beta_{1\text{-}42}$ concentration was ~30 nM in the dentate gyrus. A 2% suspension of the retrograde label FluoroGold (Fluorochrome, LLC, Denver, CO) was co-injected in all cases. Animals were sacrificed 2, 4, or 7 days *post* injection by anesthesia with ketamine and xylazine followed by transcardial perfusion with 4% PFA in PBS. Brains were post-fixed in 4% PFA for 24 hr at 4°C, followed by 30% sucrose infiltration, embedded in Tissue-Tek O.C.T Compound (Sakura Finetek, Torrance, CA), and cryostatically sectioned at 20 µm thickness. Finally, sections of the basal forebrain, which includes populations of neurons that project axons to the dentate gyrus (Amaral and Kurz 1985, Leranth and Frotscher 1989), were imaged for FluoroGold labeling.

```
      *
1728 UGAUUGAGUCACAUUUGGUUCUUCCUCCCUUUUUUCCUCUGGAUGCACUCUGAUGAUACCAUGGU
1793 UACCCCAUUUGAGCUCUGUUUAAAUAAAUAUUGUCCUUUCAUGUAAAUUAUUCUGGAUGUAGAUU
1858 GAGCUUAUUAAAUGUUACACACAAAGUAUUCAUGCAUGGUGAAUCCAAAUUGUAUACUGUAAAUU
1923 UACAUAU...AAUUGUGU 4363
```

FIGURE 2-B: Partial sequence of the 3'UTR of rat *Pafah1b1* starting at the stop codon (*). The binding regions of the CUGU and control LNA oligomers are indicated in green and yellow, respectively. The CUGU element of the putative APC-binding site is underlined.

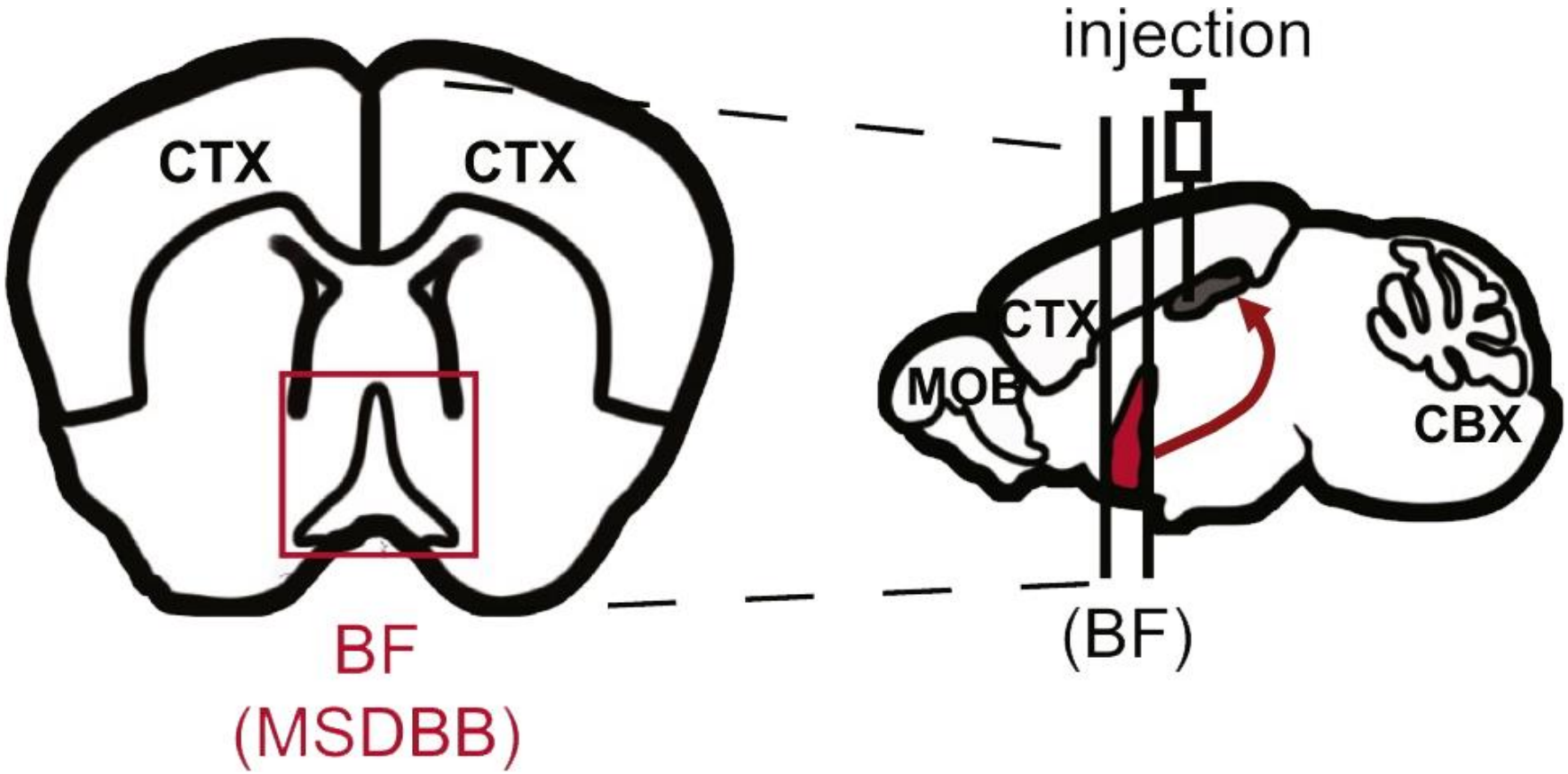

FIGURE 2-C: Representation of the mouse forebrain in coronal section (left) and injection procedure (right) for FluoroGold tracer experiments. DMSO vehicle was injected into the left hemisphere and $A\beta_{1\text{-}42}$ into the right hemisphere. The vertical solid black bars indicate the interval of brain sections adjacent to the site of the injection that were selected to analyze the basal forebrain (BF). The hippocampal formation, including dentate gyrus, is highlighted in gray and the BF in red. The red arrow indicates the course taken by axonal projections of basal forebrain cholinergic neurons to the dentate gyrus. MOB, main olfactory bulb; CTX, cortex; CBX, cerebellar cortex; BF, basal forebrain; MSDDB, complex of the medial septum-diagonal band of Broca. Figure adapted from Baleriola, Walker *et al*. 2014.

II.4. Immunocytochemistry and Functional Imaging

a. Microscope set-up and image acquisition

Neurons were imaged using a Plan-Apochromat 63x/1.40 oil objective on an Axio-Observer.Z1 motorized inverted microscope equipped with an AxioCam MRm Rev. 3 camera (Zeiss, Thornwood, NY). This microscope's filter set allows three-channel detection of fluorophores from the DAPI (Em = 461 nm), eGFP (Em = 509 nm), and Texas Red (Em = 615 nm) families. During live-cell imaging, a heat-controlled microscope chamber incubator together with a temperature-, CO_2-, and, humidity-controlled stage incubator allowed samples to be kept at 37°C and 5.0% CO_2, while a Definite Focus module (Zeiss) prevented Z-drift during the relatively long-term observation.

Exposure settings for image acquisition of stained axons were determined automatically using AxioVision 4.8 acquisition software (Zeiss) on a random axonal field of a control sample, to ensure that pixel intensities were within the linear range and avoid pixel saturation. These settings were then maintained for all samples in any given experiment. Images were focused based on the counterstain so that acquisition would be blind to the staining of interest. For fixed samples, images were acquired in Z-stacks of three slices in five random fields per cover glass; Z-slices of axonal fields were 0.1 μm apart, whereas those in somatic fields were 1 μm apart.

b. Fixation and blocking

Neurons were fixed with 4% PFA/4% sucrose in PBS for 20 min at room temperature. Afterward, cover glasses were washed three times in PBS, then blocked and permeabilized for 1 h with BGT buffer (3% BSA, 100 mM glycine, 0.25% Triton X-100).

c. Quantitative immunofluorescence

After fixation and blocking, neurons were incubated with primary antibodies against two or more of the following targets: β-III tubulin (Abcam, ab7751; 1:500), 4E-BP1, phospho-4E-BP1, S6, phospho-S6 (Cell Signaling Technology, 1:1,000), Lis1 (Sigma-Aldrich, SAB3500302, 1:400), or $p150^{Glued}$ (Abcam, ab11806, 1:500). Then, cover glasses were washed three times with TBS and incubated with fluorophore-conjugated Alexa secondary antibodies (1:2,000) for 1 h at room temperature, washed three more times with TBS, and mounted with ProLong Gold antifade reagent. Staining for β-III tubulin was used to generate a mask within which the pixel intensity of the immunofluorescence signal for the protein of interest was quantified. For each image, background pixel intensity was calculated outside the axonal mask and subtracted from the signal for the protein of interest.

d. Live-cell imaging of axonal cargoes

To assess generic axonal trafficking, 50 nM LysoTracker Green DND-26 was added to axons during an axonal medium exchange 15 min prior to the start of imaging. For examining transport specifically of NGF-containing endosomes, quantum dot-conjugated NGF was prepared by mixing mouse NGF 2.5S-Biotin (Alomone Labs, Jerusalem) and Qdot 585 Streptavidin Conjugate in a 1:1.2 molar ratio and incubating them together at 4°C with continuous inversion for 24 h. The next day, QD-NGF was diluted to 100 ng ml^{-1} and added to axons with a medium change 15 min before imaging. During imaging, neurons were kept in a CO_2- and humidity-controlled incubation chamber maintained at 37°C. Images were acquired every 13 s over a total 4-minute time period, with 3 fields of axons imaged per replicate.

For motility analysis, LysoTracker-positive particles were scored only if they were ≥1 μm in diameter, whereas QD-NGF particles were included only if they were ≤0.5 μm in diameter. Particles were scored as stationary, anterograde, retrograde, or bidirectional according the following definitions: stationary if they traveled a distance <1 μm during the observation period; anterograde or retrograde if they displaced >3 μm in one direction; and bidirectional if they traveled >3 μm in both directions.

e. Cell death and survival assays

When complete neutralization of all residual NGF activity in culture was needed, an anti-NGF antibody (10 μg ml^{-1}, ab6198, Abcam) was added with NGF-free medium to both the somatic and axonal compartments or to the somatic compartment alone.

To detect apoptotic cell death, TUNEL was performed on fixed samples using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI), consistent with the manufacturer's instructions but employing modifications for use in microfluidic devices. Cells were permeabilized for 1 h with BGT buffer, and then washed three times in PBS. Equilibration buffer was added to all compartments for 30 min to pre-equilibrate, after which incubation buffer (45 parts equilibration buffer, 5 parts nucleotide mix including fluorescein-12-dUTP, and 1 part TdT enzyme) was added only to the somatic compartment. For the TUNEL reaction, samples were incubated at 37°C for 1 h, and, to stop the reaction, the microfluidic chambers were removed and 2x SSC was added. Finally, cover glasses were washed three more times in PBS, dried, and mounted with ProLong Gold antifade reagent with DAPI prior to imaging.

Meanwhile, survival was analyzed using calcein staining of living cells. Cell bodies were incubated with 4.17 μg ml^{-1} calcein AM dye in DMSO for 40-60 min at 37°C. At that point,

calcein was quenched with 15 mg ml^{-1} bovine hemoglobin (Sigma-Aldrich), and nuclei were labeled with Hoechst stain. Cells were immediately live-imaged inside the microscope incubation chamber kept at 37°C and 5% CO_2.

TUNEL-positive nuclei with apoptotic morphology (pyknosis, karyorrhexis) and calcein-positive cells were scored in 5 fields per replicate that were proximal to the microgrooves.

II.5. Detection of RNA

a. Fluorescence *in situ* hybridization

Antisense riboprobes were transcribed *in vitro* from sense oligonucleotides containing a T7 promoter site (…GCCCTATAGTGAGTCGTATTAC-3′) at their 3′ end using the MEGAshortscript T7 Transcription Kit and digoxigenin-conjugated UTP (Roche, Indianapolis, IN), according to the manufacturer's instructions. In short, the reaction mixture was assembled with nucleotides, T7 enzyme, and 200 nM template DNA, gently mixed, and incubated at 37°C for at least 2 h. In the last 15 min of the 37°C incubation, TURBO DNase was added to degrade the template DNA. After their *in vitro* transcription, a mix of five non-overlapping riboprobes with matching GC content was used to detect each mRNA:

Gfp probe.1-

5′-GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAG-3′

Gfp probe.2-

5′-GACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTAG-3′

Gfp probe.3-

5′-ACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACG-3′

Gfp probe.4-

5′-AAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAG-3′

Gfp probe.5-

5′-AGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGG-3′;

Pafah1b1 probe.1:

5'-GCTCCGGTGGAATGAACCTTACTTGTTGACTGGTTGCTGATTGGATTCAC-3′

Pafah1b1 probe.2:

5'-GGATCTGAGACTAAAAAAAGTGGCAAGCCTGGACCCTTCTTGCTATCTGG-3′

Pafah1b1 probe.3:

5'-CTGTTGGTGCCTGACTTGATGGCCTCATTTTGGGGAAAGTGGTTATTAGG-3′

Pafah1b1 probe.4:

5′-CTAAGCTGAGAGAAAGTCACTTTATTCTCCCCTCTAATGGGCCATTCACC-3′

Pafah1b1 probe.5:

5′-TACTGTTTTCTCTGTCTGCTGTCTAACCCTGTGCCTTGCCTGGGATAAGG-3′;

Dctn1 probe.1:

5'-TTGGAGATCCTCAAGGCTGAAATTGAAGAGAAAGGCTCTGATGGGGCTGC-3′

Dctn1 probe.2:

5'-TCACCAAGGCCATCAAGTACTACCAGCATCTGTACAGCATCCACCTCGCT-3′

Dctn1 probe.3:

5'-CAACAGATATTGCTCTTCTTCTGCGAGACCTGGAAACATCCTGCAGTGAC-3′

Dctn1 probe.4:

5′-AAGGATGCTGATGAGCGAATCGAGAAAGTTCAGACTCGGCTGGAGGAGAC-3′

Dctn1 probe.5:

5′-GGCCAAGGAAGAGCAGCAAGACGACACAGTCTACATGGGCAAAGTGACCT-3′.

FISH was performed consistent with a published protocol (Femino, Fay et al. 1998), with minor modifications. Neurons grown in microfluidic chambers were fixed in 4% PFA/4% sucrose in PBS for 20 min at room temperature. Following three washes with PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS and washed twice more with PBS. The coverslips

were incubated with a total of 100 ng digoxigenin-labeled riboprobes (20 ng each of five distinct riboprobes) in 30 µl hybridization buffer (50% formamide, 2x SSC, 0.2% BSA, 1 mg ml^{-1} *E. coli* tRNA, 1 mg ml^{-1} salmon sperm DNA) overnight at 37 °C. The coverslips were washed with constant agitation at 37 °C, first with 50% formamide in 2x SSC for 30 min followed by 50% formamide in 1x SSC for another 30 min. An additional three washes were done at room temperature with 1x SSC for 15 min each. The coverslips were washed three times with PBS-T for 5 min each, blocked with 3% BSA in PBS-T for 30 min, and incubated with anti-digoxin (Sigma-Aldrich, DI-22; 1:500) and anti-β-III tubulin (Abcam, ab41489; 1:1,000) antibodies in blocking solution overnight at 4 °C. The coverslips were washed three times with PBS-T and incubated with fluorophore-conjugated Alexa secondary antibodies (1:2,000) for 1 h at room temperature, washed and mounted with ProLong Gold antifade reagent.

Staining for β-III tubulin was used to generate a mask within which the pixel intensity of the FISH signal was quantified. For each image, background pixel intensity was calculated outside the axonal mask and subtracted from the FISH signal. Finally, average fluorescence intensity of axonal fields that were incubated with a non-targeting *Gfp* probe was subtracted from the fluorescence intensities resulting from hybridization with *Pafah1b1* or *Dctn1* riboprobes.

b. RNA purification

RNA was purified from the axonal compartments of microfluidic chambers using the PrepEase RNA Spin kit (Affymetrix, Santa Clara, CA). Axons were lysed with RA1 buffer, and, for each condition, lysates from six microfluidic devices were pooled, to which 1% BME was added. After vigorous vortexing, lysates were added to filter units (purple), centrifuged at 11,000x *g* for 1 min, and equal volume of 70% EtOH was added to the flow-through. Samples

were then bound to RNA spin columns (light blue) by centrifugation at 8,000x *g* for 30 s, and eluent was discarded. Membrane desalting buffer was added to the spin column, centrifuged at 11,000x *g* for 1 min, and discarded. DNase reaction mixture (1 part rDNase stock solution to 9 parts DNase reaction buffer) was applied directly to the spin column membrane and incubated at room temperature for 15 min. The spin column was subsequently washed three times: first, with RA2 buffer, centrifuged at 8,000x *g* for 30 s; second, with RA3 buffer, centrifuged at 8,000x *g* for 30 s; and lastly, again with RA3 buffer, centrifuged at 11,000x *g* for 2 min. After air-drying the membrane, RNA was eluted by adding 100 µl of RNase-free water directly to the spin column and centrifuging at 11,000x *g* for 1 min.

A total amount of approximately 2 ng was generally isolated from axonal lysates, which was cleaned and concentrated with the RNeasy MinElute Cleanup kit (QIAGEN, Valencia, CA). 350 µl of RLT buffer was added to the 100 µl sample, followed by 250 µl of pure EtOH, and mixed well. Next, the 700 µl of diluted RNA was transferred to a spin column, centrifuged at 8,000x *g* for 15 s, and the eluent discarded. The membrane was then washed three times: twice with RPE buffer, centrifuged at 8,000x *g* for 15 s; and then with 80% EtOH, centrifuged at 8,000x *g* for 2 min. To dry the membrane, the spin column was centrifuged at full speed without its lid for 5 min. Finally, RNA was eluted by adding 10 µl of RNase-free water directly to the membrane and centrifuging at full speed for 1 min.

c. Real-time reverse transcription PCR

Concentrated RNA was reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix. Briefly, axonal RNA, 50 µM oligo$(dT)_{20}$ primers, annealing buffer, and nuclease-free water were combined, incubated in a thermal cycler at 65 °C for 5 min, and

then placed on ice for 1 min. Afterward, reaction mix and SuperScript III enzyme mix were added, and the reaction was incubated at 50 °C for 50 min before termination at 85 °C for 5 min. Real-time RT-PCR was performed using TaqMan Gene Expression master mix with TaqMan probe and primer sets for *Gapdh* (Rn01775763_g1), *Pafah1b1* (Rn00443070_m1), and *Dctn1* (Rn00577061_m1) in a StepOnePlus Real-Time PCR instrument. Amplification was accomplished using the following conditions: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 1 minute. Each condition was subjected to three technical replicates per experiment. Relative gene expression was quantified by the comparative C_T method, and *Pafah1b1* and *Dctn1* transcript levels were normalized to *Gapdh* as the endogenous control.

II.6. General Notes

a. Source of materials

All reagents or materials were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise noted.

b. Statistical analyses

Two means were compared by Mann-Whitney *U* tests, whereas multiple means were compared using Kruskal-Wallis ANOVA with Dunn's multiple comparison testing. When comparing multiple groups in experiments with more than one variable, as was most common, two-way ANOVA was performed.

c. Respective contributions

Joseph M. Villarin performed and analyzed all *in vitro* experiments, with assistance from Ethan P. McCurdy specifically for immunostaining translation markers and scoring some of the cell death and survival assays. Dr. Jimena Baleriola from the Hengst laboratory and Dr. Ying Jean from the Troy laboratory performed *in vivo* injection experiments on mice and provided unpublished data for analysis by the author, allowing us to report and discuss them here.

d. Publication status

The content of Chapters III and IV has been composed into a manuscript, and it is planned to be published soon, with a small amount of additional work. Chapter V will likely be further developed by Ethan P. McCurdy and others from the Hengst laboratory, thus forming the basis for a likely follow-up publication.

CHAPTER III. TRANSCRIPTS ENCODING DYNEIN COFACTORS ARE LOCALIZED TO AXONS AND REGULATED IN DISTINCT POOLS

III.1. Rationale and Summary

The last decade has seen mounting interest in characterizing the localized mRNA content of neuronal axons and understanding its purposes for being there (Willis, van Niekerk et al. 2007, Taylor, Berchtold et al. 2009, Zivraj, Tung et al. 2010, Gumy, Yeo et al. 2011, Baleriola, Walker et al. 2014, Minis, Dahary et al. 2014). As RNA biology continues to mature, it becomes increasingly imperative to unravel the complex web of regulatory interactions between mRNA transcripts, RNA-binding proteins, and organizing structures throughout the cell, particularly within specific subcellular compartments. Fortunately, this effort is just beginning to bear its first fruits, with the discovery of regulatory hubs that integrate the localization and translation of mRNAs (Tcherkezian, Brittis et al. 2010, Preitner, Quan et al. 2014).

In the work presented here, an unbiased review of evidence for axonally localized transcripts of the microtubule motors and their related proteins was used as a starting place in order to investigate specific candidate mRNAs in greater depth. After verifying that two mRNAs coding for the dynein cofactors Lis1 and p150^{Glued} are localized to axons, their multilevel regulation by NT signaling was investigated, concluding in the identification of part of their regulatory machinery.

III.2. Results

a. Transcripts for dynein and dynein cofactors, especially Lis1 and p150^{Glued}, localize to axons

It was evident, from reviewing the available literature, that the presence of mRNAs related to the function and regulation of the microtubule motors is a feature found repeatedly in axons of both the central and peripheral nervous system [**FIGURE 3-A**] (Gumy, Yeo et al. 2011, Baleriola, Walker et al. 2014, Minis, Dahary et al. 2014). Several members of the kinesin superfamily with recognized functions in axons are seen to possess axonally localized mRNAs, though this may vary widely among different species and neuronal subtypes. Likewise, transcripts for multiple subunits of cytoplasmic dynein 1 itself, including many of the heavy, intermediate, and light intermediate chains—essentially the core of the holoenzyme—appear to be localized to axons. By way of comparison, transcripts encoding equivalent subunits of cytoplasmic dynein 2, which is specific to intraflagellar transport (Pazour, Dickert et al. 1999), are not detected in axons with any consistency. Of the various cofactors associated with cytoplasmic dynein's activity, the ones whose transcripts were found to be localized to axons in the greatest abundance and with the most reproducibility are Lis1 and the p150^{Glued} and p50/dynamitin subunits of the dynactin complex.

Because of their characterized roles in adapting the dynein motor for different modes of transport (McKenney, Weil et al. 2011), it was decided to focus attention on Lis1 and dynactin's main functional subunit, p150^{Glued}. The chief experimental platform for the studies described here involved culturing primary rat neurons in tripartite microfluidic chambers that allow fluidic isolation of distal axons from cell bodies and dendrites [see **FIGURE 2-A**] (Taylor, Blurton-Jones et al. 2005, Hengst, Deglincerti et al. 2009). To verify the finding that transcripts for Lis1 and p150^{Glued} are localized to axons, single-molecule FISH was utilized to directly visualize the

mRNAs, *Pafah1b1* and *Dctn1* respectively, within axons of embryonic DRG neurons. Both mRNAs were readily detectable in a punctate pattern in axons and with significantly higher intensity than that obtained with a *Gfp* control probe [**FIGURE 3-B**].

b. Neurotrophin signaling regulates axonal levels of mRNAs coding for Lis1 and p150^{Glued}

It was next considered whether changes in NT signaling regulate the axonal localization of *Pafah1b1* and *Dctn1* transcripts, as it has previously been observed, in regenerating adult DRG axons, that NTs can regulate the abundance of specific mRNAs through their anterograde recruitment from the cell body (Willis, van Niekerk et al. 2007). In addition to testing the effects of NGF stimulation (100 ng ml^{-1}) on the axonal abundance of these transcripts, how axons respond to NGF withdrawal (0 ng ml^{-1}) was also examined. A third NGF concentration (5 ng ml^{-1}) was chosen as a baseline against which to compare any changes, as this concentration is low but sufficient to keep embryonic DRG neurons alive in culture. Accordingly, quantitative FISH was performed on axons kept at 5, 0, or 100 ng ml^{-1} NGF for 12 h [**FIGURE 3-B**], allowing enough time for mRNAs to be redistributed and reach a steady-state concentration in axons. Quantification revealed that neither *Pafah1b1* nor *Dctn1* mRNA was recruited in response to stimulation with NGF, but, conversely, NGF deprivation caused a significant increase in axonal Lis1 transcript levels [**FIGURE 3-C**]. The FISH signal was shown to be specific for the targeted mRNAs, given that transfection of axons with siRNAs targeting either transcript reduced the FISH signal to background levels. As an orthogonal approach to confirm the results of the FISH experiments, quantitative real-time RT-PCR was performed on RNA harvested from axonal compartments under the same conditions [**FIGURE 3-D**]. This axonal RT-PCR likewise demonstrated a significant increase in *Pafah1b1* mRNA with NGF

deprivation, and it seemed to indicate, more clearly than FISH, a trend towards an increase in *Dctn1* mRNA also when axons are kept without NGF, though this effect was not significant.

c. Lis1 and p150^{Glued} transcripts undergo differential translational activation in response to NGF stimulation or withdrawal

When axons were transfected with siRNAs targeting *Pafah1b1* or *Dctn1* mRNA, it was discerned that the efficacy of siRNA knockdown varied depending on what concentration of NGF was applied to axons [**FIGURE 3-C**]. In particular, it did not appear that either transcript could be knocked down under the baseline condition of 5 ng ml^{-1} NGF. RISC and the RNAi pathway were previously shown to be functional in developing axons (Hengst, Cox et al. 2006), and it is possible to selectively silence an mRNA in axons via local siRNA transfection without causing any decrease of the transcript's abundance in cell bodies (Hengst, Deglincerti et al. 2009, Baleriola, Walker et al. 2014, Gracias, Shirkey-Son et al. 2014, Baleriola and Hengst 2015). Unpublished observations from several members of the Hengst laboratory have suggested, however, that the susceptibility of axonal transcripts to RNAi may be linked to their accessibility to the translation machinery. According to this premise, the FISH data would imply that *Pafah1b1* mRNA is translated in the case of either NGF stimulation or withdrawal, whereas *Dctn1* mRNA is only translated due to stimulation with NGF. The consequences of this apparent differential translational regulation for Lis1 or p150^{Glued} synthesis within axons will be revisited and explored further in the succeeding chapter.

The possibility that Lis1 transcripts might be translated as a specific response to withdrawal of NGF is curious, considering that translational regulation by NT signaling has, before now, only been described as a result of NT stimulation rather than deprivation (Cox,

Hengst et al. 2008, Hengst, Deglincerti et al. 2009, Andreassi, Zimmermann et al. 2010, Melemedjian, Asiedu et al. 2010, Gracias, Shirkey-Son et al. 2014). This prompted an investigation of translational activity in NGF-deprived axons, which was assayed by performing immunofluorescence against two translation markers, the phosphorylated forms of 4E-BP1 and S6 [**FIGURE 3-E**]. 4E-BP1 functions as a translational repressor by binding to eIF4E, the rate-limiting component of the translational pre-initiation complex, but its phosphorylation forces it to dissociate from eIF4E and thus enables binding of mRNA to ribosomes (Haghighat, Mader et al. 1995). Meanwhile, S6 is a protein component of the 40S ribosomal subunit whose phosphorylation leads to increased translation (Thomas, Siegmann et al. 1980). While total staining for these markers was not significantly changed, phosphorylation of both was significantly increased upon withdrawal of NGF from distal axons within 10 min, a short time frame consistent with induction of translation by a local signaling pathway. It is known that the kinases that phosphorylate 4E-BP1 and S6 are both subject to activation by mTOR (Price, Grove et al. 1992, Hara, Yonezawa et al. 1997), so it was tested whether the application of rapamycin to axons would prevent the phosphorylation of these two proteins upon NGF withdrawal. Indeed, this treatment reversed the observed increases in 4E-BP1 and S6 phosphorlyation, indicating that translational activation upon NGF withdrawal is mTOR-dependent.

d. Association with APC characterizes a distinct pool of axonal Lis1 transcripts

Recently, *Pafah1b1* was found to be part of the interactome of APC, a novel RNA-binding protein (Preitner, Quan et al. 2014). APC is a microtubules plus-end tracking protein, also referred to as a "+TIP" (Nathke, Adams et al. 1996), and, by binding a specific cohort of mRNAs, it is proposed to be a regulatory platform for the localization and translation of

transcripts that are functionally important at the distal end of axonal microtubules. Consequently, it seemed pertinent to investigate whether association with APC was essential to *Pafah1b1* regulation in axons. To address this query, *Pafah1b1*-APC association was targeted using an LNA oligomer designed to bind and sterically block the putative APC binding site, a CUGU-motif in the mRNA's 3'UTR [see **FIGURE 2-B**]. A conceptually identical approach, utilizing a PNA instead of an LNA, was previously shown to be able to obstruct APC's interaction with one of its target mRNAs (Preitner, Quan et al. 2014). A second LNA, binding *Pafah1b1* mRNA 13 bases upstream of the CUGU LNA, was designed for use as a control. The LNAs were transfected into the cell body compartment, and mRNA levels in axons were determined by quantitative FISH 12 h after different NGF treatments (5, 0, or 100 ng ml^{-1}), as before [**FIGURE 3-F**]. The control LNA had no discernible effect when compared to naïve axons (two-way ANOVA p=0.7585), while transfection with the CUGU LNA caused a reduction in axonal *Pafah1b1* levels in all three conditions (p=0.008). The differences in *Pafah1b1* mRNA abundance at 5 and 0 ng ml^{-1} or 0 and 100 ng ml^{-1} NGF were significant in both naïve and CUGU LNA-transfected axons and extremely similar in magnitude (5 and 0 ng ml^{-1}: 0.56 vs. 0.50; 0 and 100 ng ml^{-1}: 0.58 vs. 0.68). These results suggest that interaction with APC may be responsible for the axonal localization of a fixed amount of *Pafah1b1* but that the recruitment of additional *Pafah1b1* into axons in response to NGF deprivation is APC-independent.

	protein	gene	rat DRG (Gumy, 2011)	mouse DRG (Minis, 2014)	rat hippocampal neurons (Baleriola, 2014)
kinesin motors					
kinesin-1 family	Kif5a	*KIF5A*	yes	yes	yes
	Kif5b	*KIF5B*	yes	yes	no
	Kif5c	*KIF5C*	yes	yes	yes
	Klc1	*KLC1*	no	yes	yes
	Klc2	*KLC2*	yes	yes	no
	Klc3	*KLC3*	no	no	NA
	Klc4	*KLC4*	yes	yes	NA
kinesin-2 family	Kif3a	*KIF3A*	yes	yes	no
	Kif3b	*KIF3B*	no	yes	NA
	Kif3c	*KIF3C*	yes	yes	no
kinesin-3 family	Kif1a	*KIF1A*	yes	yes	yes
	Kif1b	*KIF1B*	yes	yes	no
	Kif1c	*KIF1C*	NA	yes	no
dynein motors					
dynein 1 (cytoplasmic)	HC	*DYNC1H1*	yes	yes	yes
	TCTex1	*DYNLT1*	yes	no	no
		DYNLT3	yes	no	no
	LC8	*DYNLL1*	yes	yes	NA
		DYNLL2	yes	yes	yes
	LC7/Roadblock	*DYNLRB1*	yes	yes	no
		DYNLRB2	no	no	no
	IC	*DYNC1I1*	yes	no	yes
		DYNC1I2	yes	yes	yes
	LIC	*DYNC1LI1*	yes	yes	no
		DYNC1LI2	yes	yes	yes
dynein 2 (intraflagellar)	HC	*DYNC2H1*	no	no	NA
	LIC	*DYNC2LI1*	no	yes	no
dynein cofactors					
dynactin	p150^{Glued}	*DCTN1*	yes	yes	yes
	ARP1	*ACTR1B*	yes	yes	no
	ARP11	*ACTR3B*	no	no	no
	p50/dynamitin	*DCTN2*	yes	yes	yes
	p24	*DCTN3*	yes	yes	no
	p62	*DCTN4*	no	yes	yes
	p25	*DCTN5*	yes	yes	no
	p27	*DCTN6*	yes	yes	no
CLIP170	CLIP170	*CLIP1*	no	yes	yes
Lis1/NudE/NudEL complex	Lis1	*PAFAH1B1*	yes	yes	yes
	NudE	*NDE1*	no	yes	no
	NudEL	*NDEL1*	no	yes	NA
Bicaudal D homologues	Bicaudal D1	*BICD1*	no	no	yes
	Bicaudal D2	*BICD2*	no	yes	NA
RZZ complex	ROD	*KNTC1*	no	no	no
	ZW10	*ZW10*	no	no	no
	Zwilch	*ZWILCH*	no	no	NA
	Spindly	*SPDL1*	no	NA	no

FIGURE 3-A: Transcripts encoding microtubule motors as well as their regulatory cofactors have been found in transcriptomes derived from embryonic rat DRG axons using microarray, embryonic mouse DRG axons using RNA sequencing, and embryonic rat hippocampal axons using RNA sequencing. Transcripts seen to be localized in all three studies are highlighted in red; Lis1 and p150^{Glued}, selected for further investigation here, are enclosed in blue.

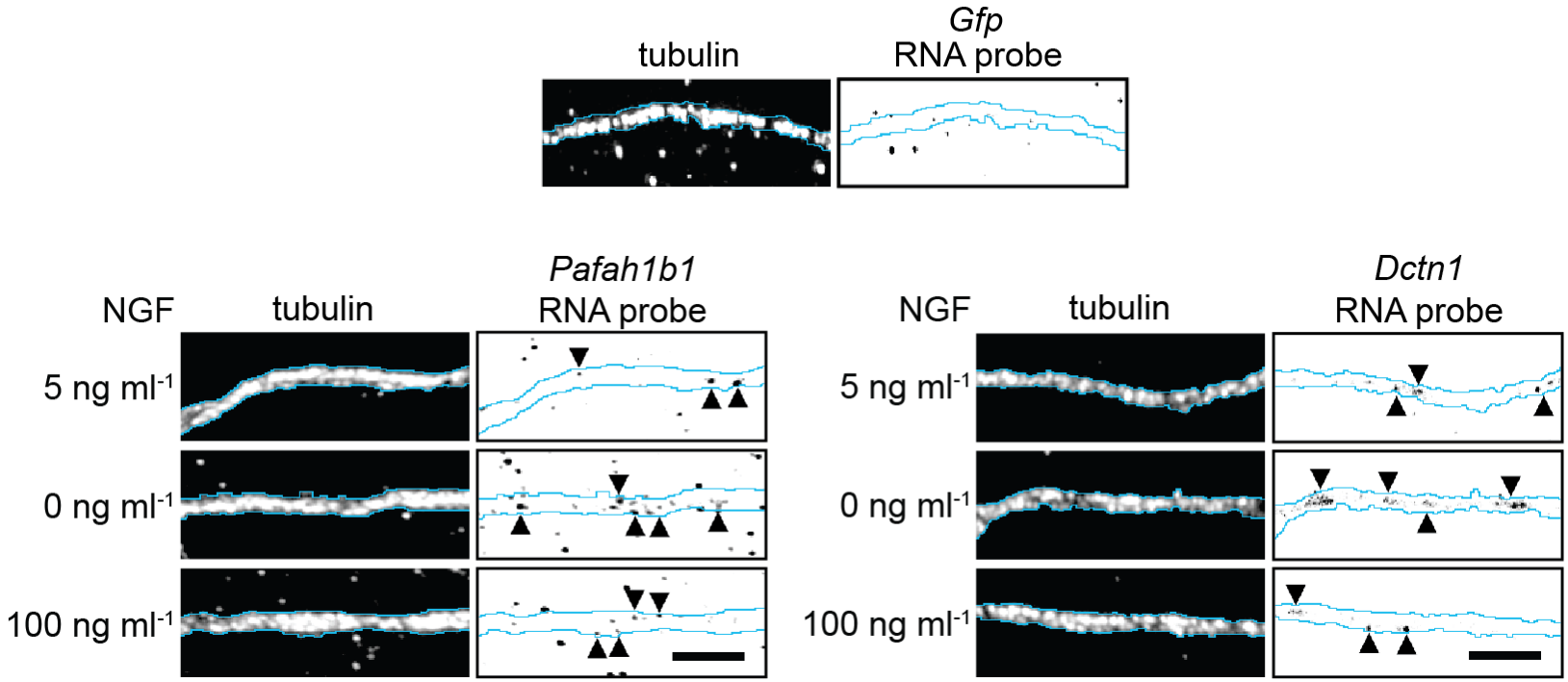

FIGURE 3-B: After growing DRG neurons in microfluidic chambers for 3 DIV, the NGF concentration in the axonal compartments was changed to 5 ng ml^{-1}, and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1* or *Dctn1*. 24 h after transfection, axons were treated with 0, 5, or 100 ng ml^{-1} NGF for 12 h, and axonal *Pafah1b1* or *Dctn1* mRNA levels were determined by FISH. Background fluorescence was determined using a *Gfp* probe. Scale bars, 5 μm.

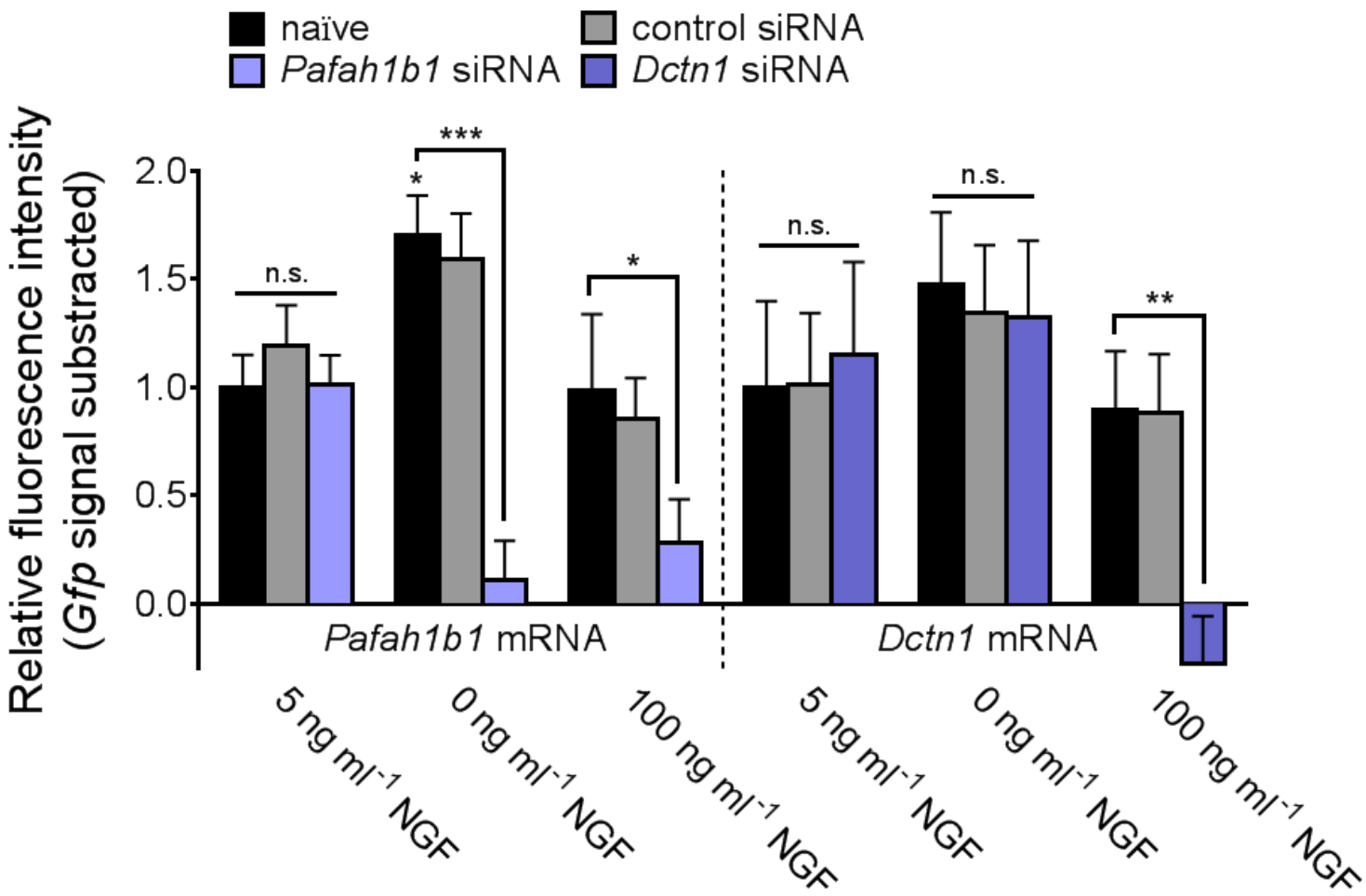

FIGURE 3-C: Quantification of FISH for *Pafah1b1* and *Dctn1* mRNA in axons [see **FIGURE 3-B**]. Background signal from using a *Gfp* probe was subtracted, and all bars were plotted relative to the first black bar in their group (*i.e.* the 5 ng ml^{-1} naïve condition for either *Pafah1b1* or *Dctn1* mRNA), which was defined as 1.0. Data represent the means ± SEM of 15-25 optical fields per condition (n = 3-5 biological replicates). *p≤0.05; **p≤0.01; ***p≤0.001. Two-way ANOVA.

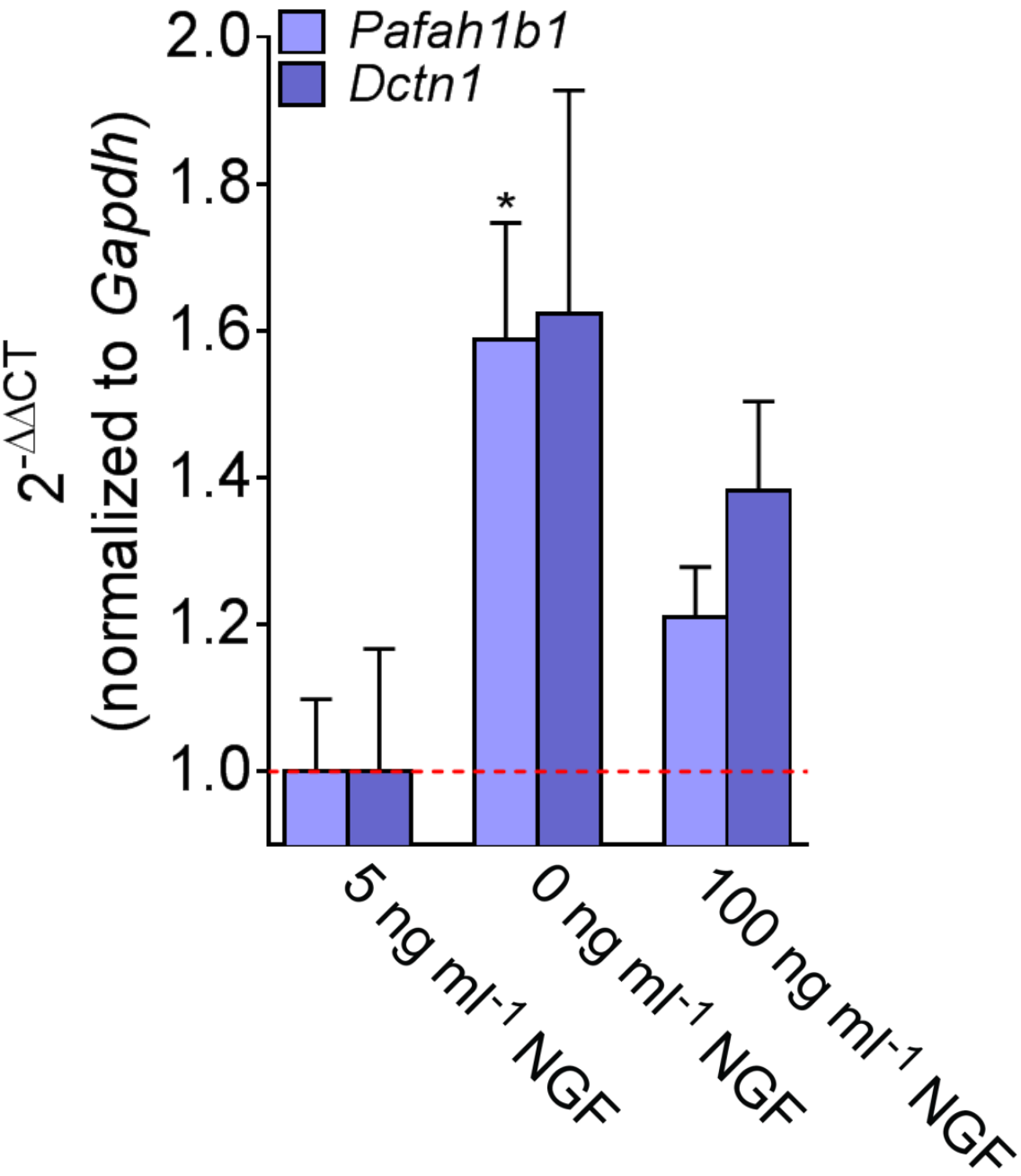

FIGURE 3-D: DRG neurons were cultured in microfluidic chambers for 3 DIV, at which time the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}. 24 h later, axons were treated with 0, 5, or 100 ng ml^{-1} NGF for 12 h, and then axonal RNA was harvested. *Pafah1b1* and *Dctn1* levels were determined by quantitative real time RT-PCR. Relative quantification with *Gapdh* as reference was done using the $2^{-\Delta\Delta CT}$ method. The means of the 5 ng ml^{-1} NGF conditions for *Pafah1b1* and *Dctn1* were defined as 1.0. Data represent the means ± SEM of 3-5 biological replicates. *$p \leq 0.05$. Kruskal-Wallis test with Dunn's multiple comparison test.

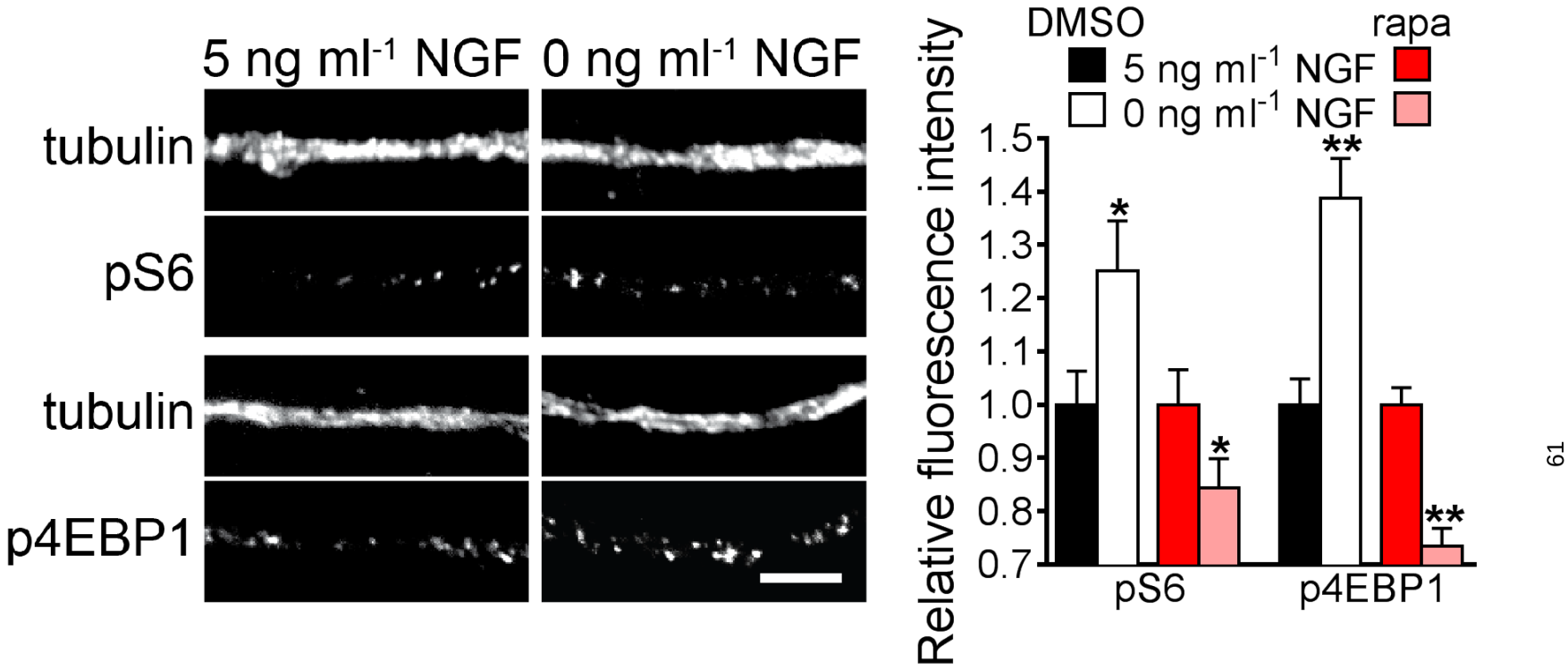

FIGURE 3-E: After growing DRG neurons in microfluidic chambers for 3 DIV, the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}. On DIV 4, axons were treated with 0 or 5 ng ml^{-1} NGF, with or without rapamycin, for 10 min. Axonal levels of p-S6 and p-4EBP1 were determined by immunofluorescence, and the mean of the 0 ng ml^{-1} NGF condition in each instance is plotted relative to its corresponding 5 ng ml^{-1} NGF condition, which was set as 1.0. Data represent the means ± SEM of 10-15 fields per conditions (n = 2-3 biological replicates). *p≤0.05; **p≤0.01. Two-tailed Mann-Whitney *U* test. Scale bar, 5 µm.

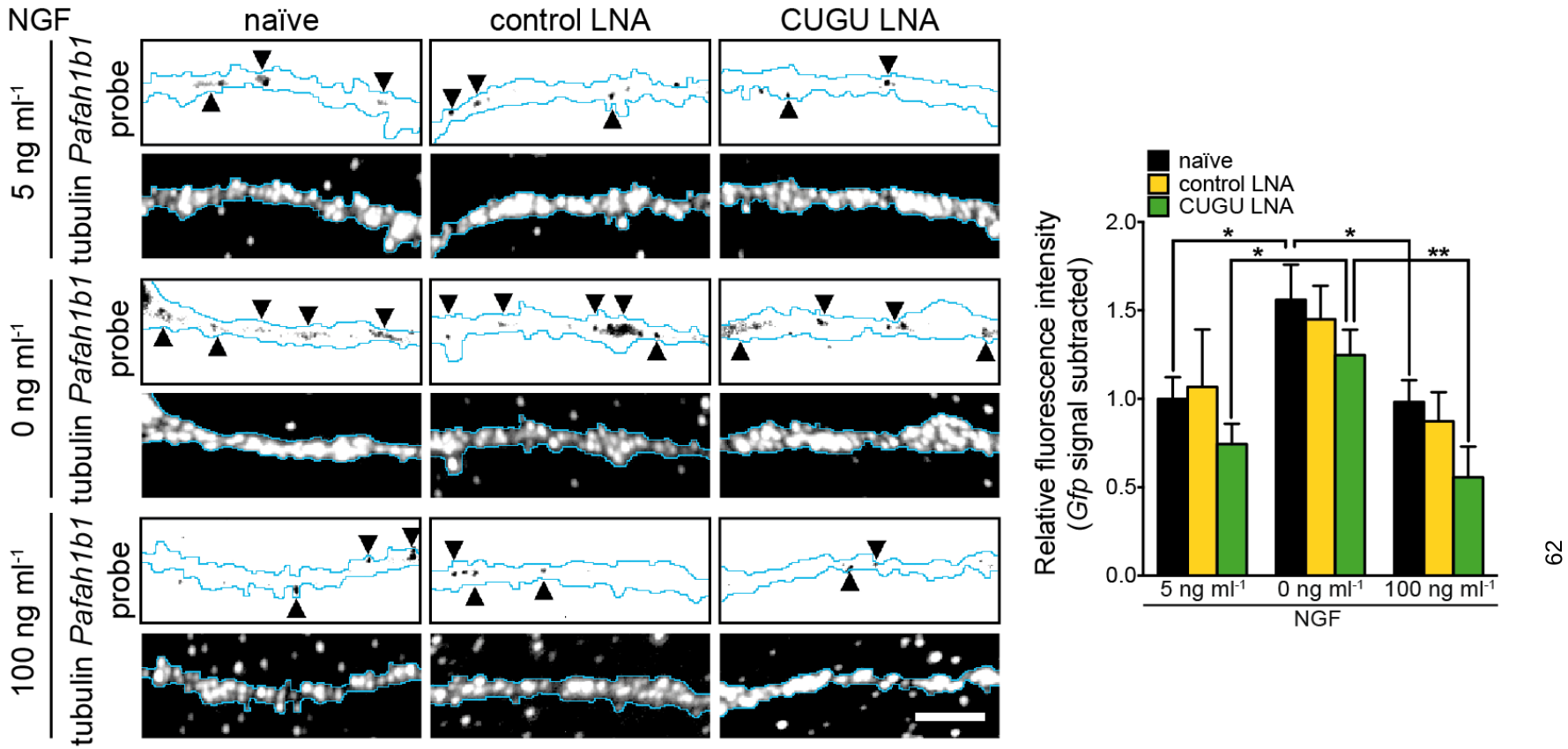

FIGURE 3-F: On DIV 3 of culturing DRG neurons in microfluidic chambers, the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}, and cell bodies were selectively transfected with the control or CUGU LNAs. 24 h after transfection, axons were treated with 0, 5, or 100 ng ml^{-1} NGF for 12 h, and axonal *Pafah1b1* mRNA levels were evaluated by FISH. Background fluorescence was determined using a *Gfp* probe and subtracted. Data represent the means ± SEM of 15 optical fields per condition (n = 3 biological replicates). *p≤0.05. Two-way ANOVA. Scale bar, 5 µm.

III.3. Interpretation

The fact that developing axons seem to be conspicuously enriched for mRNAs encoding microtubule motors and their associated proteins has been noticed before (Gumy, Yeo et al. 2011), although, as of yet, no one has put forward any hypotheses or additional data as to why this might be. The fact that several kinesin transcripts are localized to axons is not particularly surprising given the multitude of different isoforms, and, while their localization is certainly interesting, it does not appear likely that investigating their presence would lead to any real conceptual breakthrough. On the other hand, the potential local translation of mRNAs coding for cytoplasmic dynein and its cofactors opens up physiologic and regulatory possibilities that previously could not be conceived. According to our current understanding, in order to function as a minus end-directed motor in axons dynein must, itself, be transported into the periphery as a cargo by kinesins (Ligon, Tokito et al. 2004). Of course, this arrangement poses several problems, chief among which is the issue of how the cell can control, at great distances from the soma, dynein's switch from a cargo to an active motor when it is needed. Given the presence of transcripts for essentially all of dynein's major components in the available axonal transcriptomes, one plausible solution, perhaps, is that the entire dynein motor could be locally produced *de novo* as needed. However, this approach would lead to another difficulty, namely the issue of stoichiometry and ensuring that all the necessary subunits are produced in equal proportions at the same time and place in order to assemble functional motor complexes. Moreover, newly synthesized dynein would somehow have to be recruited to the cargo in need of transport. Admittedly, given the availability of the various dynein transcripts, it is a distinct possibility that the axon does have mechanisms to provide for stoichiometric assembly of dynein complexes and their linkage to cargo. One would be hard-pressed, otherwise, to explain why

mRNAs encoding dynein subunits are localized to axons at all, if not to be locally translated. By comparison, though, the prospect of axonal synthesis of dynein's cofactors from localized transcripts does not necessitate as many contingent factors. New Lis1 or p150^{Glued}, made in axons as a response to a given stimulus, could be produced alongside new motors, or they could act as the triggers needed to convert preexisting, inactive dynein complexes into motors ready for transport.

Indeed, in essentially all reported instances, localized translation has been seen to be stimulus-dependent. The finding that NGF withdrawal triggers translational activation within 10 min leads to the question as to how the absence of a ligand can be a stimulus for downstream signaling. The extremely short time needed to induce translation rules out that it might be a consequence of neuronal degeneration caused by the lack of trophic support. Rather, translation appears to be triggered by a signaling pathway that is active in the absence of NGF and suppressed by NGF binding to TrkA. As noted previously, TrkA has been proposed to act as a dependence receptor, generating different signals in the absence of NTs as compared to when it binds ligand (Nikoletopoulou, Lickert et al. 2010, Dekkers, Nikoletopoulou et al. 2013). The same hypothesis might explain why persistent recruitment of *Pafah1b1* and *Dctn1* mRNA to axons is seen only in the NGF deprivation condition. It is possible that Trk receptor activation results in immediate downstream signaling, but, with time, the cell adjusts to a new set point and any effects of NT surplus no longer endure. Surveillance of axonal mRNA 12 h after NGF stimulation, as performed here, might not catch an earlier surge in *Pafah1b1* or *Dctn1* transcripts that has, by the time of observation, faded away. In contrast, an immature DRG neuron cannot simply adjust or become tolerant to starvation from NGF, especially if, as the dependence receptor model implies, TrkA signaling occurs chiefly in just such an absence of ligand. This

study, therefore, lends additional support to the dependence receptor hypothesis of TrkA function, and, further, an experimental paradigm is provided here with which to dissect the underlying pathway downstream of this receptor.

The effects of LNA treatment blocking the putative *Pafah1b1* APC binding site, including the finding that APC might bind a specific subset of axonal *Pafah1b1* mRNAs, adds depth to previous observations of APC-dependent transcript localization. In the study initially identifying APC's function as an RBP, a similar intervention using a nucleic acid analogue to hinder APC binding to β2B-tubulin transcripts was seen to dramatically reduce that mRNA's localization to axons (Preitner, Quan et al. 2014). Some mRNA coding for β2B-tubulin continued to be detectable in axons, but this easily could have been attributed to incomplete efficacy of the PNA oligomer at blocking APC association. This previous study did not use any stimulus conditions in an attempt to regulate β2B-tubulin transcript recruitment to axons, however. As such, the evidence that a subtotal but defined amount of Lis1 transcripts is prevented from entering axons by disrupting their interaction with APC would indicate that the regulation of axonal mRNA localization is multifactorial. That is, APC is likely not the exclusive determinant of localization, even for its target mRNA species.

CHAPTER IV. LOCAL SYNTHESIS OF DYNEIN COFACTORS MATCHES RETROGRADE TRANSPORT TO ACUTELY CHANGING DEMANDS

IV.1. Rationale and Summary

Intra-axonal protein synthesis is crucial for axon development (Campbell and Holt 2001, Wu, Hengst et al. 2005, Hengst, Deglincerti et al. 2009, Gracias, Shirkey-Son et al. 2014), maintenance (Yoon, Jung et al. 2012), synapse formation (Taylor, Wu et al. 2013), and axon-to-soma communication (Cox, Hengst et al. 2008), as well as for axonal regeneration (Rishal and Fainzilber 2014) and neurodegeneration (Baleriola, Walker et al. 2014). Axonal synthesis of Lis1 itself has been demonstrated previously, but only in the context of retrograde transport of viral particles during neurotropic infection (Koyuncu, Perlman et al. 2013). From these investigations, a picture emerges in which local protein synthesis provides short-lived and spatially precise bursts of acutely needed proteins, in order to react to extracellular cues, injurious insults, or other changes in the axon's environment.

These studies sought to ascertain whether local synthesis of dynein cofactors could be a mechanism to acutely match axonal retrograde transport capabilities to changes in demand, as, for example, in response to changes in extracellular trophic support. As a result, it was discovered that intra-axonal synthesis of Lis1 and $p150^{Glued}$ is required for the adjustment of retrograde transport to acute changes in NT signaling in the periphery of neurons.

IV.2. Results

a. NGF signaling regulates retrograde transport in a protein synthesis-dependent manner

To investigate whether changes in axoplasmic transport are mediated through localized protein synthesis, embryonic rat DRG neurons were grown in microfluidic devices, and the effect of NGF stimulation or withdrawal on the transport of vesicular cargoes was assessed through live-imaging. Isolated axons were exposed to different NGF concentrations (5, 0, or 100 ng ml^{-1}) along with LysoTracker, a cell-permeable dye labeling late endosomes and lysosomes, in the presence or absence of pharmacologic inhibitors of protein synthesis, and the directional motility of LysoTracker-positive vesicles was appraised [**FIGURE 4-A**]. With either NGF stimulation or withdrawal, retrograde transport of large, LysoTracker-positive vesicles was significantly increased within 15 min, and a corresponding decrease was noted in the proportion of stationary vesicles [**FIGURE 4-B**]. The percentages of anterogradely or bidirectionally moving particles were not significantly changed with either NGF concentration. Inhibition of protein synthesis, however, completely abolished the increases in retrograde transport and significantly reduced anterograde transport upon NGF stimulation as well as NGF withdrawal. These results establish that axonal stimulation with NGF or withdrawal of NGF from axons both increase retrograde transport of large vesicular cargoes in a protein synthesis-dependent manner.

b. Lis1 and $p150^{Glued}$ are locally synthesized but differentially regulated in response to changes in axonal NGF signaling

The presence of Lis1 and $p150^{Glued}$ transcripts in axons raised the possibility that some of the observed changes in axoplasmic transport could be mediated through local synthesis of these

cofactors. As a first step, it was necessary to prove that newly made Lis1 and p150^{Glued} protein is synthesized in axons prior to or contemporaneous with the rapidly occurring changes in transport. Using quantitative immunofluorescence, the axonal abundance of Lis1 protein was found to be significantly increased upon either NGF stimulation or withdrawal for just 10 min [**FIGURE 4-C**], while, in contrast, p150^{Glued} levels were elevated only in response to NGF stimulation [**FIGURE 4-D**]. These findings are consistent with prior inferences about the translational states of *Pafah1b1* or *Dctn1* mRNAs under these conditions, as presented in Chapter III. Levels of each protein were not changed by pre-incubation with protein synthesis inhibitors at the 5 ng ml^{-1} NGF baseline condition, but the increases in their abundance seen upon NGF stimulation (Lis1 and p150^{Glued}) or withdrawal (Lis1 only) were eliminated when anisomycin or emetine were applied to the axonal compartment.

As a more specific intervention to test whether local synthesis of Lis1 and p150^{Glued} is triggered by changes in axonal NGF signaling, axons were selectively transfected with siRNAs against the *Pafah1b1* or *Dctn1* mRNAs, or with a non-targeting control siRNA, prior to subjecting them to the three experimental NGF concentrations. For either protein, no significant reduction in abundance was detected in axons kept at 5 ng ml^{-1} NGF, once more indicating that, under this condition, the transcripts are not locally translated to produce protein [**FIGURES 4-E & 4-F**]. Conversely, the significant increases in Lis1 abundance seen to result from NGF stimulation or withdrawal were totally abolished by local siRNA application, as was any increase in p150^{Glued} levels in NGF-stimulated axons. Taken together, these results demonstrate that, under certain conditions, axons have the ability to produce either Lis1 or p150^{Glued}, but local synthesis of these two dynein cofactors is differentially regulated by changes in NGF signaling.

c. Locally produced Lis1 is required for NGF-induced retrograde transport of large cargoes

To determine whether the local synthesis of Lis1 and p150^{Glued} induced by changes in NGF signaling was capable of altering retrograde transport in axons, axons were incubated with LysoTracker, and the motility of labeled vesicles was scored, as described previously. Axonal knockdown of *Pafah1b1* or *Dctn1* did not significantly affect retrograde transport in the 5 ng ml^{-1} NGF condition [**FIGURES 4-G & 4-H**], in line with the finding that neither protein is locally synthesized at this baseline condition. Axon-specific knockdown of *Pafah1b1* abolished the significant increase in retrogradely moving LysoTracker-positive particles instigated by NGF withdrawal, while, in the NGF-stimulated condition, knockdown caused a reduction in the proportion of retrograde cargoes below even the baseline levels. In contrast, knockdown of axonal *Dctn1* mRNA did not affect the movement of these large vesicular cargoes upon either NGF stimulation or withdrawal. These results establish that locally synthesized Lis1 is required for induced retrograde movement of large axonal cargoes, but p150^{Glued} is not. This conclusion is reminiscent of the finding that, with regard to their global synthesis, Lis1, but not p150^{Glued}, is essential for high-load retrograde transport (Yi, Ori-McKenney et al. 2011).

d. Transport of NGF-signaling endosomes requires local synthesis of Lis1 and p150^{Glued}

To investigate whether the requirement for local synthesis of dynein cofactor varied between differently sized cargoes, the retrograde transport of smaller NGF-containing signaling endosomes was also visualized (Delcroix, Valletta et al. 2003). Upon binding of NGF to its main functional receptor, TrkA, the receptor-ligand complex is internalized, and the resulting endosome is transported along with other downstream effector complexes to the soma by a dynein-dynactin complex (Zweifel, Kuruvilla et al. 2005). Mouse 2.5S NGF was

conjugated to fluorescent quantum dots (Cui, Wu et al. 2007), selectively applied to axons (100 ng ml^{-1}), and movement of QD-labeled NGF signaling endosomes was measured by live-cell microscopy [**FIGURE 4-I**]. The proportion of retrogradely moving particles seen under naïve and control siRNA conditions (~27%) was consistent with previous studies (Ure and Campenot 1997, Echarte, Bruno et al. 2007). Axon-specific knockdown of *Pafah1b1* or *Dctn1* significantly reduced the retrograde movement of QD-positive particles and increased the proportion of stationary particles. It thus appears that the stimulated transport of different cargoes—particularly those distinguished by size—may require the local synthesis of different regulator or adaptor proteins.

e. Retrograde transport of a death signal in NGF-deprived axons requires local synthesis of Lis1

Retrograde trafficking of NT signaling endosomes from axons to the cell body is widely considered to be required for the survival of neurons dependent upon target-derived neurotrophic support (Ye, Kuruvilla et al. 2003, Zweifel, Kuruvilla et al. 2005). Because of the observed reduction in retrogradely moving, QD-labeled NGF signaling endosomes upon axon-specific knockdown of *Pafah1b1* or *Dctn1* mRNAs [**FIGURE 4-I**], it was conceivable that survival of the DRG neurons might be impaired. To test this, cell death and survival assays were performed where NGF was withheld from the somatic and axonal compartments or applied (100 ng ml^{-1}) to the axonal compartments only. In compartments where NGF was removed, a neutralizing anti-NGF antibody was added to quench any residual NGF activity. Contrary to what was anticipated, axonal knockdown of either *Pafah1b1* or *Dctn1* in the NGF-replete condition did not induce apoptosis, as assessed by counting TUNEL-positive, dysmorphic nuclei, nor reduce the number of living neurons stained by calcein AM [**FIGURES 4-J & 4-K**]. Moreover, in the

NGF-starved condition, knockdown of *Pafah1b1* had the unexpected effect of completely preventing NGF deprivation-induced cell death. Attempted knockdown of *Dctn1* in the NGF deprivation condition did not impact cell death, consistent with the finding that NGF withdrawal does not actually lead to local synthesis of $p150^{Glued}$ [see **FIGURES 4-D & 4-F**].

Remarkably, it thus appears that, although inhibition of local Lis1 and $p150^{Glued}$ synthesis greatly impairs retrograde transport of NGF-containing signaling endosomes, their local production is not required for NGF-dependent survival. Instead, these findings are in support of an alternative explanation for the pro-survival activity of NGF, wherein it inhibits an axon-derived, retrograde pro-apoptotic signal of as-yet-undetermined identity (Mok, Lund et al. 2009, Perlson, Maday et al. 2010). Furthermore, the results of the cell death and survival assays establish that intra-axonal synthesis of Lis1 is required for the generation or propagation of this death signal.

f. Retrogradely transported GSK3β is the death signal triggered by axonal NGF withdrawal

The known requirement of Lis1 for the transport of large cargoes with greater drag forces (Yi, Ori-McKenney et al. 2011) led us to ask whether the elusive retrograde death signal was of organellar size, perhaps a type of vesicle derived from endocytosis. To test this hypothesis, the dynamin inhibitor dynasore was used to prevent endocytosis (Macia, Ehrlich et al. 2006). Application of dynasore specifically to axons completely inhibited cell death due to NGF deprivation, but it did not increase cell death in the NGF-replete condition [**FIGURE 4-L**]. For further characterization of the retrograde death pathway, attention was turned to protein kinases that have been implicated in apoptotic cell death in neurons. Whole-cell treatment with inhibitors of MLKs or p38 MAPKs has previously been shown to prevent neuronal apoptosis

induced by NT deprivation (Kummer, Rao et al. 1997, Harris, Deshmukh et al. 2002), but, in this experimental paradigm, application of these inhibitors to axons alone did not have the same effect, suggesting that these kinases act centrally rather than in the periphery. When two GSK3 inhibitors, LiCl or SB216763 (Stambolic, Ruel et al. 1996, Coghlan, Culbert et al. 2000), were applied selectively to axons, however, the induction of apoptosis with NGF deprivation was completely prevented, while neither inhibitor had any effect on cell death under NGF-replete conditions [**FIGURE 4-L**]. GSK3β previously had been proposed as a carrier of an axonally generated apoptotic signal (Mok, Lund et al. 2009). Jointly, these results indicate that the death signal, whose transport requires local Lis1 production, is likely an endocytic structure containing active GSK3β, such as an MVB (Dobrowolski and De Robertis 2012).

g. Locally translated Lis1 transcripts from APC-dependent or APC-independent pools fulfill distinct functions

The finding that a single mRNA species, *Pafah1b1*, is locally translated in response to both NGF stimulation and withdrawal, and further—as we saw in the preceding chapter—is recruited into axons only upon NGF deprivation but not stimulation, strongly suggested the existence of regulatory mechanisms that control *Pafah1b1* localization and translation under different signaling conditions. This raised the question of how interference with *Pafah1b1*-APC binding using the specially designed LNA oligomer would affect Lis1 protein abundance in axons, considering its previously seen effects at the level of transcript localization. To this end, cultured DRG neurons were transfected with the LNAs, as before, and quantitative immunofluorescence against Lis1 was performed after exposing the axons to different NGF concentrations [**FIGURE 4-M**]. No significant change in Lis1 abundance was detected at 5 ng

ml^{-1} NGF, again confirming that it is not locally synthesized under this condition [see **FIGURES 4-C & 4-E**]. The increase in axonal Lis1 abundance in CUGU LNA transfected DRGs at 0 ng ml^{-1} was indistinguishable from naïve and control LNA neurons, while, in NGF-stimulated axons, transfection with the CUGU LNA prevented the increase in Lis1 levels. Finally, the requirement of *Pafah1b1*-APC association for stimulation-induced retrograde transport of LysoTracker-positive cargos was investigated [**FIGURE 4-N**]. Transfection of the CUGU LNA had no effect on transport at 5 or 0 ng ml^{-1} but completely prevented the increase in retrograde transport triggered by NGF stimulation.

Taken as a whole, the results from the LNA experiments reveal the existence of two distinct modes of *Pafah1b1* localization in axons: one that is constitutively active and APC-dependent, and another that is APC-independent and particularly responsive to NGF deprivation. The transcripts that are localized in the APC-dependent mode are translated in response to stimulation with NGF, while the APC-independent pool is locally translated with NGF withdrawal and is boosted by recruitment into NGF-starved axons. Thus, the two diametrically opposed triggers of axonal Lis1 synthesis, NGF withdrawal and stimulation, act on two separate pools of *Pafah1b1* mRNA that each are solely responsible for the increase in local Lis1 levels under either condition.

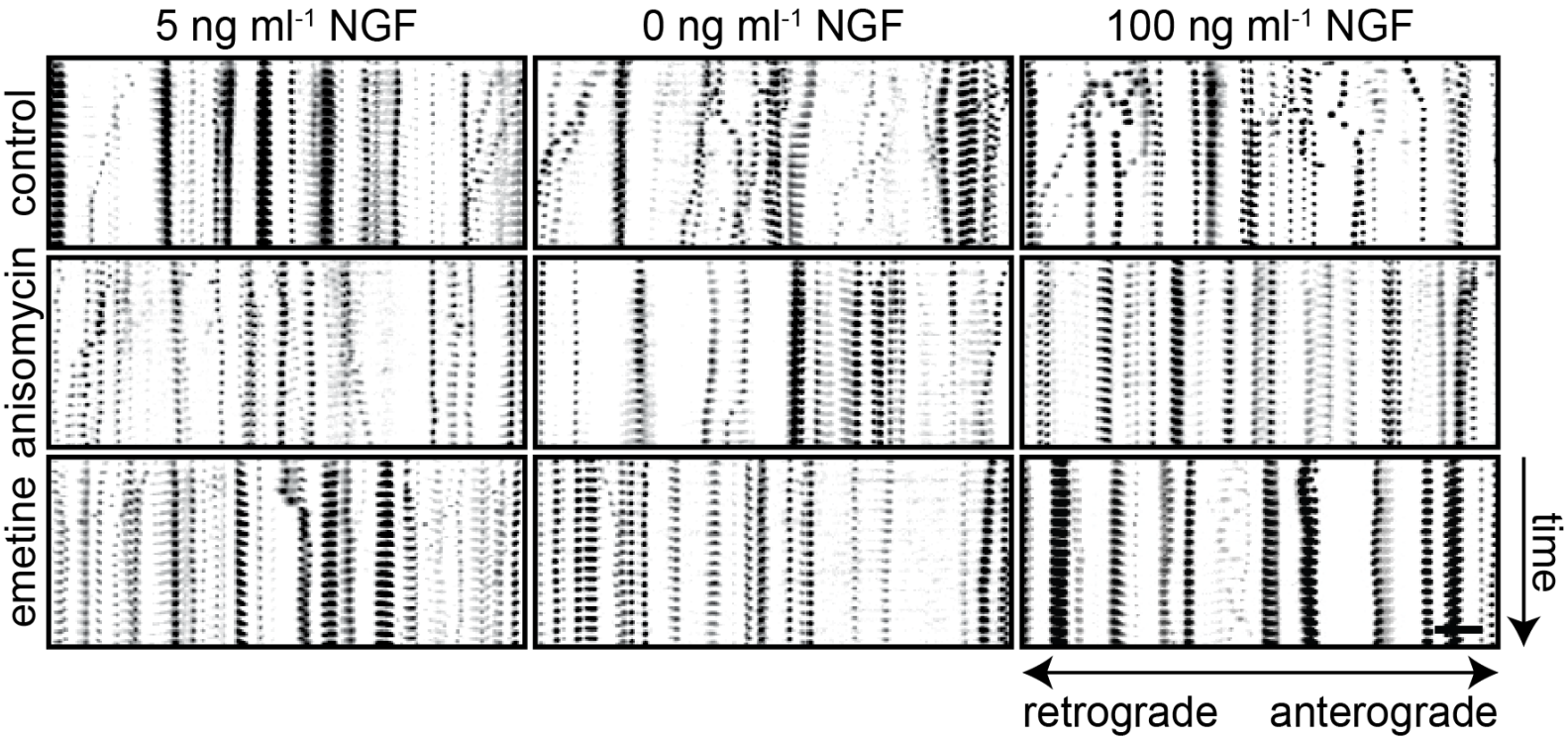

FIGURE 4-A: DRG neurons were cultured in microfluidic chambers for 3 DIV, at which point the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1} for 24 h. On DIV 4, axons were pretreated with protein synthesis inhibitors (anisomycin, emetine) or vehicle (DMSO) for 2 h before addition of 0, 5, or 100 ng ml^{-1} NGF with LysoTracker Green for 15 min. Live-imaging time-lapse series of axonal fields were acquired, with images being taken every 13 s for 4 min. Kymographs of representative 100-μm-long axonal segments are shown. Scale bar, 10 μm.

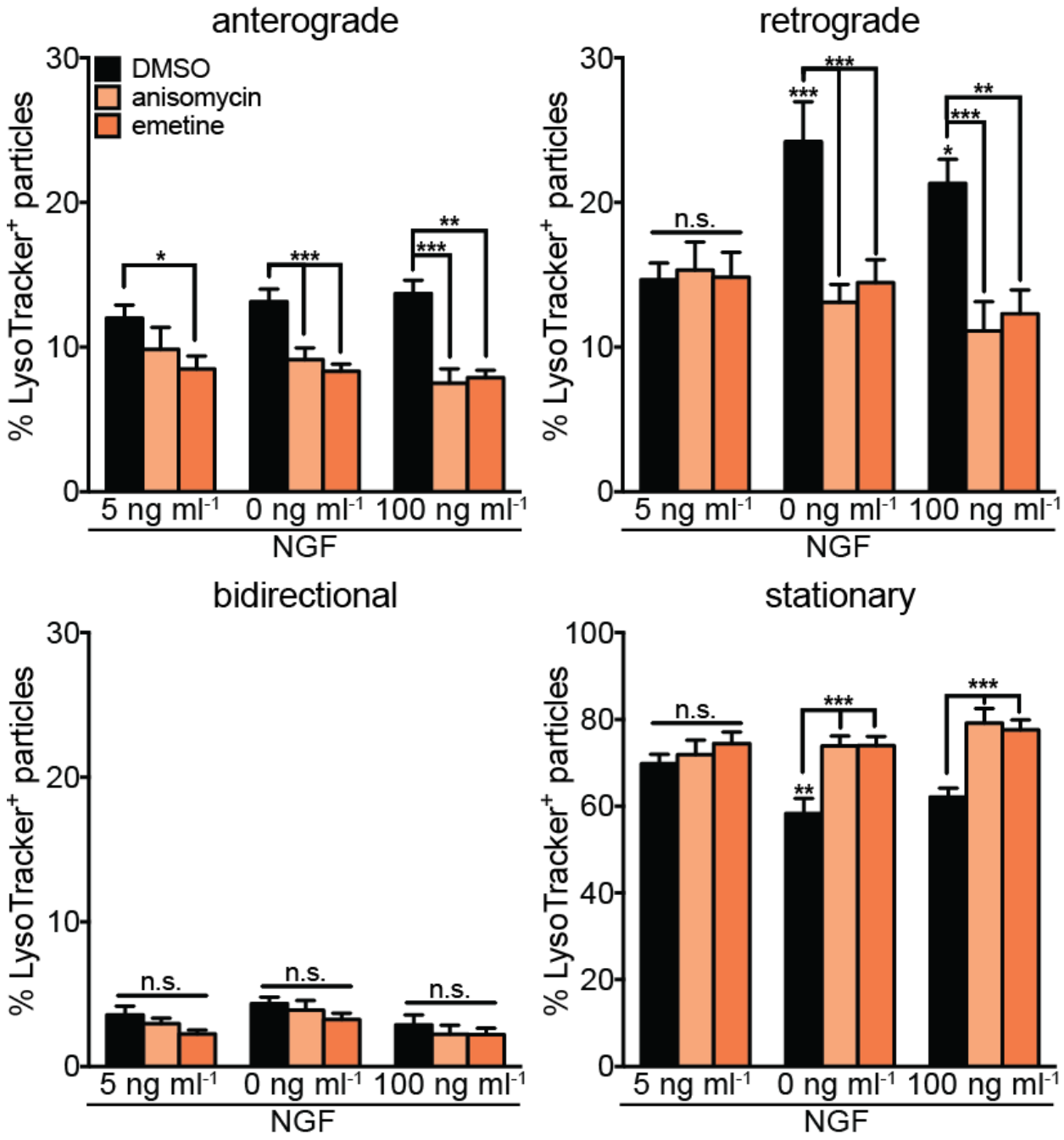

FIGURE 4-B: Quantification of vesicular motility analysis in axons treated with protein synthesis inhibitors [see **FIGURE 4-A**]. LysoTracker-positive particles with diameters ≥1 μm were scored as anterograde, retrograde, bidirectional, or stationary. Data represent the means ± SEM of 9 fields per conditions (n = 3 biological replicates). *p≤0.05; **p≤0.01; ***p≤0.001. Two-way ANOVA with Dunnett's multiple comparison test.

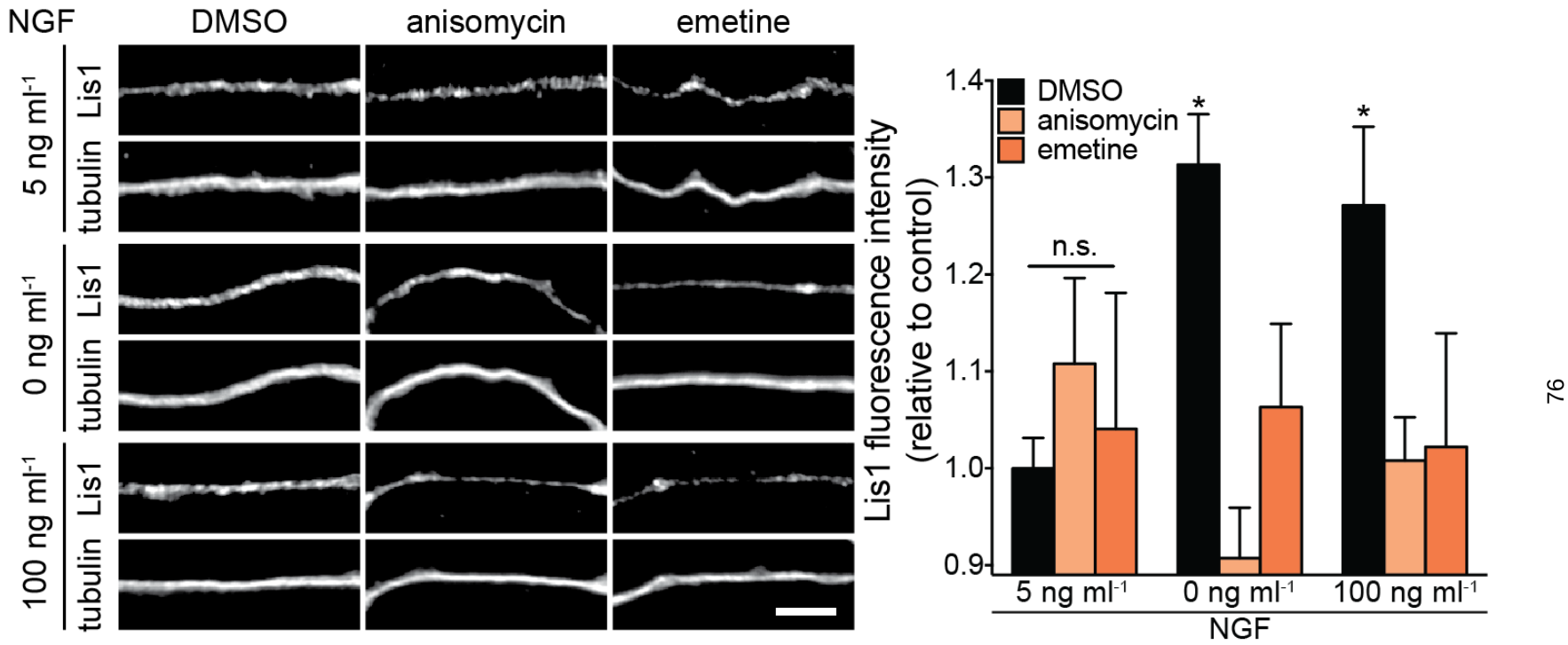

FIGURE 4-C: After growing DRG neurons in microfluidic chambers for 3 DIV, the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1} for 24 h. On DIV 4, axons were pretreated with protein synthesis inhibitors (anisomycin, emetine) or vehicle for 2 h, followed by exposure to different concentrations of NGF (0, 5, or 100 ng ml^{-1}) for 10 min. Axonal Lis1 levels were measured by quantitative immunofluorescence. Data represent the means ± SEM of 15-20 optical fields per conditions (n = 3-4 biological replicates). *$p \leq 0.05$. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 5 µm.

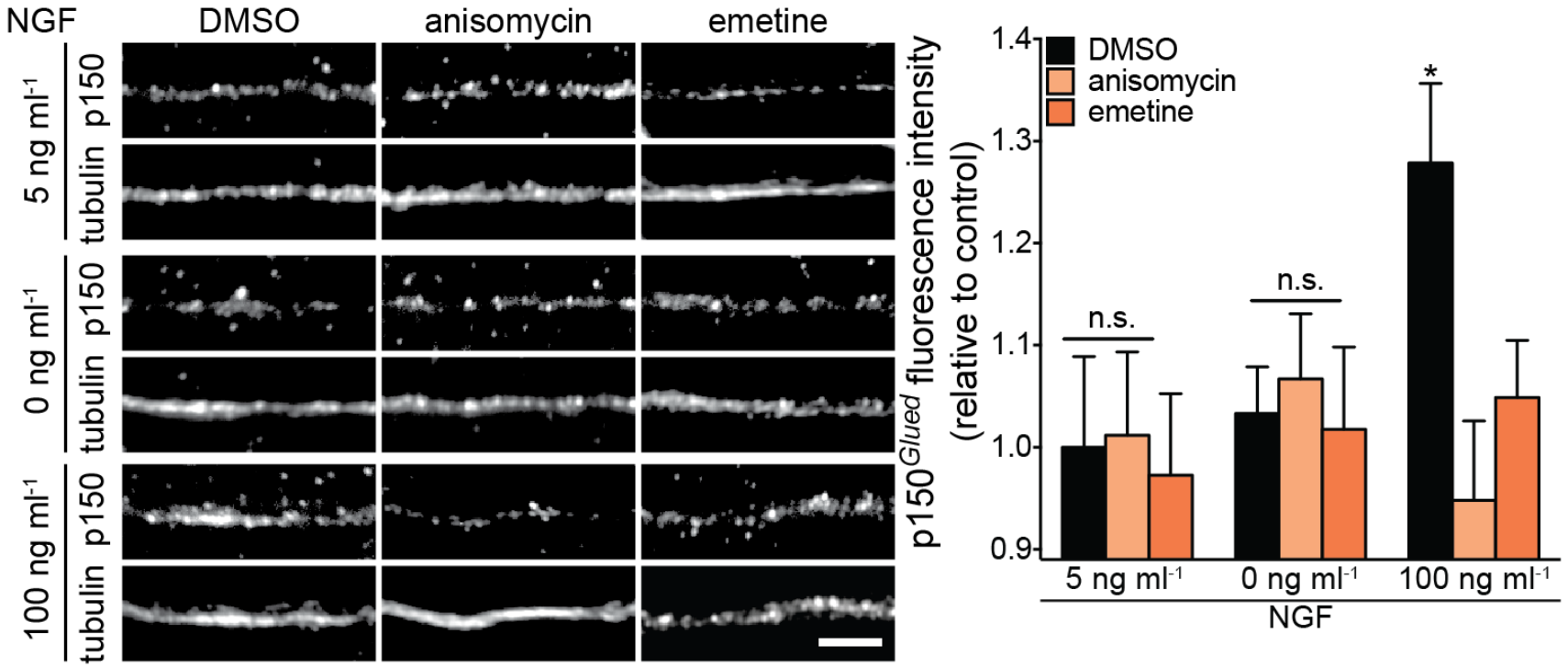

FIGURE 4-D: DRG neurons were cultured and treated with protein synthesis inhibitors (anisomycin, emetine) as in **FIGURE 4-C**. Axonal $p150^{Glued}$ levels were measured by quantitative immunofluorescence. Data represent the means ± SEM of 15 optical fields per conditions (n = 3 biological replicates). *p≤0.05. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 5 µm.

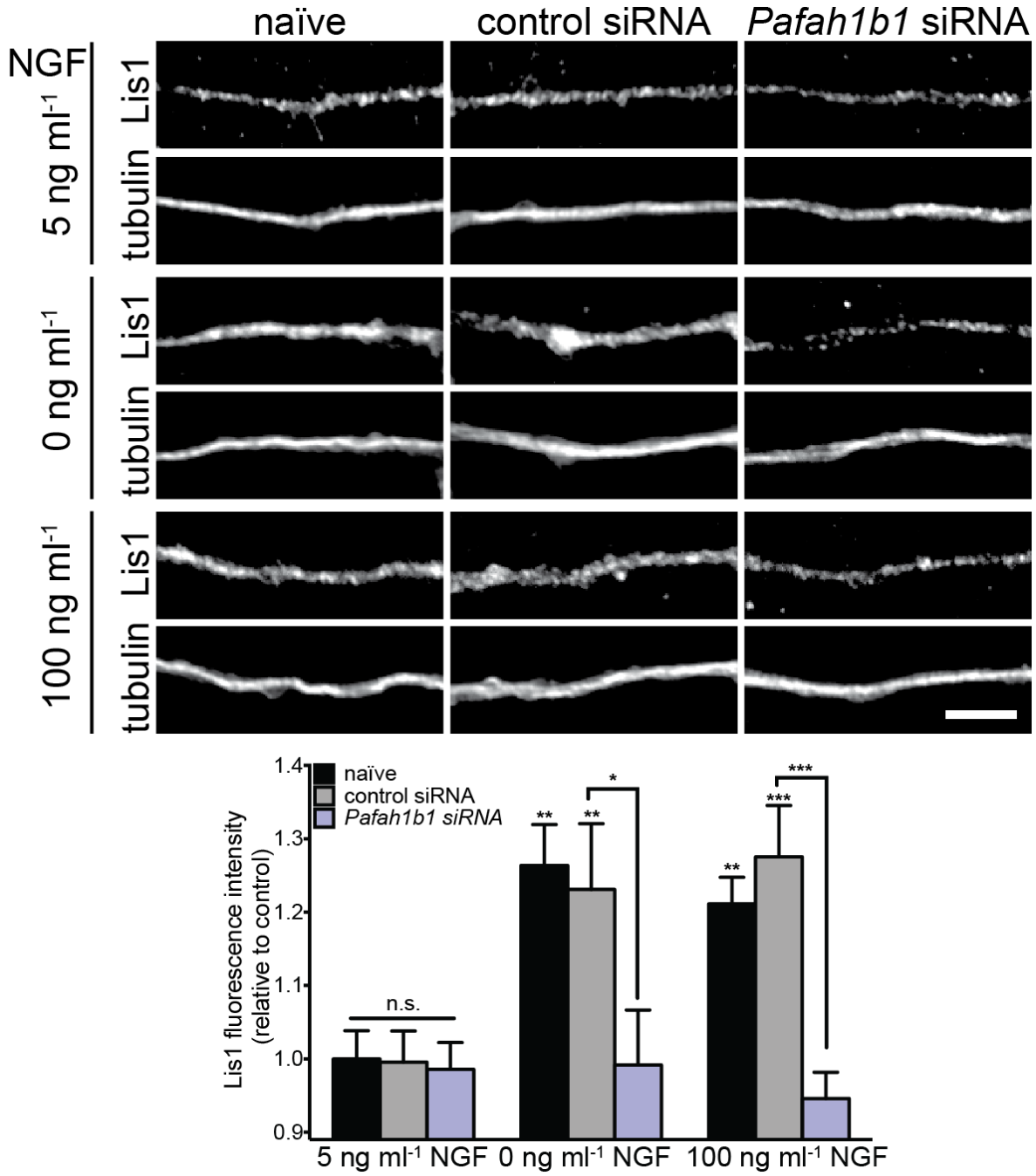

FIGURE 4-E: DRG neurons were cultured in microfluidic chambers for 3 DIV, at which point the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}, and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1*. 24 h after transfection, axons were treated with 0, 5, or 100 ng ml^{-1} NGF for 10 min, and axonal Lis1 levels were determined by immunofluorescence. Data represent the means ± SEM of 20-75 optical fields per conditions (n = 4-15 biological replicates). *p≤0.05; **p≤0.01; ***p≤0.001. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 5 µm.

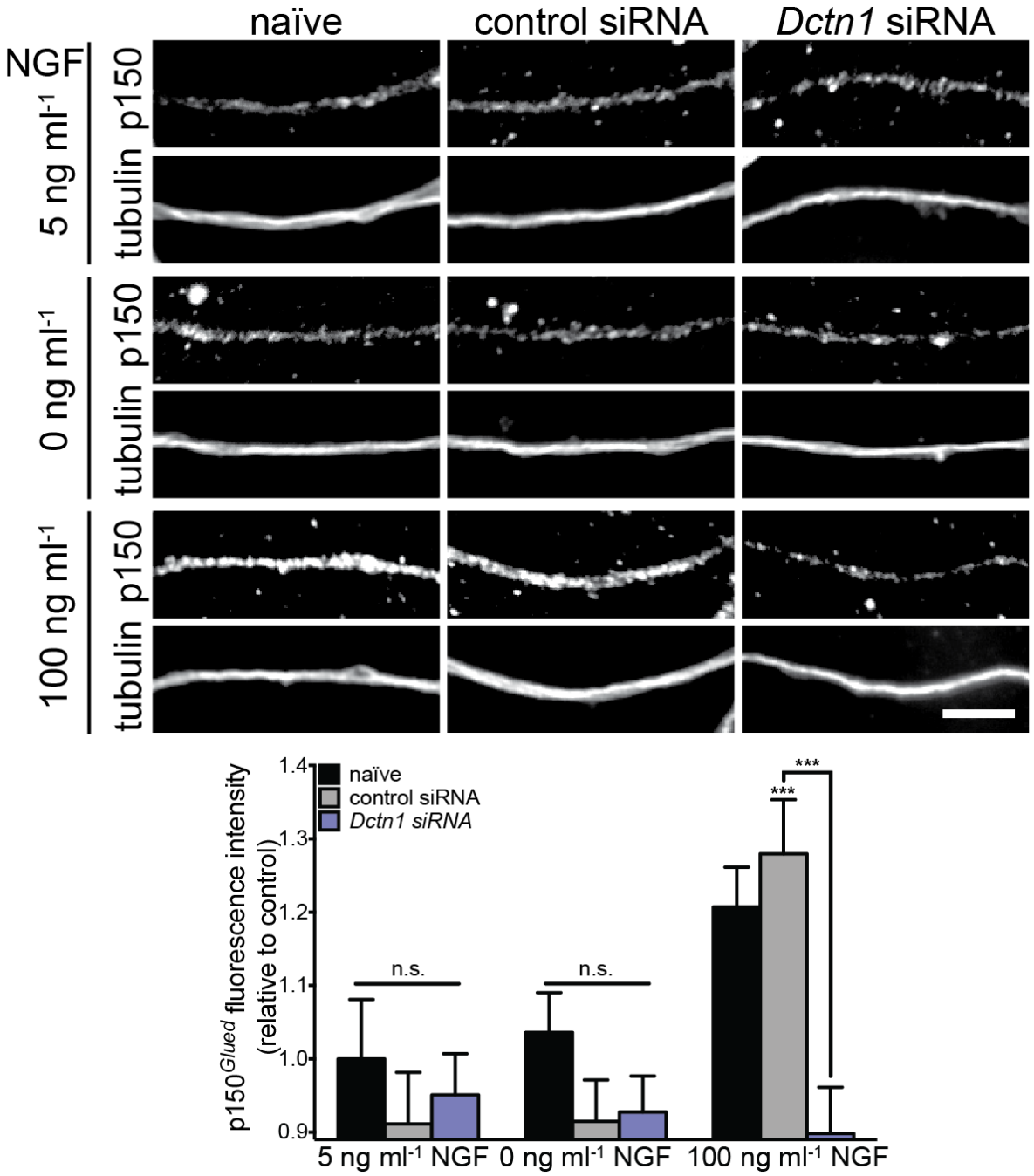

FIGURE 4-F: DRG neurons were cultured and prepared for transfection as in **FIGURE 4-E**, and, on DIV 3, axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Dctn1*. 24 h after transfection, axons were treated with 0, 5, or 100 ng ml^{-1} NGF for 10 min, and axonal $p150^{Glued}$ levels were determined by immunofluorescence. Data represent the means ± SEM of 20-40 optical fields per conditions (n = 4-8 biological replicates). ***$p \leq 0.001$. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 5 μm.

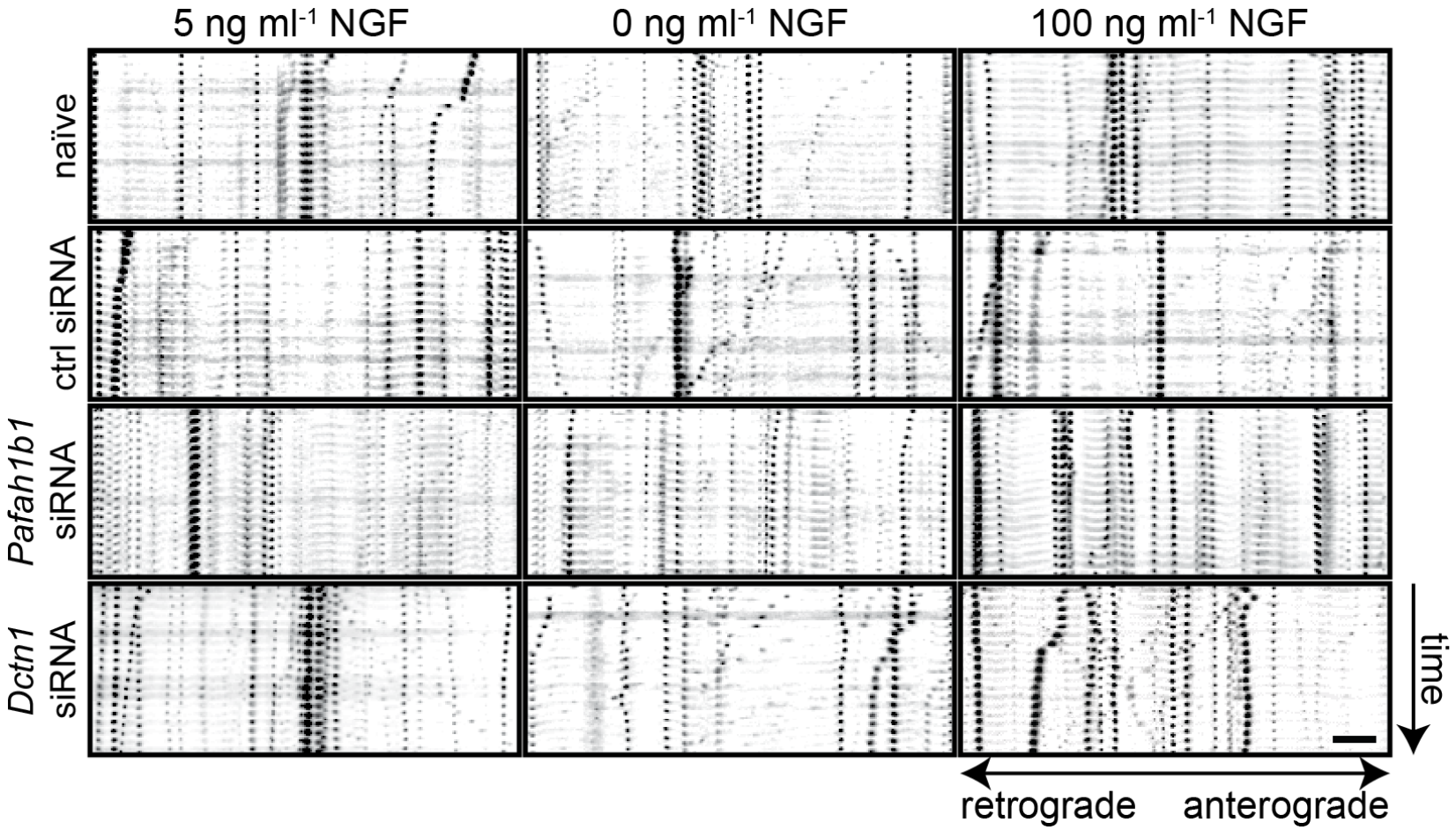

FIGURE 4-G: On DIV 3 of culturing DRG neurons in microfluidic chambers, the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}, and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1* or *Dctn1*. On DIV4, axons were treated with 0, 5, or 10 ng ml^{-1} NGF with LysoTracker Green for 15 min. Live-imaging time-lapse series of axonal fields were acquired, with images being taken every 13 s for 4 min. Kymographs of representative 100 μm segments of axons are shown. Scale bar, 10 μm.

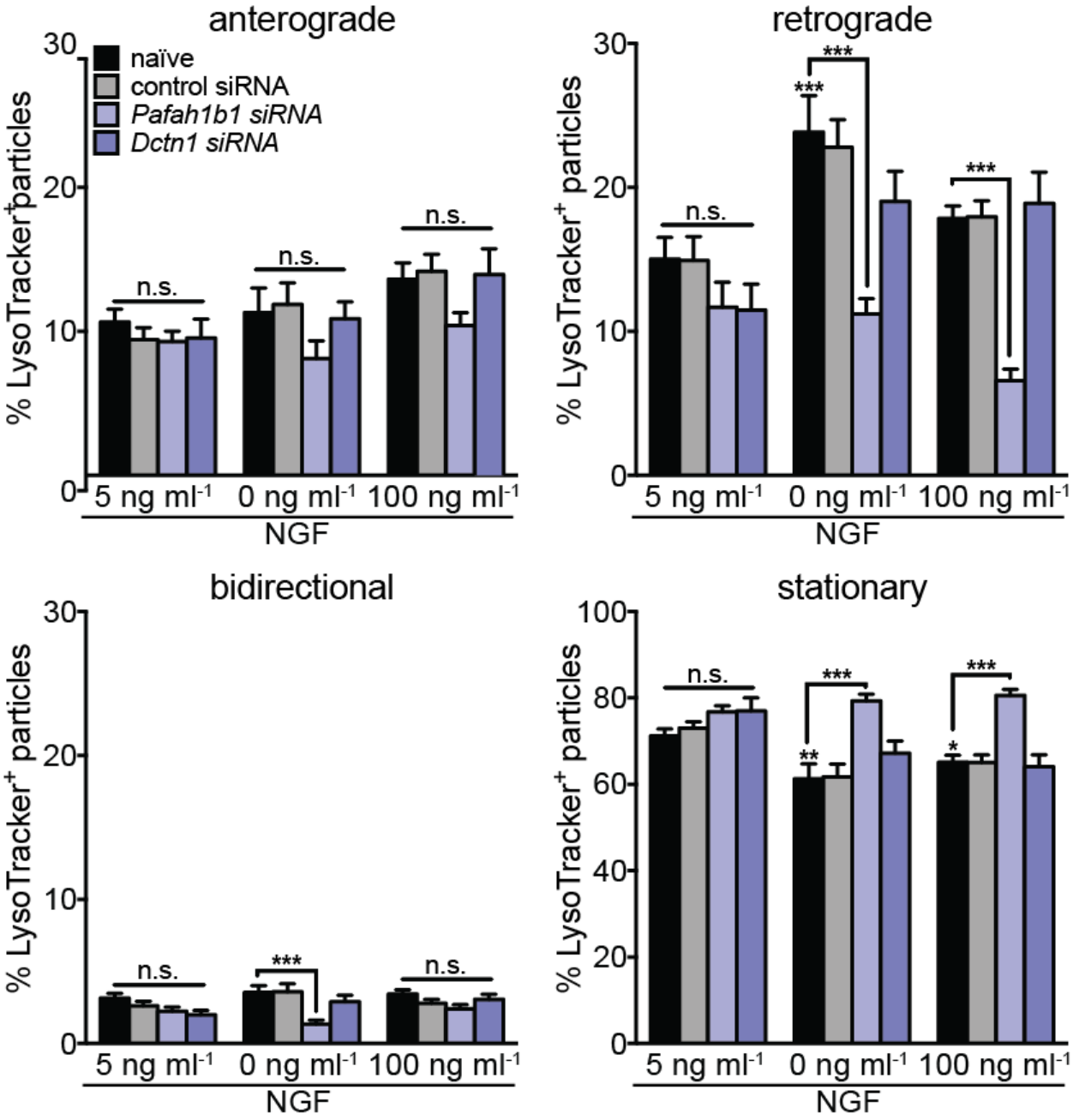

FIGURE 4-H: Quantification of vesicular motility analysis in axons treated with siRNAs against *Pafah1b1* or *Dctn1* [see **FIGURE 4-G**]. LysoTracker-positive particles with diameters ≥1 μm were scored as anterograde, retrograde, bidirectional, or stationary. Data represent the means ± SEM of 12-18 fields per conditions (n = 4-6 biological replicates). *p≤0.05; **p≤0.01; ***p≤0.001. Two-way ANOVA with Dunnett's multiple comparison test.

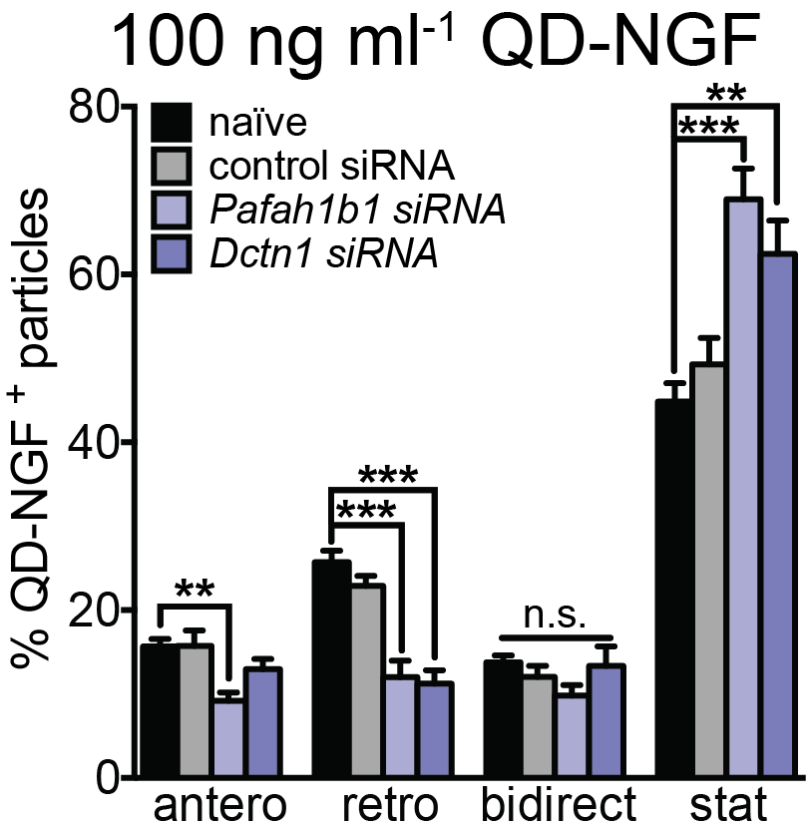

FIGURE 4-I: DRG neurons were cultured in microfluidic chambers for 3 DIV, at which point the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}, and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1* or *Dctn1*. On DIV 4, axons were treated with 100 ng ml^{-1} QD-NGF for 15 min and live-imaged. Live-imaging time-lapse series of axonal fields were acquired, with images being taken every 13 s for 4 min. QD-labeled particles <1 μm diameter were scored as anterograde, retrograde, bidirectional, or stationary. Means ± SEM of 9 optical fields per conditions (n = 3 biological replicates). **p≤0.01; ***p≤0.001. Kruskal-Wallis test with Dunn's multiple comparison test.

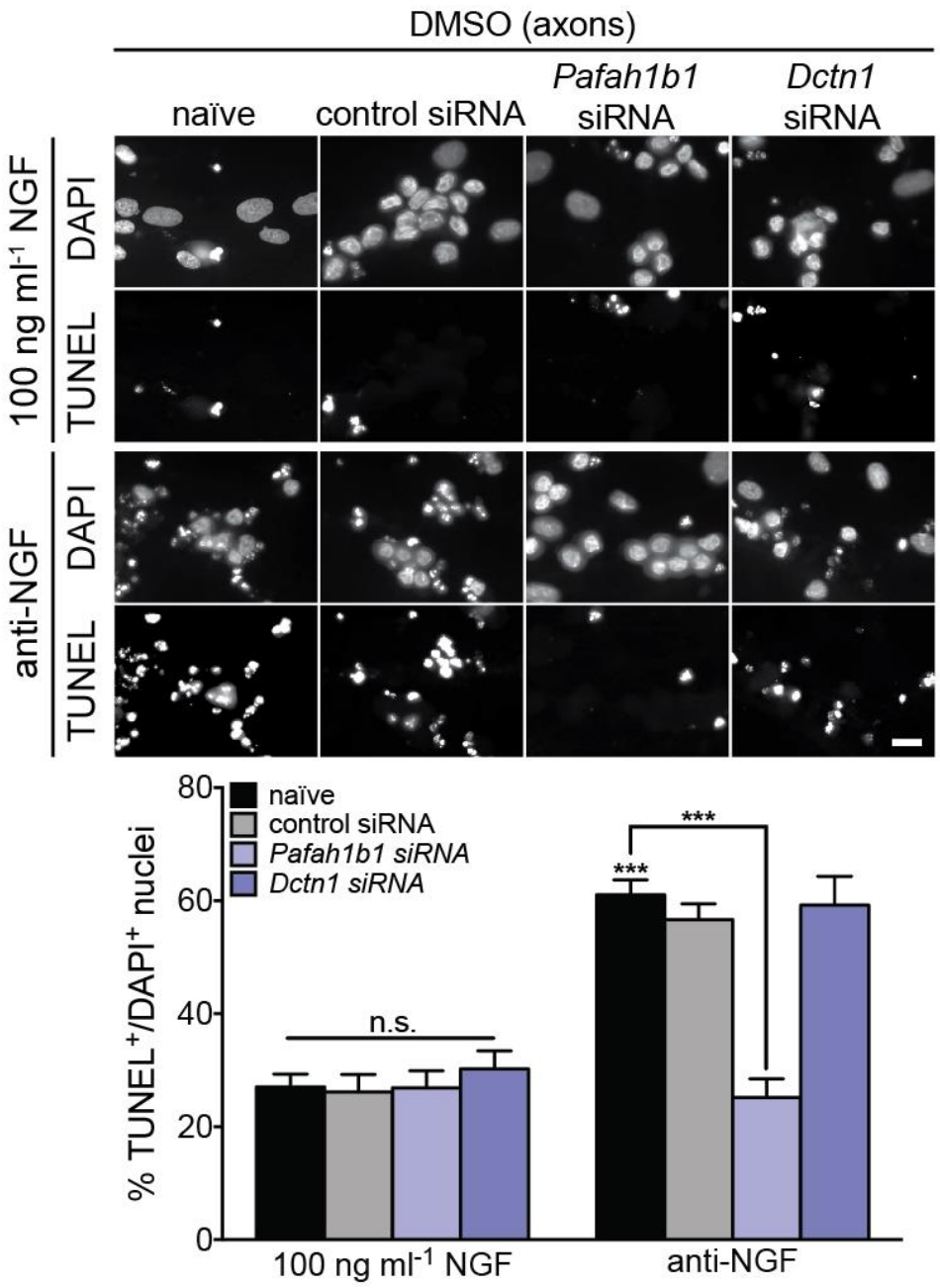

FIGURE 4-J: After growing DRG neurons in microfluidic chambers for 3 DIV, the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}, and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1* or *Dctn1*. On DIV 4, the medium in the somatic compartment was changed to NGF-free medium containing NGF-neutralizing antibody, and axonal compartments were changed to either 100 ng ml^{-1} NGF or NGF-free medium with NGF-neutralizing antibody plus vehicle for 24 h. Cell death was assessed by TUNEL assay. Data represent the means ± SEM of 15-25 optical fields per conditions (n = 3-5 biological replicates). ***$p \leq 0.001$. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 20 μm.

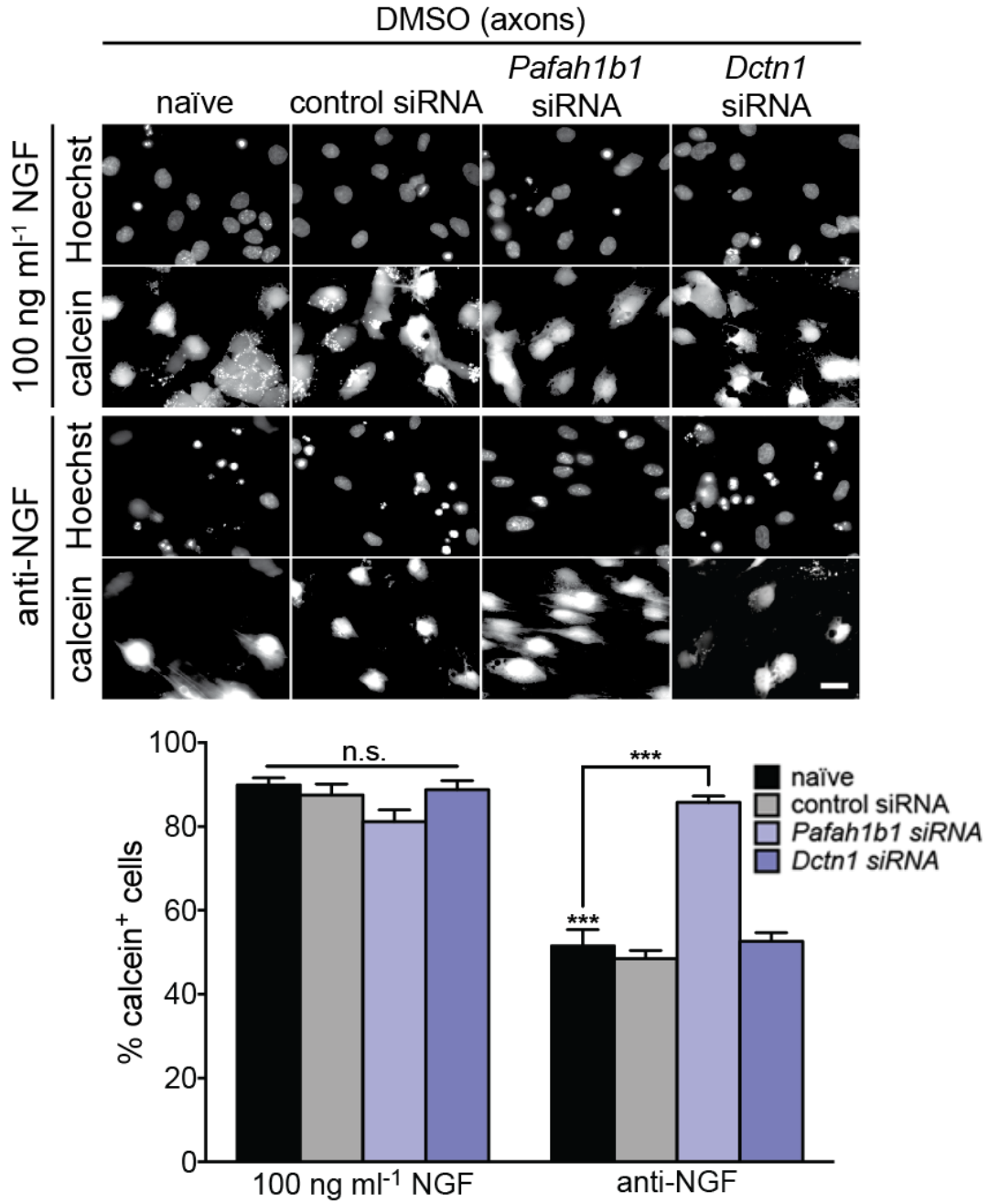

FIGURE 4-K: DRG neurons were cultured and transfected with siRNAs as in **FIGURE 4-J**. On DIV 4, the medium in the somatic compartment was changed to NGF-free medium containing NGF-neutralizing antibody, and axonal compartments were changed to either 100 ng ml^{-1} NGF or NGF-free medium with NGF-neutralizing antibody plus vehicle for 24 h. Survival was assessed by calcein AM staining. Data represent the means ± SEM of 15 optical fields per conditions (n = 3 biological replicates). ***$p \leq 0.001$. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 20 μm.

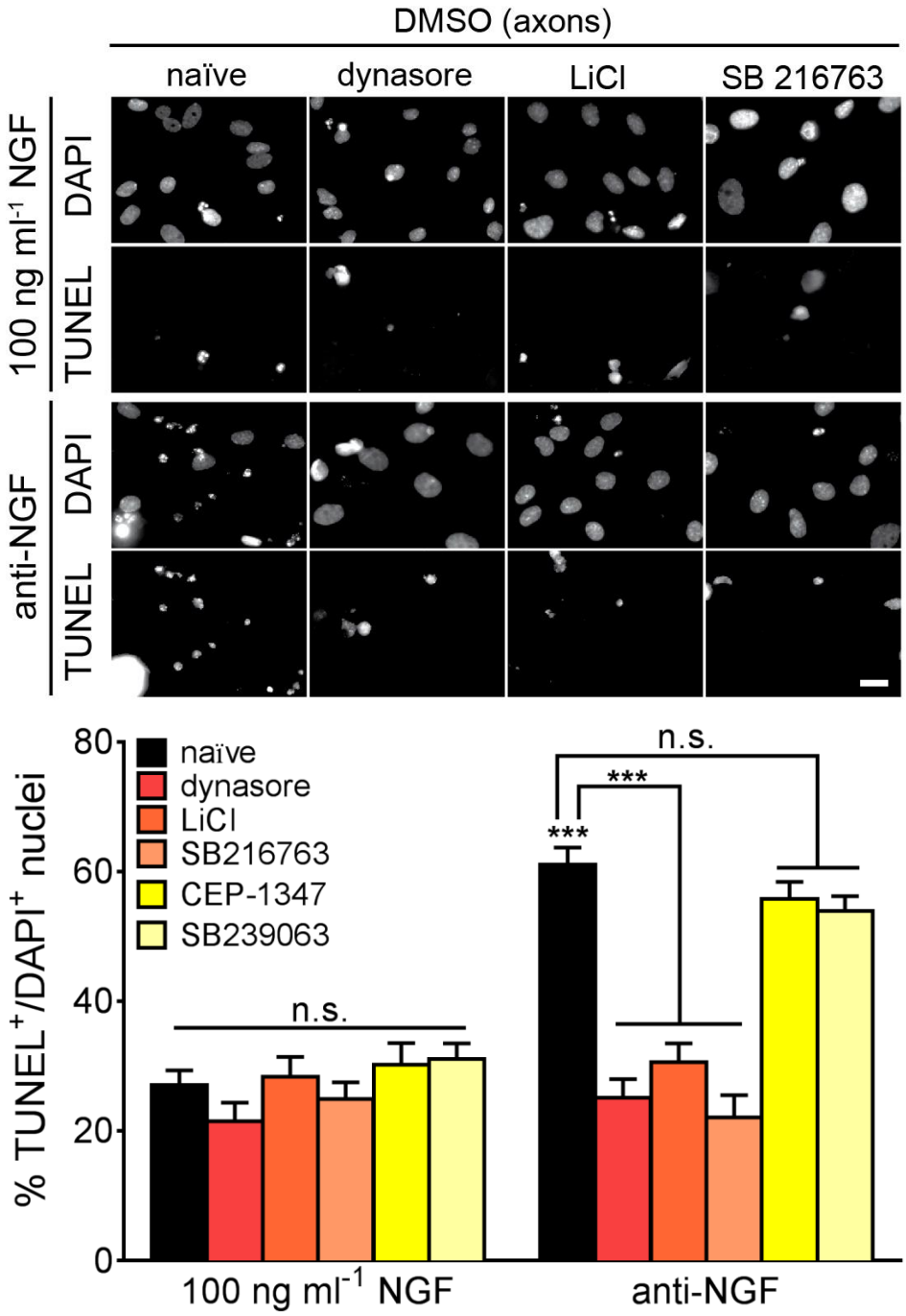

FIGURE 4-L: After growing DRG neurons in microfluidic chambers for 3 DIV, the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}. On DIV 4, the medium in the somatic compartment was changed to NGF-free medium containing NGF-neutralizing antibody, and the medium in the axonal chamber was changed to 100 ng ml^{-1} NGF or NGF-free medium with NGF-neutralizing antibody plus the indicated inhibitors or vehicle for 24 h. Cell death was assessed by TUNEL assay. Means ± SEM of 15-25 optical fields per conditions (n = 3-5 biological replicates). ***$p \leq 0.001$. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 20 μm.

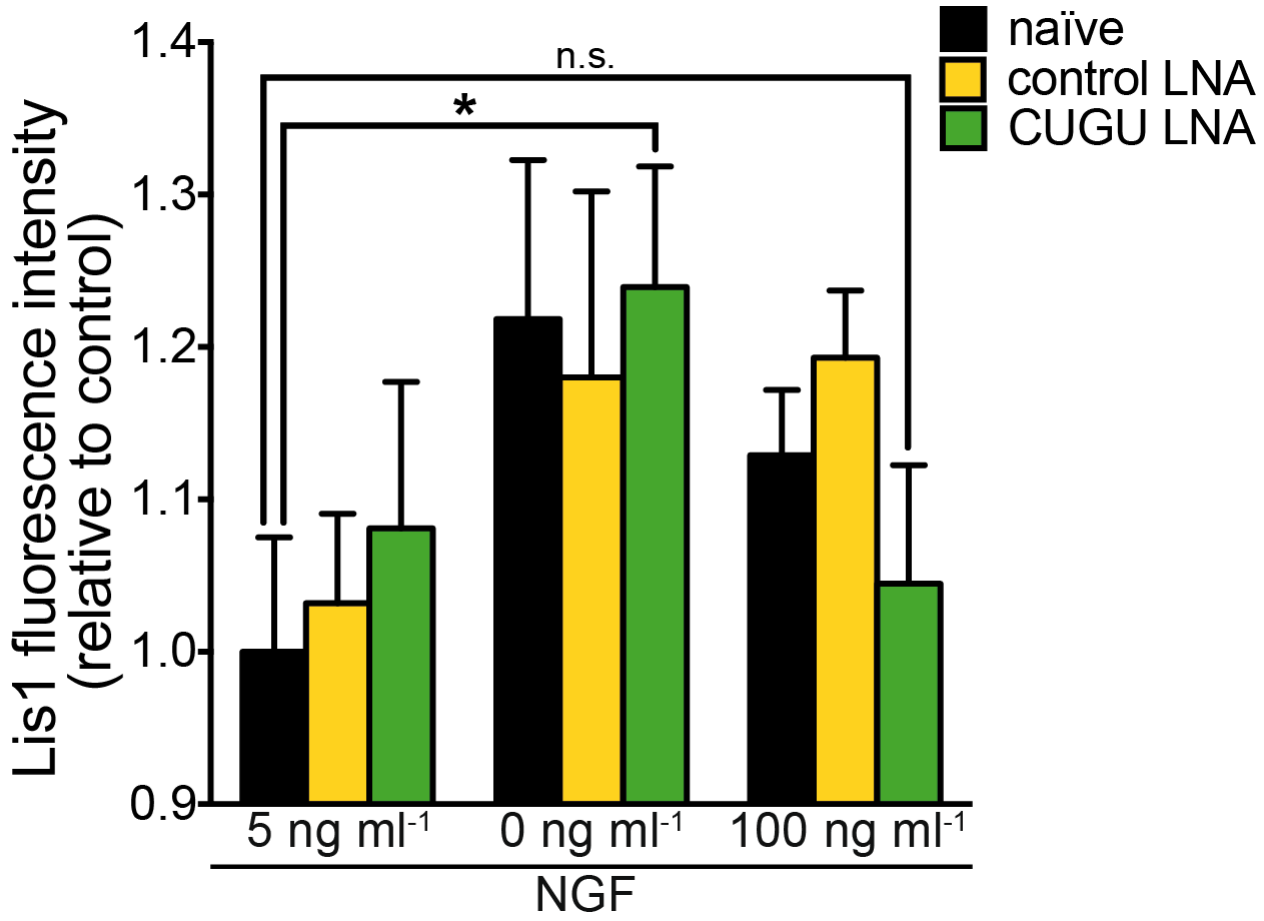

FIGURE 4-M: DRG neurons were cultured in microfluidic chambers for 3 DIV, at which point the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}, and cell bodies were selectively transfected with the control or CUGU LNAs. 24 h after transfection, axons were treated with 0, 5, or 100 ng ml^{-1} NGF for 10 min, and axonal Lis1 protein levels were measured by quantitative immunofluorescence. Data represent the means ± SEM of 20-30 optical fields per conditions (n = 4-6 biological replicates). *$p \leq 0.05$. Two-way ANOVA.

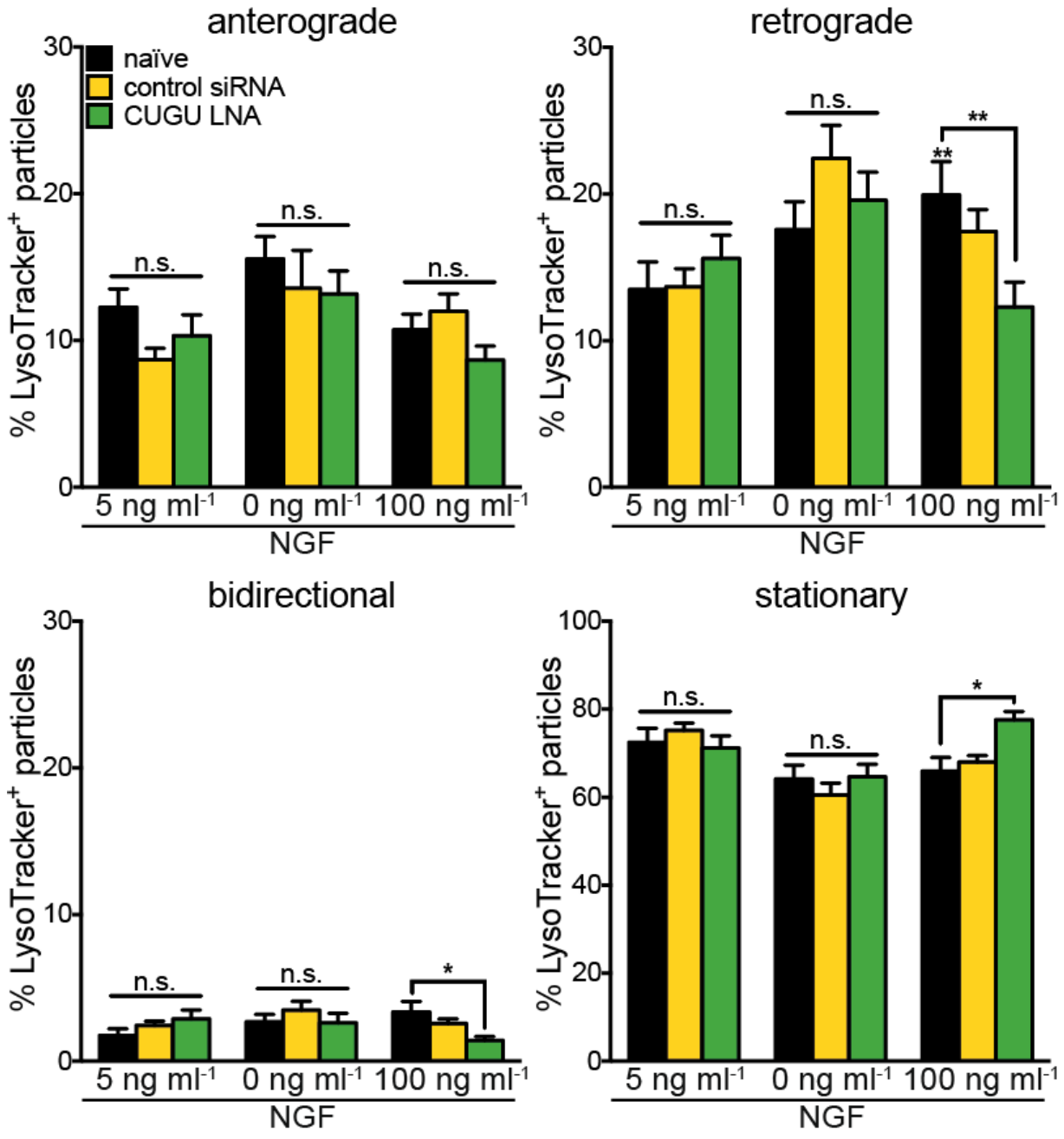

FIGURE 4-N: On DIV 3 of culturing DRG neurons in microfluidic chambers, the NGF concentration in the axonal chamber was changed to 5 ng ml^{-1}, and cell bodies were selectively transfected with the control or CUGU LNAs. 24 h after transfection, axons were treated with 0, 5, or 100 ng ml^{-1} NGF together with LysoTracker Green for 15 min. Live-imaging time-lapse series of axonal fields were acquired, with images being taken every 13 s for 4 min. LysoTracker-positive particles with diameters ≥1 μm were scored as anterograde, retrograde, bidirectional, or stationary. Data represent the means ± SEM of 9 optical fields per conditions (n = 3 biological replicates). *p≤0.05; **p≤0.01. Two-way ANOVA with Dunnett's multiple comparison test.

IV.3. Interpretation

Association with various accessory proteins enables cytoplasmic dynein to fulfill a multitude of cellular functions and to transport a wide variety of different cargoes (Vallee, McKenney et al. 2012, Maday, Twelvetrees et al. 2014). In these studies, evidence is provided that, within distal axons, dynein is regulated through local synthesis of Lis1 and $p150^{Glued}$ in response to changes in local NGF signaling. The unidirectional nature of microtubules in axons poses special challenges for the acute regulation and initiation of dynein-dependent transport, and local translation of its adaptor proteins can solve this problem. As the unidirectional orientation of microtubules is not unique to axons but also occurs in distal dendrites (Baas, Deitch et al. 1988) or during neuronal cell migration (Rakic, Knyihar-Csillik et al. 1996), it is possible that such a mechanism is utilized in these circumstances, as well.

Meanwhile, local synthesis of motor complex proteins is unlikely to be restricted to Lis1 and $p150^{Glued}$. The effect on transport observed with protein synthesis inhibitors is more severe than with axon-specific knockdown of the two transcripts individually. Notably, anterograde transport of LysoTracker-positive vesicles in axons appears to be dependent on local protein synthesis even under baseline conditions, indicating that kinesins might also be regulated by local translation. Further, $p150^{Glued}$ is only one of eleven subunits of dynactin. The transcript for another subunit, p50/dynamitin, is also consistently found in axonal transcriptomes, while the localization of transcripts coding for other subunits is less clear. It remains unknown whether the entire dynactin complex can be locally synthesized or whether it locally assembles with on-demand synthesis of $p150^{Glued}$ and, potentially, p50/dynamitin.

Thoughtful consideration of the evidence concerning axonal protein synthesis makes it clear that locally produced protein does not merely supplement the bulk synthesis of protein that

occurs in the cell body. Rather, locally synthesized protein is normally made to meet a specific, emergent need, and this same pattern appears to hold true for the dynein cofactors that have been studied here. For instance, neuron-wide knockdown of Lis1 has been seen to reduce retrograde axonal transport of LysoTracker-positive vesicles without the need for any stimulus condition (Pandey and Smith 2011), while the present studies found that axon-specific knockdown of Lis1 or p150^{Glued} could only prevent stimulation-induced changes in transport. Why is it that stimulated changes in transport require localized synthesis of new proteins, when constitutive or ongoing transport apparently does not? One possible explanation might be that, in response to environmental stimuli, previously inactive dynein motor complexes get activated and coupled to cargoes. In this regard, it is especially interesting that axonal Lis1 synthesis induced by NGF stimulation was found to require the association of *Pafah1b1* mRNA with APC. As a +TIP, APC is ideally situated to mediate the activation of the dynein motor through local production of its regulatory proteins. In fact, the recruitment of dynactin by +TIPs has been found to be required for the initiation of retrograde axonal transport of various cargoes (Moughamian, Osborn et al. 2013). In *Aspergillus nidulans*, the Lis1 homologue has also been described as an initiation factor for dynein-mediated transport that is be absent from and unnecessary for dynein-cargo complexes once they are in motion (Egan, Tan et al. 2012). Local synthesis of Lis1 or p150^{Glued} at precise loci in axons or growth cones could therefore act as a tuning or initiation mechanism for dynein-based transport.

Contrary to expectations, it was seen that neither a reduction of NGF signaling endosome transport towards the cell body nor the inhibition of endocytosis in axons caused cell death of DRG neurons. These results are in stark contrast to the proposed signaling endosome mechanism of NTs' effects, in which internalization and retrograde transport of ligand-receptor

complexes is requisite. On the other hand, these findings are in accordance with an alternative theory explaining the action of NTs, in which NGF, for example, promotes survival by locally suppressing an intrinsic death pathway originating in axons (Mok, Lund et al. 2009) without the need for trafficking of NGF or TrkA from axons to cell bodies (MacInnis and Campenot 2002, MacInnis, Senger et al. 2003, Mok and Campenot 2007). If neurotrophic signaling endosomes truly are not responsible for carrying pro-survival signals from the axon to the cell body, as was assumed, then their functional significance is difficult to explain and may require thorough reexamination in order to understand.

The present finding that association with APC establishes distinct pools of axonally localized *Pafah1b1* mRNA that differ as to whether they are translated in response to NGF stimulation or withdrawal provides mechanistic insight into the differential regulation of axonally localized mRNAs. In addition to regulating localization, APC acts as a "translational hub" for its associated mRNAs, spatially orchestrating protein synthesis in axons and growth cones (Preitner, Quan et al. 2014). It remains unknown how many translational hubs might exist in axons, or where they are to be found. A recent report that the netrin receptor, DCC, binds components of the protein synthesis machinery and regulates local translation (Tcherkezian, Brittis et al. 2010) suggests that APC is certainly not unique.

In conclusion, through these studies, a mechanistic explanation is provided for how a unidirectional motor can be tuned to fulfill changing transport needs far away from the cell body, and it is further revealed that, within subcellular compartments like axons, transcripts of the same gene can exist in functionally distinct pools based on their association with different RBPs or regulatory hubs.

CHAPTER V. EVIDENCE FOR AXONALLY SYNTHESIZED DYNEIN COFACTORS CONTRIBUTING TO AMYLOID-INDUCED NEURODEGENERATION

V.1. Rationale and Summary

Ongoing research seeks to illuminate the mysterious etiology and pathogenetic processes of AD, but it has not escaped notice that several of the hallmarks of early disease progression involve microtubule-based transport. Disorganization of the tubulin cytoskeleton due to hyperphosphorylated tau aggregation along with frank deficits in organelle trafficking are among the earliest manifestations of AD pathology (Ebneth, Godemann et al. 1998, Ishihara, Hong et al. 1999, Stokin, Lillo et al. 2005), and, intriguingly, these problems first begin to appear not in somata or dendrites but in axons (Iqbal, Liu et al. 2009, Perlson, Maday et al. 2010). Recent work has also implicated localized protein synthesis as an essential early step in the axonal response to Aβ (Baleriola, Walker et al. 2014), the neurotoxic peptide that aggregates in AD's characteristic neuritic plaques.

Accordingly, the possibility was considered that AD-associated deficits in axonal transport might involve changes in intra-axonal synthesis of dynein cofactors, similar to what we have observed in the case of developing axons' responsiveness to NTs. In model systems of amyloidopathy, it was found that Aβ exerts a complex and dynamic effect on axonal transport, which seems likely to be correlated with the peptide's regulation of the axonal localization and translation of transcripts coding for dynein regulatory proteins.

V.2. Results

a. Local application of amyloid-β oligomers induces recruitment of Lis1 transcripts into hippocampal axons

Previous work has established that exposing cultured hippocampal axons to oligomers of the $A\beta_{1-42}$ peptide triggers the recruitment into the axonal transcriptome of a specific cohort of mRNAs (Baleriola, Walker et al. 2014). Indications from RNA-sequencing suggested that *Pafah1b1* may be among the mRNAs regulated by $A\beta_{1-42}$, so FISH was performed on axons exposed to $A\beta_{1-42}$ or DMSO vehicle for 24 h in order to confirm [**FIGURE 5-A**]. Quantification of this data showed that *Pafah1b1* abundance was robustly increased 24 h after $A\beta_{1-42}$ application, as compared to treatment with the vehicle control. This recruitment effect was apparent in either naïve or control siRNA-transfected axons but was prevented by axonal transfection with siRNAs specifically targeting *Pafah1b1* mRNA, which resulted in FISH signal comparable to the vehicle-treated control axons. Meanwhile, within the DMSO condition, there were no significant differences between naïve axons or axons transfected with a control siRNA or even the *Pafah1b1*-targeting siRNAs.

b. Axonal synthesis of Lis1 shuts down in the short term after amyloid-β exposure

Given Aβ's evident effect stimulating the localization of Lis1-encoding transcripts to hippocampal axons, the question arose as to whether this neurotoxic peptide also regulated axonal Lis1 transcripts at the level of translation. By quantitative immunofluorescence, it was observed that, compared to vehicle-treated conditions, Lis1 protein abundance tended to be lower in distal axons treated for 24 h with $A\beta_{1-42}$ oligomers, although this trend was not significant [**FIGURE 5-B**]. Moreover, axon-specific transfection of siRNAs against *Pafah1b1* was not

effective at reducing Lis1 levels any further in the Aβ-treated condition, even though these siRNAs did significantly decrease axonal Lis1, as compared to the naïve and control siRNA-transfected samples, in the DMSO-treated condition. While these results indicate that, under the conditions described here, pervasive local synthesis of Lis1 occurs in axons of cultured hippocampal neurons, the inefficacy of RNAi at least within the first day after Aβ exposure suggests, based on what we have seen before, that this condition leads to a suspension of axonal *Pafah1b1* translation concomitant with inaccessibility to RISC.

c. Chronic amyloid-β exposure has a biphasic effect on axonal transport

To determine whether axonal siRNA-mediated silencing of *Pafah1b1* or Aβ's similar effect in reducing Lis1 produced changes in retrograde transport in axons, the transport assay used extensively in previous work, involving the incubation of axons with LysoTracker and scoring the motility of labeled lysosomes and late endosomes, was employed once more. In the vehicle-treated condition, where it was previously seen that application of *Pafah1b1*-targeting siRNAs significantly decreases Lis1 in axons, the present attempts at Lis1 knockdown yielded only a modest reduction in retrogradely moving particles and corresponding increase in stationary particles [**FIGURE 5-C**]. An equally mild effect was seen in $A\beta_{1-42}$-treated axons transfected with *Pafah1b1*-targeting siRNAs. However, besides the decrease in retrograde particles with axonal *Pafah1b1* knockdown compared to naïve samples in the DMSO condition, none of these results featured statistically significant differences.

As a gauge of retrograde axoplasmic transport of such cargoes *in vivo*, FluoroGold, a fluorescent tracer that accumulates in lysosomes (Persson and Havton 2009), was co-injected along with DMSO or oligomeric $A\beta_{1-42}$ into the dentate gyrus of living mice [see **FIGURE 2-C**].

The dentate gyrus contains axons and synaptic terminals of certain cholinergic neurons residing in septal nuclei of the basal forebrain (Amaral and Kurz 1985, Leranth and Frotscher 1989), so the number of FluoroGold-labeled neurons found in the basal forebrain at given points in time after injection into the dentate gyrus was taken as a readout of retrograde lysosome flux. At 2 days *post* injection, there was a slight paucity of FluoroGold-positive basal forebrain neurons in the Aβ-treated condition relative to the vehicle control [**FIGURE 5-D**], suggesting a transport deficit in line with the *in vitro* results presented here and previous studies showing that short-term Aβ exposure rapidly inhibits axonal transport (Hiruma, Katakura et al. 2003, Pigino, Morfini et al. 2009). Nonetheless, by 4 days *post* injection, this trend had reversed, with more FluoroGold-positive cells actually found in the Aβ-treated condition; the situation at 7 days *post* injection showed an even wider gap, as FluoroGold labeling in the basal forebrains of Aβ-treated animals continued to increase relative to what was seen in vehicle-treated cases. Taken together, these findings strongly indicate that unalleviated exposure of axons to Aβ oligomers causes long-term induction of retrograde vesicular trafficking, after an initial period of inhibiting transport.

d. Axonally produced Lis1 regulates multiple cell death and survival pathways in cultured hippocampal neurons

Finally, it seemed pertinent to examine whether the regulation of retrograde transport by intra-axonal synthesis of Lis1 might be involved in neuronal survival or apoptosis pathways, as seen in the case of DRG neurons. Thus, TUNEL assays were performed on cultured hippocampal neurons whose axons were incubated with $A\beta_{1-42}$ or vehicle for 48 h, and which were previously transfected with *Pafah1b1*-targeting or control siRNAs or left untransfected. As would be expected, exposure to $A\beta_{1-42}$ caused a significant increase in the number of apoptotic cells

detected in naïve or control siRNA-transfected axons [**FIGURE 5-E**]. Interestingly, axonal knockdown of *Pafah1b1* was sufficient to prevent this Aβ-induced cell death. However, in stark contrast, transfection with *Pafah1b1*-targeting siRNAs in the DMSO-treated condition was itself the cause of significantly greater cell death than was observed in controls. The seeming contradiction of axonal Lis1 knockdown leading to apoptosis in one circumstance while preventing it in another would appear to indicate that several distinct pathways governing survival and death in these neurons share a need for locally produced Lis1.

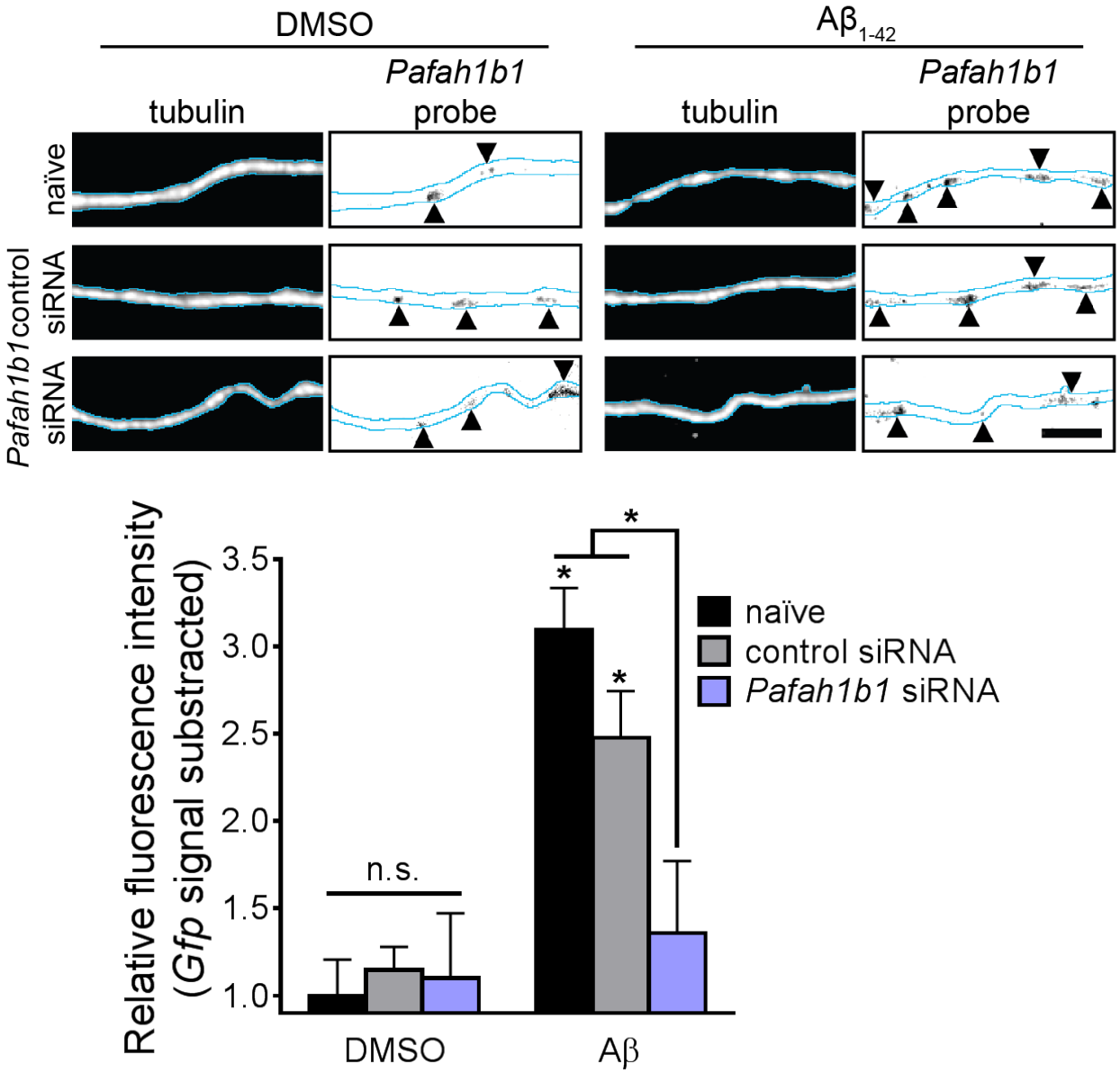

FIGURE 5-A: Hippocampal neurons were cultured in microfluidic chambers. On DIV 8, axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1*. On DIV 9, axons were treated with vehicle or $A\beta_{1-42}$ oligomers for 24 h. Axonal *Pafah1b1* mRNA levels were evaluated by FISH. Data represent the means ± SEM of 10-15 optical fields per conditions (n = 2-3 biological replicates). *p≤0.05. Two-way ANOVA. Scale bar, 5 µm.

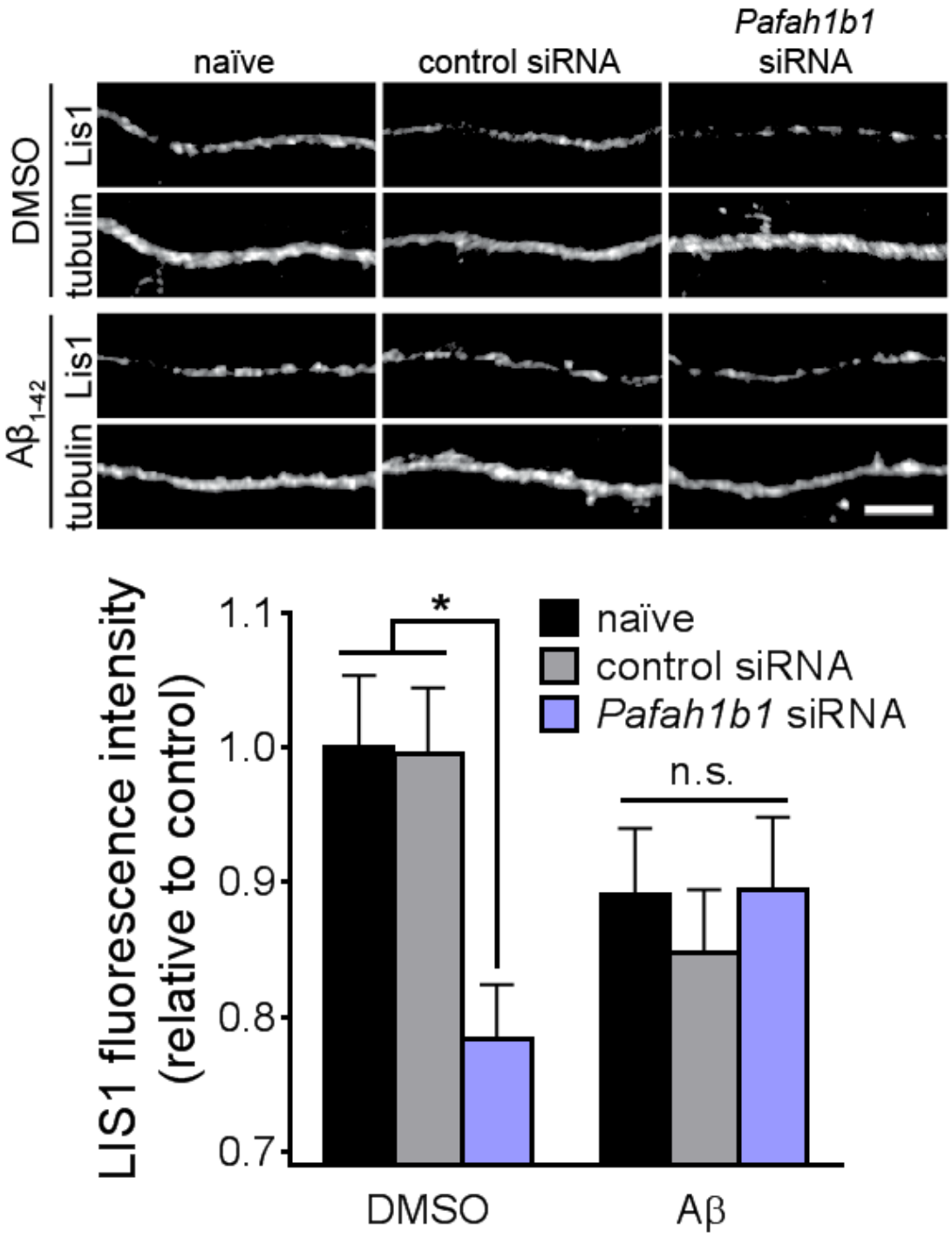

FIGURE 5-B: After growing hippocampal neurons in microfluidic chambers for 8 DIV, axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1*. On DIV 9, axons were treated with vehicle or $A\beta_{1-42}$ oligomers for 24 h. Axonal Lis1 protein levels were then measured by quantitative immunofluorescence. Data represent the means ± SEM of 25-40 optical fields per conditions (n = 5-8 biological replicates). *p≤0.05. Two-way ANOVA. Scale bar, 5 µm.

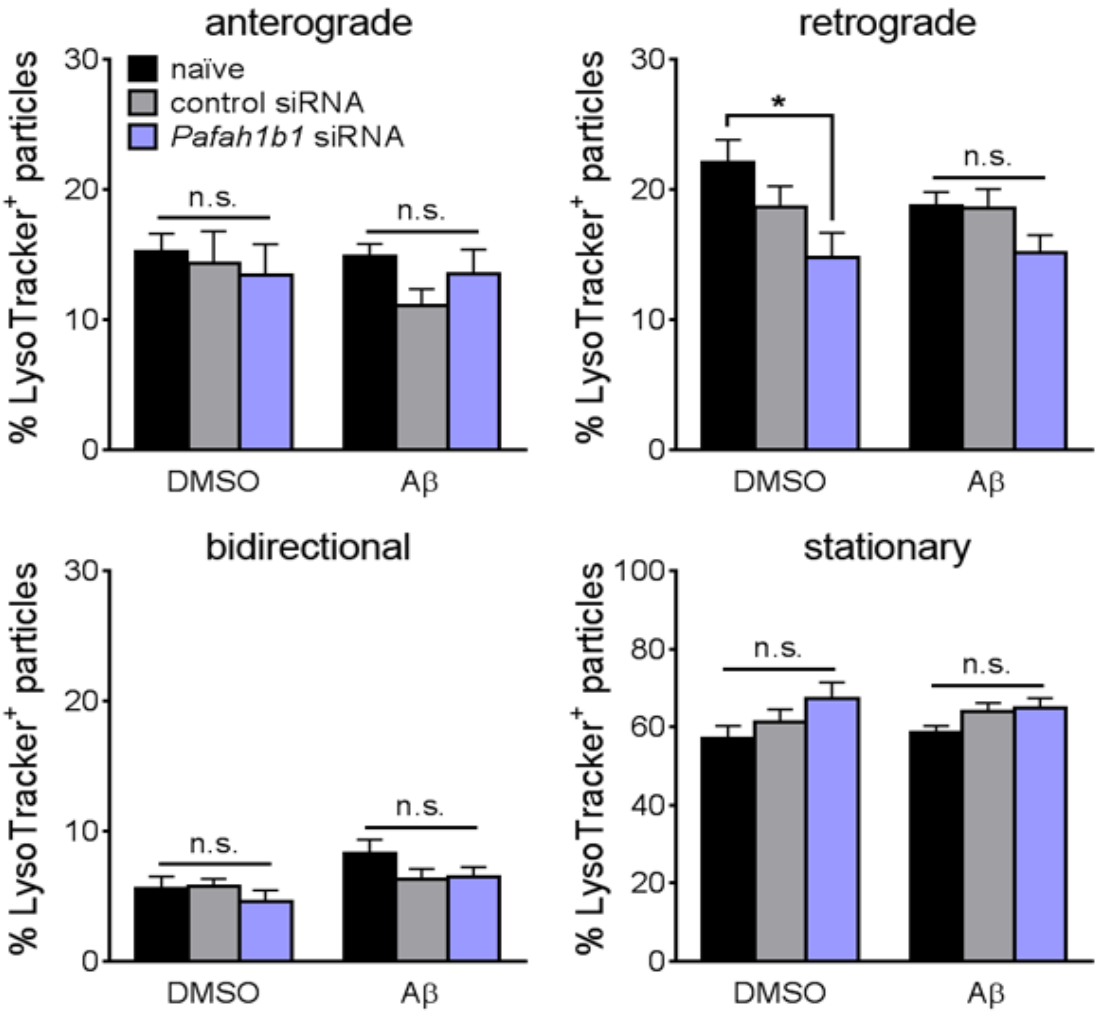

FIGURE 5-C: On DIV 8 of culturing hippocampal neurons in microfluidic chambers, axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1*. On DIV 9, axons were treated with vehicle or $A\beta_{1-42}$ oligomers for 24 h. On DIV 10, axons were supplied with LysoTracker Green for 15 min and live-imaging time-lapse series of axonal fields were acquired, with images being taken every 13 s for 4 min. LysoTracker-positive particles with diameters ≥1 μm were scored as anterograde, retrograde, bidirectional, or stationary. Data represent the means ± SEM of 12 optical fields per condition (n = 4 biological replicates). *p≤0.05. Two-way ANOVA with Dunnett's multiple comparison test.

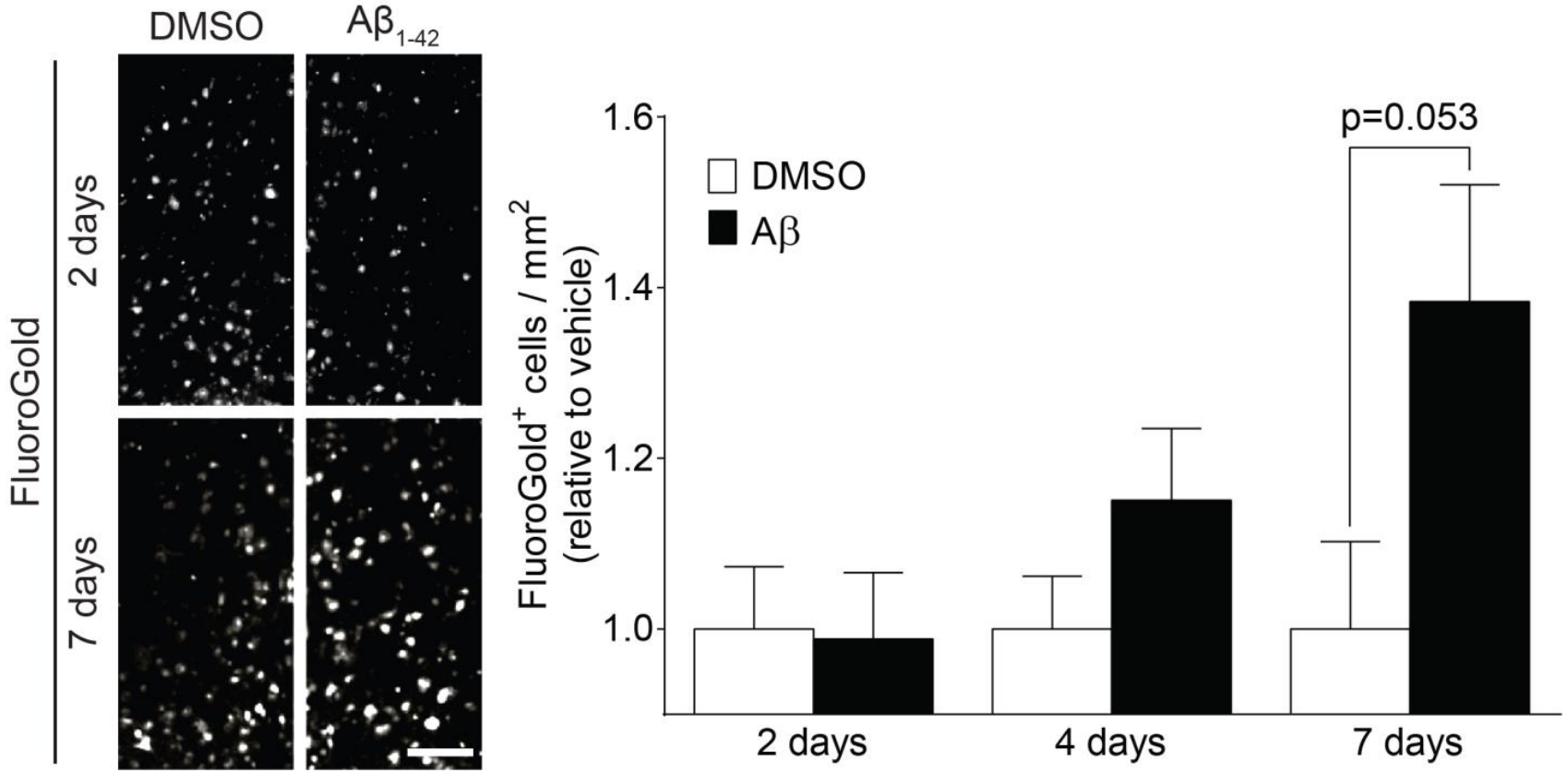

FIGURE 5-D: Mice were stereotactically injected in each dentate gyrus with vehicle or $A\beta_{1\text{-}42}$ oligomers, as well as the retrograde tracer FluoroGold. Animals were sacrificed 2, 4, or 7 days *post* injection, and the brains were sectioned. Representative images are of matched sections from the vehicle- and Aβ-treated hemispheres of a single animal's brain. FluoroGold is visible in neurons of the basal forebrain within the nucleus of the diagonal band, which project axons to the site of injection. The density of FluoroGold-positive cells was determined by counting at each time point. Data represent the means ± SEM of brain slices from 3-5 animals per condition. Mann-Whitney *U* test. Scale bar, 100 μm.

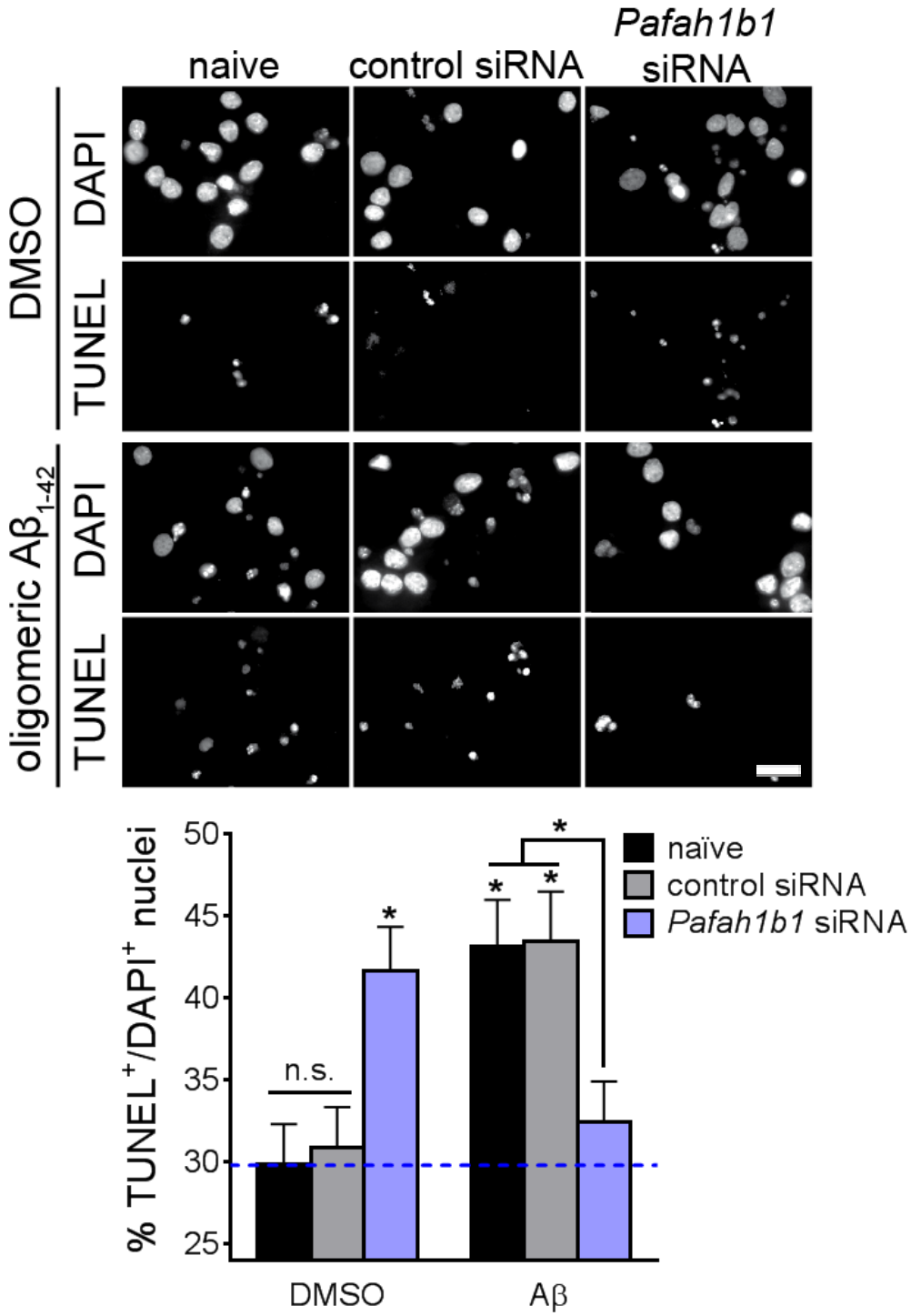

FIGURE 5-E: Hippocampal neurons were cultured in microfluidic chambers for 8 DIV, at which point axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1*. On DIV 9, axons were treated with vehicle or $A\beta_{1-42}$ oligomers for 48 h. Cell death was assessed by TUNEL assay. Means ± SEM of 50-60 optical fields per conditions (n = 5-6 biological replicates). *$p \leq 0.05$. Two-way ANOVA with Dunnett's multiple comparison test. Scale bar, 20 µm.

V.3. Interpretation

The immediate-early signaling pathways activated in axons by exposure to extracellular Aβ are very poorly understood. Several Aβ-binding sites on the plasma membrane have been identified, but their involvement in mediating Aβ neurotoxicity has been difficult to establish conclusively. For example, although Aβ is known to interact with $p75^{NTR}$, whether this binding is toxic or protective in nature is disputed (Perini, Della-Bianca et al. 2002, Zhang, Hong et al. 2003). Similarly, the low density lipoprotein, NMDA, and α7 nicotinic acetylcholine receptors have all been reported to be involved in Aβ internalization into neurons (LaFerla, Troncoso et al. 1997, Bi, Gall et al. 2002, Nagele, D'Andrea et al. 2002), and yet what second messengers or downstream effectors are involved in the response to either intracellular or extracellular Aβ remains largely a mystery. Attention has been drawn sequentially to reactive oxygen species (Behl, Davis et al. 1994), nitric oxide (Vodovotz, Lucia et al. 1996), and calcium release from the endoplasmic reticulum (Suen, Lin et al. 2003), without a clear picture emerging as to what effects of Aβ are attributable to which pathway, if any, of those studied.

It is important to recognize this relative paucity of knowledge, as it has shaped the design and interpretation of the experiments utilized here. While the results of these experiments are undoubtedly informative, much of the information they provide is, in a sense, pending better understanding of the pathophysiologic effects of Aβ in order to be evaluated with complete clarity. The chief stumbling block put forward by the present data lies in the apparent discrepancy between Aβ's effects on Lis1 at the levels of transcript localization and translation in axons. Whereas *Pafah1b1* mRNA appears to be recruited to axons *en masse* within one day of their incubation with Aβ, all indications from Lis1 immunofluorescence and *in vitro* vesicular transport assays are that, within this timeframe, these transcripts are not being translated to

generate protein. Despite this being somewhat counterintuitive, it may prove to be extremely revealing. Since the initial discovery that Aβ oligomers are potently toxic to neurons, researchers have speculated that the progression of AD could be shaped by the "biphasic impact of Aβ-derived diffusible ligands acting upon particular neural signal transduction pathways" (Lambert, Barlow et al. 1998). Lacking any information about the signaling pathways in question, it was still natural to infer that Aβ must have a biphasic effect, because, even as Aβ begins to accumulate, neuronal death is not at all characteristic of AD pathology until its later stages (Bredesen, Rao et al. 2006). Remarkably, we have now seen this kind of biphasic effect as it pertains to retrograde axonal transport, which is inhibited in the short-term by exposure to Aβ oligomers but, based on the *in vivo* FluoroGold tracing, later appears to undergo a major induction, at least with regard to degradative cargoes like lysosomes. Therefore, a simple explanation for the results we previously found to be incongruous might be that, by conducting our observations after one day of exposure to Aβ, we are catching the tail end of Aβ's inhibitory effect on axoplasmic transport, even as the axon is stocking itself with Lis1 transcripts and other apparatus for the transport induction phase to follow. Indeed, through the cell death assays, where neurons were incubated with Aβ for a longer time, we can begin to see an effect of siRNA-mediated knockdown in the Aβ-treated condition, implying that Lis1 transcripts are being locally translated.

The fact that intra-axonal synthesis of Lis1 seemed to be detected here in vehicle-treated axons of cultured hippocampal neurons and could be effectively targeted by RNAi poses another problem. As a reminder, in practically every circumstance in which localized protein synthesis has been described, it has been in response to a specific stimulus or change. It may be that growing hippocampal axons, in contrast, more or less constitutively require locally synthesized

Lis1, or, potentially, something about the utilized culture conditions, such as the laminin coating of the substrate, provided an unrecognized stimulus for local synthesis of this protein. However, it is also conceivable that the relatively long period that the neurons were kept in culture—and, especially, in a compartmentalized culture situation—might have led them to become stressed, and this stress enabled local synthesis of certain proteins as a response. The length of keeping neurons in culture was dictated by the time that it took for their axons to grow past both sets of microgroove barriers in the tripartite microfluidic chambers used for experiments, and this took significantly longer for hippocampal neurons than for the DRG neurons employed in preceding chapters.

Although valuable information was certainly gleaned from these studies in cultured hippocampal neurons, if the experiments were to be redesigned and attempted again it might be beneficial to use bipartite microfluidic devices instead, so that axons could be isolated and tested more quickly. Of course, it would also be interesting to expand the investigation beyond Lis1, as dynactin subunits may very well have their own part to play in modulating axonal transport in response to extracellular Aβ.

CHAPTER VI. SIGNIFICANCE AND CONCLUSIONS

VI.1. Conceptual Significance

a. Coordinate control of mRNA localization and translation

It has been proposed that functionally related cohorts of mRNAs bound to particular RBPs could represent "post-transcriptional operons," analogous in purpose to the polycistronic genetic regulatory structures seen in prokaryotes (Keene and Tenenbaum 2002). Recent work on the interactome of APC has provided an example lending credibility to this model (Preitner, Quan et al. 2014), and the studies presented here have shown that, in the case of *Pafah1b1* mRNA found in axons, association with APC facilitates the localization and translation of a subpopulation of this transcript species that has a situation-specific functional relevance. Blocking the interaction between APC and *Pafah1b1* reduces the mRNA's abundance in axons by only a fraction, but removal of this APC-dependent fraction of *Pafah1b1* completely prevents certain stimulus-induced transport effects that rely upon axonal synthesis of Lis1 protein. Although the potential for a single mRNA species to be packaged into multiple different RNPs had been noticed previously, before now it was difficult to demonstrate that distinct functional consequences were attributable to subsets of transcripts distinguished by their associated RBPs. Interestingly, the cases of both *Pafah1b1* and *Dctn1* mRNA show that the regulation of transcript localization and translation do not necessarily go hand-in-hand. Even if both processes are controlled by association with a single RBP, a transcript might not be translated under a condition where it is recruited into axons, or *vice versa*. Explaining why this might be would require further investigation, but, based on what we have seen so far, it may indicate a difference between the acute and chronic responses to a given stimulus.

b. Motor regulation throughout subcellular compartments

Through these studies, a previously unrecognized mechanism has been uncovered for acute tuning of the dynein motor via localized synthesis of its regulatory cofactors. While we have focused on dynein motor control in axons as a model system, the findings of the present research may be generalizable to subcellular locales throughout different kinds of cells. Cytoplasmic dynein together with dynactin and Lis1 are known to localize to the leading cell cortex of directionally migrating cells, where their inhibition leads to impaired cell movement (Dujardin, Barnhart et al. 2003). At the same time, several mRNAs essential to cell migration have been seen to localize to and undergo local translation at the leading edge (Shestakova, Singer et al. 2001, Willett, Brocard et al. 2013, Maizels, Oberman et al. 2015), perhaps hinting that the same might occur for dynein and its cofactors. Such findings make it clear that localized expression of protein takes place in cells far less morphologically complex than neurons.

Considering the established importance of dynein's interaction with Lis1 for the development and patterning of the cerebral cortex, it might be fruitful to explore whether Lis1's roles in radial glia and migrating cortical neuron progenitors involve localized synthesis of the protein. It has been observed that the lissencephaly phenotype classically associated with Lis1 haploinsufficiency is enhanced by mutation of APC (Hebbar, Guillotte et al. 2008), and conditional knockout of APC in neural progenitor cells actually results in severely disrupted cortical lamination on its own (Yokota, Kim et al. 2009). As opposed to global Lis1 abundance, the recruitment of transcripts encoding Lis1 specifically to microtubule plus-ends and their local expression there might thus be a critical but as yet unappreciated step in normal cortical migration, which, when disrupted, produces the devastating effects of lissencephaly.

Finally, these results might help to resolve a considerable point of argument about several dynein cofactors, concerning their role in the initiation of dynein-driven transport. This controversy has proven especially problematic for Lis1, which appears to produce physical effects on the motor that imply its persisting attachment (McKenney, Vershinin et al. 2010, Huang, Roberts et al. 2012) yet is found, by some groups, to only be necessary when initiating the motor's activity from a standstill (Egan, Tan et al. 2012). The results presented here may offer a sort of compromise, wherein stimulus-induced, intra-axonal synthesis of dynein cofactors is specifically responsible for the observed effects on initiation of transport for certain cargoes.

c. A "retrosome" for axon-to-soma signaling

On the basis of the findings that NGF deprivation-induced cell death depends upon a retrograde signaling complex produced through endocytosis and requiring GSK3 activation, it was postulated that this death signal might be a GSK3β-containing endosome or MVB. It has long been appreciated that MVBs represent a sizable portion of retrogradely transported cargoes *in vivo* (Tsukita and Ishikawa 1980), and GSK3 sequestration into MVBs has been reported to take place in other cell signaling pathways (Taelman, Dobrowolski et al. 2010). Interestingly, Weible and Hendry, two of the originators of the signaling endosome hypothesis of NT function, previously proposed that MVBs, by enclosing and preserving a representative sampling of signaling molecules from the axon prior to transport, could "allow the cell body to receive a snapshot of the events occurring" in the periphery (Weible and Hendry 2004). Accordingly, they dubbed the MVBs seen in axons "retrosomes." It seems that the results are very consistent with this idea of retrosomes as a constitutive signaling platform for axon-to-soma communication.

VI.2. Technical Applications

a. Approaches to localized translational profiling

By informing us as to what proteins can be produced locally *versus* those which must be trafficked from the cell body, subcellular transcriptomes have proven to be an indispensable resource in uncovering the various roles of localized protein synthesis. Yet, as we have seen, not all of the transcripts that are present in a subcellular locale like the axon will be undergoing translation at the same point in time or in a given situation. In some cases, what we really wish to know is which localized mRNAs are actively associated with polyribosomes and being translated under the circumstances that we are studying. Methods for gathering this information about the "translatome"—such as the extension of RNA sequencing into what is known as ribosome profiling, or Ribo-Seq—are just beginning to be applied to whole cells (Ingolia, Ghaemmaghami et al. 2009, Ingolia, Lareau et al. 2011, Brar, Yassour et al. 2012). Notably, there is already interest building in applying these kinds of techniques to subcellular compartments, especially in neurons (Kitchen, Rozowsky et al. 2014), but, thus far, there has been little in the way of concrete results (Kratz, Beguin et al. 2014).

In this respect, the finding that the ability to silence axonally localized mRNA through RNAi may be tied to the translation state of the transcripts in question presents an unexpected methodological opportunity. RNA sequencing is relatively difficult and time-consuming, and preparing RNA, particularly from isolated axons, requires a tremendous amount of biological material. However, this observation implies that a western blot or immunostaining of an axonal protein of interest after local siRNA treatment could be a valid approach to determining whether the mRNA coding for that protein is being actively translated. That is, if axonal knockdown is

seen to be effective in reducing the protein's abundance, the result indicates that the protein is being translated.

Contrary to the findings presented here and the hypothetical approach just expounded, it is worth pointing out that RNAi is conventionally understood not to be coupled by necessity to translation (Gu and Rossi 2005). In whole *Drosophila* embryo lysates, for instance, the application of protein synthesis inhibitors was not seen to perturb efficient RNAi (Zamore, Tuschl et al. 2000). Nevertheless, components of RISC, which is the molecular machinery for RNAi, have repeatedly been found to associate with ribosomal proteins and RNAs (Hammond, Boettcher et al. 2001, Caudy, Ketting et al. 2003, Pham, Pellino et al. 2004), as well as with whole polyribosomes (Djikeng, Shi et al. 2003). The purpose of this association between RISC and ribosomes is not presently understood, but it is conceivable that what is true for RNAi globally might not be so in subcellular compartments like axons. The unique RNP packaging and regulatory systems that control mRNA localization and expression in these compartments might mean that specially localized mRNAs are generally less accessible than their cell body counterparts to both RISC and ribosomes. Accordingly, since each process requires mRNAs to become accessible to their respective machinery, localized translation and RNAi would be correlated even if not mechanistically linked.

b. Targeting localized protein synthesis *in vivo*

A long-standing criticism of research into localized protein synthesis has involved the doubt as to whether this phenomenon is actually relevant to biological systems at work in living tissues and organisms, or if it is just an unphysiologic artefact of cells in culture. While *in vivo* demonstrations have been provided for the functions of a few locally synthesized proteins, such

as importin β1 in axonal injury signaling (Perry, Doron-Mandel et al. 2012) or β-actin in supporting axon regeneration after injury (Donnelly, Willis et al. 2011), in many other cases the methods needed to generate such proof presently do not exist. Not long ago, two colleagues eloquently summarized the distinctive challenge of devising interventions to target localized protein synthesis, saying, "Conclusive evidence in support of an *in vivo* physiological role for local translation ideally requires *some way of selectively perturbing translation in a subcellular compartment of interest while not affecting production of the same gene product elsewhere in the cell*" (*emphasis added*, Perry and Fainzilber 2014). Most of the pharmacological or molecular biological tools sufficient for work *in vitro*, with the aid of compartmentalized culture techniques and the like, cannot manage such selectivity *in vivo*. Provided that anatomy conveniently affords a spatial separation between the somata of the neurons of interest and their projecting axons, an inhibitor compound or siRNA could be applied locally in the region of the axons, but this approach cannot absolutely rule out that any resulting effects are due to the involvement of glia or other neurons surrounding those axons.

For β-actin and importin β1, selective perturbation of their synthesis in axons was made possible by exceptionally detailed knowledge about the mechanisms of their mRNA localization. Preventing β-actin mRNA's binding to the RBP responsible for its localization or genetically deleting the preferentially localized long 3' UTR isoform of importin β1 mRNA produced, in either case, what amounted to a subcellular knockdown (Donnelly, Willis et al. 2011, Perry, Doron-Mandel et al. 2012). Of course, this is precisely what the APC-blocking LNA oligomer has already been employed to do *in vitro*, with the added qualification that it appears to target only a functional subfraction of axonal Lis1 transcripts. Applying this LNA or others like it for use *in vivo* will be more or less trivial. *In utero* electroporation techniques have been used

effectively for some time to introduce molecular biological reagents to the central nervous systems of mammalian embryos, which would be a prime setting in which to utilize LNAs for interfering with mRNA localization and local translation events (Tabata and Nakajima 2001, Takahashi, Sato et al. 2002). Because such LNAs would selectively block the targeted transcripts' localization to axons or another subcellular compartment as desired, there should be less potential for off-target effects, and the delivery system for the reagent need not distinguish between neurons and glia or other surrounding cells that do not share neuronal morphology. The tantalizing prospect of being able to finely dissect out different cellular roles for a single protein through methods of function-specific inhibition thus appears to be just entering our grasp. Moreover, the applicability of these tools to *in vivo* studies represents an extraordinary opportunity for future research into localized protein synthesis and cell biology in general.

VI.3. Future Directions

a. Follow-up studies

In order to validate that the effects of the CUGU LNA treatment are, in fact, due to inhibited binding of APC to the Lis1 transcript, several additional experiments are necessary. To begin with, the physical interaction between APC and this particular mRNA should be proven more directly, beyond relying upon the apparent APC binding motif in the mRNA's 3' UTR. One well-established method is to use ultraviolet light to crosslink RNAs with their interacting RBPs *in situ*, then perform immunoprecipitation for the protein of interest—in this case, APC. RNA that is pulled down with APC can be purified from the protein and analyzed by RT-PCR to see if it contains *Pafah1b1* transcripts. The inverse approach, of pulling down *Pafah1b1* mRNA to detect what RBPs associate with it, has also been made possible quite recently by the exploitation of nuclease-deficient Cas9 protein, which can be targeted to *Pafah1b1* by a specific guide RNA (Qi, Larson et al. 2013). Using this technique, which is currently being evaluated for this application by other members of the Hengst laboratory, has the distinct advantage of yielding information specific to *Pafah1b1*, and it could therefore reveal what other RBPs this transcript associates with, beyond merely confirming its binding to APC. After it is shown definitively that APC binds the transcript, it should be straightforward, using one of the two outlined techniques, to prove that application of the CUGU LNA reduces this association.

Another line of inquiry that would be suitable to follow up on concerns the retrograde apoptotic signaling complex induced by NGF deprivation. Although it is parsimonious to infer that endocytosis and GSK3 activation are both involved in the generation of the same signal, strictly speaking this cannot be deduced based on the data collected so far. It would strengthen our conclusion to visualize GSK3 within axons to ascertain whether this kinase localizes to any

particular type of endocytic compartment, which often can be distinguished on the basis of their morphology and characteristic Rab membrane proteins. Of course, it would be especially informative to see if GSK3's localization bears any relation to that of Trk receptors, or if its localization changes along with its activation state given different conditions of NGF signaling.

b. Neuronal maturation and the downregulation of axonal protein synthesis

The observation that embryonic DRG neurons can be made insensitive to NGF deprivation simply by inhibiting axonal synthesis of Lis1 raises fundamental questions about the transient NT dependence of many developing neurons. Upon reflection, it is curious that intra-axonal translation apparently undergoes quiescence over the course of neuronal maturation, in parallel to the neurons themselves becoming less dependent upon neurotrophic support. It could therefore be hypothesized that protein synthesis-dependent mechanisms within axons, such as the Lis1-mediated signaling pathway here identified, are what confer temporary dependence upon NTs to developing neurons. Previous work investigating the basis of neuronal maturation and NT independence has noted that TrkA receptors display more persistent phosphorylation in mature neurons, which endures even in the complete absence of NGF (Tsui-Pierchala and Ginty 1999). This would suggest that, as the neuron matures, TrkA is tuned to be less sensitive for distinguishing between NGF's presence and absence, instead being set by default to the mode of signaling normally associated with its ligand-bound state. Having reached its innervation target and completed its morphological elaboration, it makes sense for the mature neuron to settle into a more maintenance-focused state, which involves re-tuning of its receptors for extrinsic signals and downregulation of the localized protein synthesis pathways that were appropriate for more dynamic circumstances where acute responses were constantly needed. Yet, if later on the axon

faces a dramatic change, such as physical injury or neurotoxic stress, it can reactivate its quiescent mechanisms of intra-axonal protein synthesis in order to mount a rapid and effective response.

c. Differential post-translational modifications of locally synthesized proteins

Although here it has been taken for granted that locally synthesized proteins should be molecularly identical to their cell body-produced counterparts and thus function mechanistically in the same way, it might not be true that axonally and somatically generated versions of the same protein would be entirely equivalent. Theoretically, it would seem practical for the cell to have means of distinguishing proteins based on where they were produced so that it can regulate their activity and lifespan appropriately. One feasible prospect for organizing such control is through differential post-translational modification of proteins depending on their point of origin—the application of a sort of "MADE IN SOMA" or "MADE IN AXON" label to the newly synthesized protein. Lis1 may prove to be an instructive case in this regard. The yeast homologue of Lis1 is a known target of SUMOylation (Alonso, D'Silva et al. 2012), and there are indications of late that this modification may be conserved in mammals (Alonso, Greenlee et al. 2015). While it is not exclusive to axons, covalent attachment of SUMO to proteins has already been shown to occur in axons and may preferentially mark proteins undergoing retrograde transport towards the cell body (van Niekerk, Willis et al. 2007). Given the already-exploited ability to perform axon-specific knockdown of Lis1, it should be straightforward to determine through biochemical methods whether axonally synthesized Lis1 has a preferential disposition to be SUMOylated or not.

Even if there is no detectable difference between the fractions of axonally *versus* somatically derived Lis1 that are marked by SUMOylation, there is another conceivable scenario in which this modification could be particularly important to the regulation of axonally produced protein. Unlike the related ubiquitin modification, SUMO is not conventionally associated with marking proteins for degradation (Mahajan, Delphin et al. 1997), but SUMOylation can occasionally help to recruit specialized ubiquitin ligases and thus initiate ubiquitin-mediated proteolysis (Prudden, Pebernard et al. 2007). Only a few mammalian SUMO-targeted ubiquitin ligases have been identified thus far (Tatham, Geoffroy et al. 2008, Poulsen, Hansen et al. 2013), and, while at least one appears to be expressed in certain types of neurons (Kelly, Thymiakou et al. 2013), there is no indication that these enzymes are localized to axons. Lis1 protein produced in either somata or axons might therefore undergo SUMOylation, but axonal SUMO-Lis1 could be more stable due to the absence of SUMO-targeted ubiquitin ligases in axons. However, if SUMO-Lis1 were carried with motor-cargo complexes back to the cell body, it would then be subjected to ubiquitylation and consequent degradation. Of course, such a system remains speculative and might not be widely generalizable to different axonal proteins. Still, it is clear that, alongside transcript localization and local translation, post-translational modification and the control of protein lifespan merit further investigation as they pertain to the regulation of protein abundance and function within subcellular compartments.

d. Beyond "local" translation: protein synthesis-dependent regulation at a hyperlocal scale

Referring to protein synthesis as "localized" if it occurs somewhere within a subcellular compartment instead of the cell body has been convenient, but it is increasingly becoming obsolete. Axons, for instance, are often upwards of twenty times more voluminous than the cell

body itself (Daroff and Aminoff 2014), and, although "hot spots" for protein synthesis have been visualized within axons (Aronov, Aranda et al. 2002), it has been difficult to establish with certainty the distinguishing characteristics of these hot spots or what cellular substructures they represent. The discovery that APC mediates mRNA localization specifically to microtubule plus-ends and the exploitation of this fact to selectively inhibit an apparent functional subfraction of locally produced protein may therefore represent the crossing of a significant threshold. We are encouraged by these findings to conceive of evermore-defined cellular landmarks, ranging from vesicles and organelles to the plasma membrane to cytoskeletal formations and more, as potential platforms for "hyperlocal" protein synthesis. Consistent with this perspective, a recent report has already implicated mitochondria in coordinating nearby protein synthesis machinery for active translation that is needed at sites of axon branch formation (Spillane, Ketschek et al. 2013).

Probing hyperlocal translation could have a profound impact on our understanding of physiologic systems, including the intracellular transport regime that so much time has been devoted to considering here. The characterization of molecular motors fundamentally changed how we thought about motility in cells, and, for the most part, it drew our attention away from the cargoes themselves. However, the association of protein synthesis machinery with some cargoes might suggest that they can "hitch a ride" with dynein or kinesin motors, effectively regulating their own motility by locally synthesizing adaptor proteins on their surface. In the fungus *Ustilago maydis*, polyribosomes have been seen to be transported on the surface of early endosomes and are likely translationally active there (Higuchi, Ashwin et al. 2014), suggesting the real possibility of such a phenomenon. As our ability to detect and intervene in these kinds of hyperlocal processes improves, it seems quite likely that their study will become an increasingly important part of cell biology.

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