Riding the rails – different modes for RNA complex transport in axons

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Neurons are highly polarized cells with axons that innervate distant targets. The distance of subcellular compartments from the nucleus requires sophisticated transport mechanisms and local action of vital processes for proper function and rapid responses to local stimuli (Terenzio et al., 2017). This is partially achieved by transport of mRNAs to subcellular locations and regulation of local translation for axonal growth, branching, synaptic plasticity, and regeneration, among other needs. Axonally synthesized proteins support neuronal survival, and axonal development, maintenance, and growth (Rishal and Fainzilber, 2014; Dalla Costa et al., 2021). Thus, understanding the mechanisms that promote RNA transport to subcellular locations in neurons will contribute to the development of novel strategies to enhance axon regeneration and survival.

In the axon, a variety of cargos are transported bi-directionally along microtubules to control neuronal functions such as neurite elongation and neuronal polarization. Microtubules are intrinsically polar, with a fast-growing plus end and an opposite slow-growing minus end. In axons, microtubule plus ends extend to the axon terminal while minus ends align towards the cell body. This polarity allows unidirectional movement in the axons with kinesins moving towards the plus end, mediating anterograde transport to axon tips, and cytoplasmic dynein moving towards the minus end, enabling retrograde signaling to cell bodies (Terenzio et al., 2017). The number of cargos far exceeds the number of molecular motors and ranges from membrane organelles through large macromolecular complexes to viruses (Salogiannis and Reck-Peterson, 2017). In addition, defects in microtubule-based transport and mutations in the motors can themselves lead to neurodevelopmental and neurodegenerative disorders (Sleigh et al., 2019).

The canonical view of microtubule-based transport is that specific cargo adaptors recruit molecular motors to cargos. However, an alternative mechanism of cargo motility, termed hitchhiking, was recently described. Evidence suggests some cargos can achieve motility by hitchhiking on organelles that are already linked to motors, rather than directly binding molecular motor complexes themselves. Thus, motile membranous organelles can provide platforms for the movement of other cargos (Salogiannis and Reck-Peterson, 2017). Here we review recent studies on RNA transport in neurons, revealing multiple modes of motility employed by different RNAs and RNA binding proteins (RBPs).

Hitchhiking modes of transport have been described for diverse types of RNAs in axons. microRNAs (miRNAs) are small noncoding regulatory RNAs, which have been identified in the axonal compartment together with their precursor miRNAs (pre-miRNA) (Corradi and Baudet, 2020). How are miRNAs transported and localized to axons? Gershoni-Emek et al. (2018) identified miRNAs, Dicer, and Argonaute-2 in motor neuron axons far from the perinuclear region and demonstrated that these silencing machinery components can be associated with mitochondria. Furthermore, live-cell imaging revealed that miR-124 is actively transported with acidic compartments in axons, and associates with stalled mitochondria at growth cones and axonal branch points, where local translation was shown to occur (Gershoni-Emek et al., 2018). Another recent publication described axonal transport of pre-miRNAs on late endosomes/lysosomes (Corradi et al., 2020). These findings suggest that miRNAs and their precursors, and perhaps also other non-coding RNAs, can be transported to axons in association with different membranous organelles.

mRNAs are transported together with RBPs as ribonucleoprotein particles (RNPs) to distal subcellular locations for local translation. A recent study suggested that certain axonal RNA granules are also transported by associating with endosomes in axons (Cioni et al., 2019). Cioni et al. (2019) reported that RNPs associate with motile Rab7a endosomes along retinal ganglion cell axons, and that these RNP-bearing Rab7a endosomes also associate with ribosomes. In addition, they used live imaging to show that Rab7a endosomes often pause on mitochondria and that these contacts coincide with translational hotspots, suggesting that the RNP-bearing late endosomes are sites of local protein synthesis. Endosomes traffic diverse cargos within axons (Terenzio et al., 2017), and their usage as a platform for transport and localization of RNPs may integrate different pathways.

A third example of a hitchhiking mechanism proposed lysosomes as another transport platform for RNA granules in neurons (Liao et al., 2019). These authors used a combination of proximity labeling proteomics, live-cell microscopy, and *in vitro* biophysical modeling to identify Annexin A11 as a molecular tether that can dynamically couple RNA granules with lysosomes in primary cortical neurons. Annexin A11 possesses an N-terminal low complexity domain, which promotes its phase separation into membrane-less RNA granules, and a C-terminal membrane-binding domain, which enables interactions with lysosomes. The findings suggest that lysosomes may also provide platforms for neuronal RNA transport, recruiting RNA granules through a molecular tether that links the granule to lysosome membranes. Moreover, since the endolysosomal system is very dynamic, the findings of Cioni et al. (2019) and Liao et al. (2019) may reflect a continuum of related membrane-bound organelles as platforms for RNA transport (**Table 1**). These platforms may enable the convergence of miRNAs, mRNA, and mitochondria for regulation of local translation by specific stimuli. The functional roles of such complexes will be of interest for future studies.

In contrast to the above examples of hitching a ride on organelles, other recent studies have described direct interactions of RNAs and their carriers with motors for axonal transport. Baumann et al. (2020) showed that adenomatous polyposis coli protein is linked to the heterotrimeric kinesin-2 KIF3A/B/KAP3 through the cargo adaptor KAP3 to drive the transport of specific axonal mRNA packages (Baumann et al., 2020). Using microscale thermophoresis and *in vitro* motility assays together with TIRF microscopy, Baumann et al. (2020) identified a minimal complex of proteins that can transport mRNAs with 3'UTRs enriched in guanine. Moreover, these G-motif–containing RNA sequences increase transport efficiency and balance access of different mRNAs to the transport system. Although this study has not yet been confirmed *in vivo*, the *in vitro* findings suggest that adenomatous polyposis coli can drive the transport of specific mRNA packages in axons by direct binding to motor proteins. It will be of interest to see if this mechanism is also exploited by other RBPs that bind guanine-rich RNA sequences.

A second motor-based transport system was identified in hippocampal neurons, where the APP tail-1 (PAT1) protein was shown to be involved in the transport of β-actin mRNA into dendrites, by direct binding to the kinesin-I motor complex and the RBP zipcode-binding protein 1 (ZBP1) (Wu et al., 2020). ZBP1 is the main RBP for β-actin mRNA, and the study used yeast two-hybrid to identify PAT1 as an adapter linking ZBP1 and KLC1 or KLC2 (KLC1/2), the cargo-binding subunits of kinesin. ZBP1 binds to the β-actin mRNA 3'UTR concomitantly with N terminal binding to PAT1, thereby bridging the complex to KLC1/2 and activating the kinesin-I motor complex for microtubule-dependent β-actin mRNA transport. This study shows that motor proteins can transport RNA via membrane-free direct protein-protein and protein-mRNA binding interactions.

Fukuda et al. (2021) identified another system of RNA granule transport by direct binding to a kinesin motor. They used live-cell imaging of dorsal root ganglion sensory

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Table 1 | **Transport modes employed by different RNAs and RBPs**

Transport molecules	Mechanism	Main cell type tested	Adaptor/platform	Reference
miR-124, Dicer, Ago2	Hitchhiking	Mouse motor neurons	Acidic compartments	Gershoni-Emek et al., 2018
Axonal RNA granules	Hitchhiking	Xenopus retinal ganglion cells	Endosomes	Cioni et al., 2019
Axonal RNA granules	Hitchhiking	Rat cortical neurons	Lysosomes via ANXA11	Liao et al., 2019
pre-miRNAs	Hitchhiking	Xenopus retinal ganglion cells	Late endosomes/ lysosomes	Corradi et al., 2020
APC and associated mRNAs	Direct	Mammalian cell lines	KAP3 to kinesin-2	Baumann et al., 2020
β-Actin mRNA, RBP ZBP1	Direct	Mouse hippocampal neurons	PAT1 to kinesin-I	Wu et al., 2020
Nucleolin and associated mRNAs	Direct	Mouse DRG sensory neurons	KLC2 and Kif5A	Doron-Mandel et al., 2021
SFPQ and associated mRNAs	Direct	Rat DRG sensory neurons	KLC1 and Kif5A	Fukuda et al., 2021

Ago2: Argonaute-2; ANXA11: Annexin A11; APC: adenomatous polyposis coli; DRG: dorsal root ganglion; KAP3: kinesin-associated protein 3; KLC: kinesin light chain; PAT1: APP tail-1; RBPs: RNA binding proteins; SFPQ: splicing factor proline/glutamine-rich; ZBP1: zipcode-binding protein 1.

neurons to show that splicing factor proline/glutamine-rich (SFPQ) - containing transport granules move in axons with microtubule motors. SFPQ co-immunoprecipitation and mass spectrometry analyses identified KIF5A and KLC1 as the major molecular motors responsible for anterograde axonal transport of SFPQ. Both KLC1 and KIF5A have highly divergent C-terminal regions, which ensures specificity of binding in this case. Furthermore, these authors showed that RNA molecules are required for SFPQ-RNA interaction with KIF5A/KLC1 (Fukuda et al., 2021). The findings suggest that binding to RNA cargos may induce a conformational change in SFPQ that facilitates axonal transport of SFPQ RNA granules by binding to KIF5A and its cargo adaptor KLC1, a mechanism that may be mirrored by other membrane-free RBP-kinesin complexes. Structural studies on SFPQ complexed with different RNA cargos will be of interest to shed further light on this mechanism.

We recently described the motor-binding mechanism for the RBP nucleolin in axons, establishing another example of a membrane-less RBP-kinesin complex (Doron-Mandel et al., 2021). Nucleolin is a multifunctional RBP, which we previously showed to be important for axonal transport of mRNAs that regulate neuronal growth and survival (Perry et al., 2016; Terenzio et al., 2018). Our recent study used biochemical assays and proteomics to show that kinesin-mediated transport of nucleolin is dependent on its Glycine-Arginine-Rich (GAR) domain in dorsal root ganglion sensory neurons. The nucleolin-kinesin interaction is direct, mediated by binding of the GAR domain to KLC2 (and likely also other KLCs). We generated a nucleolin GAR deletion mouse model by gene editing, and although the homozygous mutant is embryonic lethal, experiments with heterozygotes confirmed that the GAR domain is required for axonal localization of nucleolin and its cargo mRNAs (Doron-Mandel et al., 2021). Moreover, heterozygous GAR deletion neurons show accelerated outgrowth in culture, and expression of the GAR domain as a dominant-negative has similar effects, in line with the predictions of our previously proposed length sensing and neuron growth control model (Rishal et al., 2012). Finally, the GAR domain also functions in plasma membrane association and nucleolar localization of nucleolin. We suggest that GAR domains may act as multifunctional subcellular localization elements for a range of RBPs, potentially explaining their implication in different neurodegenerative diseases.

In summary, a flurry of recent studies has reported multiple modes of transportation employed by different RNAs and RBPs in axons (**Table 1**). The studies highlight diverse carriers for mRNA transport to axons, utilizing both membrane-associated and membrane-free transport modalities. A number of questions arise, for example, if and how local translation might be regulated on or within these carriers? How do axons avoid or regulate the degradation of RNA or nascent proteins by lysosomes when those are part of the carrier complex? How can neurons regulate the recruitment of RBPs to motor proteins? Future studies on these and related questions will shed light on the dynamics and specificity of these different transport complexes and will be critical to devise specific perturbations to address their physiological importance in axonal maintenance and regeneration.

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