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The travels of mRNAs in neurons: *Do they know where they are going?*

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Abstract

Neurons are highly polarized cells that can extend processes far from the cell body. As such, transport of messenger RNAs serves as a set of blueprints for the synthesis of specific proteins at distal sites. RNA localization to dendrites and axons confers the ability to regulate translation with extraordinary precision in space and time. Although the rationale for RNA localization is quite compelling, it is unclear how a neuron orchestrates such a complex task of distributing over a thousand different mRNAs to their respective subcellular compartments. Recent single-molecule imaging studies have led to insights into the kinetics of individual mRNAs. We can now peer into the transport dynamics of mRNAs in both dendrites and axons.

The local transcriptome

Advances in sequencing technology have revealed thousands of different messenger RNAs in neuronal processes [1-5]. The significance of this is that much of the entire transcriptome is present in dendrites and axons, and that RNA localization is a prevalent method to achieve protein sorting. How such a large pool of transcripts is trafficked through extensive neuronal processes, arrive at the correct location and are translated there in response to some cue remains to be elucidated [6,7]. The synergy of endogenous mRNA labeling techniques concurrent with high-resolution microscopy have been essential in expanding our understanding of the kinetics of mRNA transport in the neuron.

RNA transport: structure and composition of the granule

RNA transport granules are higher-order assemblies of RNAs and proteins representing functional collections of multiple messenger ribonucleoprotein (mRNP) complexes traveling along neuronal processes [8,9]. The exact size and composition of these transport granules remain largely undetermined and their role in mRNA localization and translational regulation has been discussed extensively [10,11]. In the mammalian brain, increasing numbers of mRNAs containing localization elements or ‘zipcodes’ have been identified in

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neuronal processes including those encoding structural proteins (β -actin, MAP2, PSD-95) [12-14], receptors (GluA1, GluA2) [15], and signaling molecules (BDNF, CaMKII α , mTOR) [16-19]. These *cis*-acting sequences usually located in the 3'UTR of mRNAs are recognized by specific RNA-binding proteins (RBPs) or *trans*-acting factors and assemble into an mRNP. Next, the RNA-protein complex can mediate interactions with the translational machinery and self-assemble into transport granules [16,20]. One of the most abundant and well-characterized mRNP in both axons and dendrites is β -actin; and its association with zipcode binding protein 1 (ZBP1/IGF2BP1) is critical for transport and proper localization [12,21-23].

Once packaged, mRNPs are transported along the neuronal cytoskeleton by a superfamily of molecular motors: kinesins, dyneins and myosins [24,25]. In both axons and dendrites, most anterograde transport along microtubules (MT) is performed by kinesins and particularly by the plus-end directed motor, KIF5 [26]. Cytoplasmic dyneins are the minus-end directed motors, mediating retrograde transport back to the cell body [27] in axons and distal dendrites. Dyneins have been observed performing movements in both directions in proximal dendrites due to the mixed polarity of MT. Although the cytoskeletal polarity is an important determinant, often the net movement of a particular mRNP is dependent on the stoichiometry of the motors bound to it. The combined forces of the motors result in 'a tug of war' where the mRNP moves in the direction of the greater net force. Such scenarios have been extensively explored for various cargo transport [28] including mRNPs in oligodendrocytes [29] and seems to be a general rule for trafficking along processes (Figure 1).

RNA transport: the granular conundrum

Since the characterization of transport granules from the mouse brain, attempts to develop a unified model of how mRNAs, RBPs and motors associate has been challenging due to the intrinsic complexity and heterogeneity of transport granules [26,30]. With so many RBPs capable of recognizing RNA, it is difficult to establish clear rules regarding RNA-protein interactions [31]. According to the 'RNA granule hypothesis,' mRNAs have sequences that are recognized by a set of RBPs in the nucleus that changes composition as the mRNP travels to its cytoplasmic destination [32]. To complicate matters further, each RBP can bind to multiple transcripts simultaneously—for instance, the Fragile X mental retardation protein (FMRP) is a component of multiple mRNPs and binds to mRNAs such as Arc, CaMKII α , PSD-95 [33]. RBPs such as Staufen and FMRP can bind to complex secondary or tertiary RNA structures in the form of stem-loops [30,34,35]. This presents a combinatorial problem where each mRNA is capable of binding a discrete subset of RBPs with different affinities and where each RBP is shared by multiple transcripts.

An unresolved question in mRNP formation is whether RBP binding to the zipcode of mRNAs is sufficient to package them into 'transport-ready' granules and where in the cell this happens. In other words, the exact mechanism by which RBPs engage the motor protein is still not clear. Two possibilities exist: the RBPs interact directly with the motor or indirectly through an adapter protein as part of an mRNP complex [36]. Previously, a direct association between FMRP and kinesin had been shown [37], suggesting that transcripts

bound by FMRP are capable of transport along MT tracks. A different approach utilized *In vitro* reconstituted mRNPs to determine the relative contributions of individual components in transport. This demonstrated that the mRNA can play a pivotal role in enhancing the processivity of mRNPs on MT [38]. Although adaptors are necessary to mediate interactions with motors, perhaps the RNA cargo can contribute to the efficiency of the motor.

The heterogeneity and diversity of mRNPs segues into important questions about assembly—whether different mRNA species co-assemble into the same granule, share a core subset of RBPs, or if granules are homotypic and contain the same mRNA species in single or multiple copies. There is growing evidence for the latter, with every species of mRNA traveling singly and independently as shown by single-molecule fluorescence in situ hybridization (FISH) studies of different dendritically localized mRNAs [39,40] and by real-time imaging of endogenous β -actin mRNA in axons [23]. From the neuron's perspective, transporting mRNAs individually over long distances is not the most parsimonious strategy, and it would be energetically favorable to package multiple copies into a single mRNP. Simultaneous imaging of different mRNAs and RBPs at various stages of the mRNA life cycle as it gets exported from the nucleus would be highly informative. Recently, a super-registration method to identify mRNA-protein interactions *In situ* has been developed, enabling characterization of the RBPs of β -actin mRNA, and their degree of association [41]. Such approaches combined with biochemical studies (e.g. CLIP) [33] of other dendritic mRNAs in the future can provide insights into the RNP composition of each mRNA with high spatial resolution along the length of the dendrite.

Although we know much about the components of mRNPs, questions about granule dynamics and maintenance persist—do mRNAs or RBPs play an instructive role in assembly and higher-order clustering of multiple mRNPs together into a single granule? Recent work on stress granules highlighted the presence of intrinsically disordered regions on RBPs that can phase separate into liquid droplets [42]. Phase separation into granule-like structures was greatly facilitated by the presence of RNA [42–44]. One can envision that interactions between disordered regions of RBPs coupled with multiple RNAs can contribute to granule dynamics *In vivo*. Such findings underscore the importance of the fine control exerted by all the different components of the mRNP in determining the precise localization of mRNA for neuronal gene expression.

mRNA transport in dendrites

The dendritic tree with its branched morphology represents a complex maze for mRNA transport. Real-time imaging and tracking of mRNAs have allowed us to understand the basic rules of transport behavior (Box 1). Interestingly, endogenous and reporter mRNAs containing zipcodes in their 3'UTRs exhibited similar velocities during directed motion—which is consistent with the *cis*-acting element being the critical determinant of dendritic transport. Movement of mRNA appear confined at first with the majority of the molecules exhibiting either stationary or corralled motion at steady-state. This is best illustrated by β -actin mRNA, where 80% of which were largely stationary over a 5 second time window [45]. However, this observation was dependent on the timescale of detection, since longer imaging intervals (minutes timescale) revealed that 90% of the mRNAs had the capacity to

move [21]. When the mRNA particles were in motion, they would either move processively (0.5–2.0 $\mu\text{m/s}$) or in a series of short distances (few microns) intervened by short pauses (<10 s), or remain corralled (diffusion within a small volume of space). The directed movement is indicative of motor-driven transport along MT tracks, with instantaneous velocities ranging from 0.5–5 $\mu\text{m/s}$ [46].

Dendritic mRNAs can move in either direction, or switch directions—depending on the combined force of the bound motors (see previous section) and the orientation of the MT. Similar bidirectional movement has been observed for Arc, another activity-regulated mRNA with reported velocities similar to β -actin [47]. Interestingly, the authors found that inhibiting neuronal activity did not alter the directionality or velocity of Arc mRNAs, suggesting that once mRNPs are loaded onto MT tracks, the processive movements are determined by the balance of the motors. Collectively, all dendritic mRNAs exhibit bidirectional motion, but with a slight bias towards the anterograde which allows them to be delivered to the distal dendrite to participate in local translation when needed.

mRNA transport in axons

Axons exemplify the need for localized RNA as they can project over long distances and can reach a meter long in mature mammals. To characterize the kinetics of endogenous mRNA movement in axons, Holt and coworkers used molecular beacons (MB) to label individual β -actin mRNAs at single-molecule resolution [23]. MB are short antisense oligonucleotides with a fluorophore and a quencher on either end. When the MB is unbound, it forms a stem-loop which brings the fluorophore and the quencher in close proximity and does not emit light (dark); when the MB is bound to its target, the stem-loop opens up and separates the fluorophore from the quencher and allows it to emit light. The specificity of binding results in very low background and when many MB are bound to the mRNA, it yields sufficient signal for single-molecule tracking for analyses within axons. They observed directional anterograde transport ($1.1 \pm 0.08 \mu\text{m/s}$) followed by pausing or retrograde transport ($0.81 \pm 0.05 \mu\text{m/s}$) which resulted in a slight bias towards the anterograde. Intriguingly, two different modes of movement were detected for both directions. The complex motions of endogenous mRNA were modeled using a Hidden Markov-Bayesian algorithm (Box 1) to elucidate β -actin mRNA displacements [48]. They concluded that about 14% of the observed β -actin mRNA trajectories exhibited directed transport in axonal shafts.

How is RNA transport and translation regulated by synaptic activity and local signaling?

To date, the most well-characterized species of localized mRNP is β -actin, where the mRNA is associated with ZBP1 and ribosomes in a translationally repressed granule. Upon stimulation, the mRNPs become unmasked and release a translatable pool of β -actin mRNA in distal dendrites [45,49]. Moreover, it has been shown that neuronal activity increases the frequency of anterograde movement for more efficient localization of mRNAs to dendrites. This has been observed for CaMKII α and β -actin mRNA containing mRNPs [50], and is dependent on neuronal depolarization and NMDA receptor activation. Similar regulation of increased dendritic trafficking was observed for mRNAs encoding AMPA receptor subunits

GluA1 and GluA2 upon metabotropic glutamate receptor activation [15]. However, it is not obvious from these studies whether enhanced movement enabled proper localization of mRNAs and productive translation to influence synaptic plasticity. A recent study using glutamate uncaging to locally activate a subset of dendritic spines has shed new insight into this question [21]. Upon local glutamate release by UV-photouncaging, endogenous β -actin mRNAs localized to the stimulated dendritic spines with high efficiency, resulting in an accumulation of mRNAs over two hours. This phenomenon was sensitive to NMDA receptor antagonists, suggesting that Ca^{2+} influx through NMDA receptors signals to the base of the spine to capture transporting mRNPs. The findings in this study provide evidence in favor of the ‘sushi belt model’ of mRNA localization [51], where β -actin mRNAs are cruising along the dendrites and patrolling through multiple synapses until they are captured by the recently activated synapses to anchor and translate (Figure 2). Finally, using a Halo-tagged β -actin reporter, the authors showed that once an mRNA localizes to an activated spine, it persists there for hours undergoing multiple rounds of translation. This newly synthesized pool of β -actin protein may play a distinct role in spine enlargement and synaptic consolidation. Although it supports the ‘patrol and local entrapment’ model as a general mechanism of how local activity can capture mRNAs, further studies are needed to prove its applicability and physiological relevance to other dendritically localized mRNAs with varying levels of abundance.

On the other hand, it has been challenging to study presynaptic RNA localization due to the difficulty of manipulating and imaging discrete axonal projections in the brain. Nevertheless, new methods are being developed to study presynaptic protein synthesis. Through the use of slice electrophysiology, combined with membrane-permeable and -impermeable translation inhibitors, it was recently shown that long-term decreases in neurotransmitter release required newly synthesized proteins [52]. Detection of ribosomes in the presynaptic compartments using super-resolution imaging supported this interpretation, although the presence of mRNA was inferred. Development of a calcium indicator targeted to the axonal endoplasmic reticulum [53] could be used to ask whether local calcium plays a role in presynaptic plasticity and correlate with axonal RNA trafficking. Taken together, these tools offer new strategies to address whether activity-dependent RNA localization occurs on both sides of the synapse.

The Last Mile Problem

The last mile is a term in supply chain logistics which describes the final leg of transportation or movement of goods that is disproportionately less efficient. How each species of mRNA reaches its destination presents a similar problem. The complex organization of the cytoskeleton in dendrites and axons (i.e. the mixed polarity of microtubules and polarized actin filaments) along with the engagement of the many different motors provides a complicated distribution model. Over a thousand different mRNAs need to find their way through distal locations within axons and dendrites. These imply that the mRNAs search for their final destination by moving on and off the tracks, parking in specific locations and translating relevant proteins there. There are also technical limitations to observing these events in living cells. To visualize the RNA localization machinery such as mRNAs, adaptor proteins, molecular motors and translated proteins simultaneously at high

resolution, novel orthogonal tagging systems beyond fluorescent proteins are needed for multiplex imaging of many different components [21,23,54-56]. Such advances will aid in the development of strategies to see how a neuron handles the constant delivery of multiple mRNPs to its processes.

Perspectives

The relevance of transport mRNPs *in vivo* and their role in synaptic tagging still remains to be addressed [57]. It has been almost 20 years since Arc/Arg3.1 mRNA was shown localized to dendritic fields after high-frequency stimulation of the perirhinal path synapses [58]. This and subsequent studies in dissociated culture and *in vivo* have unambiguously highlighted the importance of NMDA receptor-mediated Ca^{2+} influx in driving mRNA localization to activated dendritic spines. Various subtypes of glutamate receptors and their differential contributions to long-lasting forms of plasticity imply how these receptors individually or in concert could influence RNA localization. But, real-time imaging of mRNA transport in awake animals has not been possible. Thus, direct evidence linking the specific role of RNA transport to behavior or brain function is lacking. Innovative brain tissue and *in vivo* imaging methods are needed to elucidate the functional role of RNA localization within intact circuits, as well as in behaving animals to provide the physiological context to local protein synthesis.

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Box 1:**Analyzing mRNA trajectories and inferring dynamics**

Direct visualization and tracking of single mRNAs are routinely performed in laboratories to understand the dynamics of mRNAs with high spatiotemporal resolution. Improvements in imaging technology has enabled tracking of mRNAs (or mRNPs) with unprecedented temporal resolution. Widely used methods for analyzing mRNA movement primarily relies on either generating kymographs for individual trajectories or calculating mean square displacements (MSD) from pooled trajectories. From the kymographs, instantaneous velocities can be measured by determining the slope of the run phases. In contrast, the MSD analyses provide a time-averaged estimate of the diffusion behavior of the mRNAs. Although the analysis is based on individual trajectories, the final readout of the MSD is indicative of the population behavior. Both kymographs and MSD plots can qualitatively confirm stationary and active transport of mRNPs, however detailed quantification of the exact mRNA diffusion along the length of the trajectory is lost. Because a high degree of heterogeneity exists in mRNA movements, where they can stochastically switch between several diffusive states, methods like Hidden Markov Modeling (HMM) provide powerful means to infer transient states from complex trajectories. By applying Bayesian statistics, one can determine the best possible stochastic motion model to describe a given trajectory (i.e. 2-state vs 3-state, so on) and has led to its prominence in analyzing single-molecule tracks of RBPs and mRNPs, as well as surface dynamics of receptors and channels. Using a carefully annotated HMM approach, Bathe and colleagues [48] have applied the Bayesian procedure to correctly identify and characterize directed transport (both retrograde and anterograde) interspersed with random pausing events—a characteristic feature of mRNPs. Besides, one can also calculate the lifetime of each annotated motion state, providing high-resolution temporal insights into the transient nature of the mRNP transport. Finally, the distributions of state lifetimes, the diffusion and the velocity magnitudes across a pool of mRNPs from multiple neurons can be determined to get the average behavior in a population.

Figure Box1:

mRNP trajectory analysis methods, as adapted from Monnier *et al.* Nature Methods 2015 [48]. (A) Snapshot from live imaging of MS2-tagged β -actin mRNAs in dendrites. Particle trajectory of a single mRNP and its kymograph. (B) Analysis of the mRNP trajectory with a diffusive-only HMM approach versus HMM-Bayes approach, which accounts for both diffusive state (D, blue) and active transport state (DV, pink). The states are annotated along the entire trajectory with the time spent in each state.

Figure 1.

Snapshot of mRNA transport in neuronal processes. In both dendrites and axons, mRNPs (complexes of mRNAs with RBPs) are actively transported along MT tracks (minus-ends depicted as boxes). The MT are of mixed orientation in proximal dendrites and more uniform polarity in distal dendrites or axons. The oscillatory behavior of actively transported mRNAs is depicted with the arrows indicating the possible movement directions, anterograde in green versus retrograde in red.

The mRNPs traveling in axons and dendrites share common features and the inset shows a magnified view of an mRNP associated with the motors dynein and kinesin. Different ratios of the motors associated with the mRNP represent the ‘tug of war’ scenario where the movement is determined by the net combined force. In dendrites, mRNAs can be localized along the entire length of the dendrite often near the base of a stimulated dendritic spine. For axons, the final destination is the growth cone, where the mRNAs undergo corralled diffusion.

Figure 2.

Model of mRNA localization in dendrites following local synaptic stimulation. (A) Dendritic mRNPs (exemplified by β -actin) can exhibit a scanning behavior like a circling conveyor belt referred to as the ‘sushi belt model’. (B) Following stimulation, these mRNPs localize to the activated spines with high efficiency. This represents the ‘synaptic tagging and capture’ scenario where β -actin mRNAs are specifically anchored near activated synapses, and undergo local translation to generate a pool of new β -actin proteins which can be incorporated into the expanding dendritic spine structure. This model of structural plasticity underscores the importance of mRNAs localization and local translation. The exact mechanism of how the capture occurs, such as the presence and the role of an anchoring protein (i.e. ZBP1/IGF2BP1) in localization and persistence of the mRNA at the designated dendritic spine needs further elucidation.