Dynein-Dependent Transport of *nanos* RNA in *Drosophila* Sensory Neurons Requires Rumpelstiltskin and the Germ Plasm Organizer Oskar

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Intracellular mRNA localization is a conserved mechanism for spatially regulating protein production in polarized cells, such as neurons. The mRNA encoding the translational repressor Nanos (Nos) forms ribonucleoprotein (RNP) particles that are dendritically localized in *Drosophila* larval class IV dendritic arborization (da) neurons. In *nos* mutants, class IV da neurons exhibit reduced dendritic branching complexity, which is rescued by transgenic expression of wild-type *nos* mRNA but not by a localization-compromised *nos* derivative. While localization is essential for *nos* function in dendrite morphogenesis, the mechanism underlying the transport of *nos* RNP particles was unknown. We investigated the mechanism of dendritic *nos* mRNA localization by analyzing requirements for *nos* RNP particle motility in class IV da neuron dendrites through live imaging of fluorescently labeled *nos* mRNA. We show that dynein motor machinery components mediate transport of *nos* mRNA in proximal dendrites. Two factors, the RNA-binding protein Rumpelstiltskin and the germ plasm protein Oskar, which are required for diffusion/entrapment-mediated localization of *nos* during oogenesis, also function in da neurons for formation and transport of *nos* RNP particles. Additionally, we show that *nos* regulates neuronal function, most likely independent of its dendritic localization and function in morphogenesis. Our results reveal adaptability of localization factors for regulation of a target transcript in different cellular contexts.

Introduction

Intracellular mRNA localization is a conserved mechanism for spatially regulating protein production in polarized cells, such as neurons (Holt and Bullock, 2009; Martin and Ephrussi, 2009). In neurons, mRNA localization targets the expression of specific proteins to dendrites or axons to confer the unique structural and functional properties of these compartments. Requirements for localization of mRNAs to neuronal processes have been demonstrated in dendrite morphogenesis, growth cone navigation, synaptogenesis, and synaptic plasticity (Holt and Bullock, 2009; Doyle and Kiebler, 2011).

We previously identified a role for mRNA localization in dendrite morphogenesis in *Drosophila* larval class IV dendritic arborization (da) neurons. These sensory neurons elaborate highly branched dendritic trees that innervate the larval epidermis and function in nociception and light avoidance (Hwang et al., 2007; Xiang et al., 2010). Maintenance of these complex dendritic trees requires the translational repression complex formed by Nanos (Nos) and Pumilio (Pum; Ye et al., 2004; Brechbiel and Gavis,

2008; Olesnick et al., 2012) and consequently *nos* and *pum* mutants exhibit reduced dendritic branching complexity. *nos* mRNA forms particles that are dendritically localized and the *nos* mutant dendritic defect can be rescued by transgenic expression of wild-type *nos* mRNA but not by a localization-compromised *nos* derivative, indicating that dendritic localization of *nos* and local Nos synthesis are essential for dendrite morphogenesis (Brechbiel and Gavis, 2008). Nos and Pum also have non-neuronal functions, including embryonic abdomen formation and germline development. These, too, require spatially regulated Nos synthesis, which is achieved through localization of *nos* mRNA to the specialized germ plasm at the posterior of the embryo (Becalska and Gavis, 2009; Lasko, 2011).

Targeting and transport of mRNAs to their proper destinations is a highly regulated process. Studies in various cell types have shown that mRNAs are transported as ribonucleoprotein (RNP) particles, most often by motor proteins traveling on microtubules or actin filaments. These particles are assembled through the recognition of *cis*-acting localization signals by RNA-binding proteins, and the recruitment of additional factors, including translational repressors and motor proteins (Martin and Ephrussi, 2009). Several RNA-binding proteins, including Staufen (Stau) and Fragile X mental retardation protein (FMRP), have been identified in neuronal granules and have been shown to play roles in dendritic mRNA transport in cultured mammalian hippocampal neurons (Tang et al., 2001; Duchaine et al., 2002; Dichtenberg et al., 2008). These granules also contain kinesin motors, which are thought to mediate their transport into the dendrites (Jeong et al., 2007; Dichtenberg et al., 2008). FMRP par-

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ticle trafficking has also been observed in *Drosophila* neurons, in this case requiring components of the dynein motor machinery (Bianco et al., 2010). Less is known about the requirements for transport of specific mRNAs, however.

We have previously shown that localization of *nos* occurs before fertilization, with *nos* accumulating in the germ plasm at the posterior of the oocyte through a diffusion and entrapment process independent of microtubules (Forrest and Gavis, 2003). This localization requires the *Drosophila* heterogeneous nuclear RNP (hnRNP) M homolog Rumpelstiltskin (Rump), which binds directly to *nos* mRNA, and the germ plasm organizer Oskar (Osk; Wang et al., 1994; Sinsimer et al., 2011). Localized *nos* RNP particles are subsequently segregated to the germ cell progenitors as they form at the posterior of the embryo, through microtubule-dependent transport (Lerit and Gavis, 2011).

Here we have investigated the mechanism of dendritic *nos* mRNA localization by analyzing requirements for *nos* RNP particle motility in class IV da neuron dendrites of intact larvae, through live imaging of fluorescently labeled *nos* mRNA. We show that dynein motor machinery components mediate transport of *nos* mRNA in proximal dendrites. In addition to their roles in the diffusion/entrapment-mediated localization of *nos* in the oocyte, Rump and Osk function in da neurons for the formation and transport of *nos* RNP particles. Additionally, we show that *nos* regulates neuronal function, most likely independent of its dendritic localization and function in morphogenesis. Our results reveal adaptability of localization factors for regulation of a target transcript in different cellular contexts.

Materials and Methods

Fly strains and genetics. The following transgenic strains were used: *UAS-MCP-RFP*; *nos-(ms2)₁₈* (Brechtel and Gavis, 2008); *osk-(ms2)₆* (Lin et al., 2008); *UAS-lacZ-MS2bs* (van Gemert et al., 2009), and *UAS-RFP.nls* (Lipsick and Bilder, personal communication to FlyBase). The following mutant alleles and allelic combination were used: *nos^{RC}/nosRD* (Curtis et al., 1997); *pum^{ET9}/pum^{ET7}* (Lehmann and Nüsslein-Volhard, 1987); *Dhc⁶⁻¹²/Dhc⁸⁻¹* (McGrail et al., 1995; Gepner et al., 1996); *BicD^{18a}/BicD²⁶* (Mohler and Wieschaus, 1986); *egl^{wu50}/egl^{3e}* (Mach and Lehmann, 1997; Navarro et al., 2004); *Khc¹⁷/Khc⁸* (Saxton et al., 1991; Brenda et al., 1999); *osk⁵⁴/osk⁸⁴* and *osk⁵⁴/osk^{A87}* (Lehmann and Nüsslein-Volhard, 1986, 1991; Vanzo and Ephrussi, 2002); *rump¹* (Jain and Gavis, 2008). Mutant analysis necessitated the use of two different combinations of class IV da neuron GAL4 drivers recombined with *UAS-mCD8:GFP* transgenes. *GAL4⁸⁻¹²³*, *UAS-mCD8:GFP* on the third chromosome (Ye et al., 2004) was used in combination with mutants on the second chromosome; *GAL4⁴⁷⁷*, *UAS-mCD8:GFP* on the second chromosome (Grueber et al., 2003) was used in combination with mutants on the third chromosome. *UAS-RNAi* lines were obtained from the Vienna *Drosophila* RNAi Center (*Dhc*: 28054; *BicD*: 27683; *egl*: 21779; *osk*: 107546; *rump*: 44659) and the Harvard Transgenic RNAi Project collection (*pum*: JF02267; *Khc*: HMS01519). The *UAS-nosRNAi* transgenic lines are described by Menon et al. (2009). *UAS-KhcRNAi* was expressed using the *GAL4⁴⁷⁷* driver, and *ppk-mCD4-GFP* (Han et al., 2011) was simultaneously used to label the neurons. All other *UAS-RNAi* lines were expressed using *pickpocket1.9-GAL4* (*ppk-GAL4*; Ainsley et al., 2003) together with *UAS-dicer2* (nociception assay) or a recombinant *ppk-GAL4*, *UAS-mCD8:GFP* chromosome together with *UAS-dicer2* (morphology analysis) at 25°C (Zhong et al., 2010).

Live imaging and analysis of particle motility. Larvae were anesthetized and mounted for imaging as described in Brechtel and Gavis (2008) except that a 1:5 chloroform/halocarbon oil 700 mixture was used. Neurons were imaged within 15 min of exposure to anesthesia on a PerkinElmer spinning disc microscope equipped with an EM-CCD camera (Hamamatsu), used with a 60×/1.4 numerical aperture (NA) oil-immersion objective. Movies were captured at 400 ms/frame rate in a single focal plane over a range of 30–120 s as necessary to minimize photobleaching and photo-

toxicity. The dorsal-most class IV da neuron, ddaC, from abdominal segments 4–6 was imaged. Particles were tracked semimanually with the Volocity software program (PerkinElmer). Average particle velocities were measured for individual runs of single particles that were visible in at least three consecutive movie frames and the mean average velocity was calculated. Static and motile particle number was quantified from the first 75 frames of each movie and normalized to dendrite length. Statistical significance was determined by the two-tailed Student's *t* test. Net displacement of a motile particle was measured by determining the vector between its positions at the start and end of the imaging period. The χ^2 goodness-of-fit test was applied to test a 1:1 ratio of anterograde-to-retrograde displacement.

Imaging and quantitation of dendrite morphology. Dendrite morphology in late (wandering) third instar larvae was analyzed by direct fluorescence imaging of anesthetized larvae, prepared as described above. Neurons were imaged using a Leica SPE microscope with a 20×/0.7 NA air objective. For quantitation of dendrite termini, neurons were either imaged using a Zeiss LSM510 microscope with a 40×/1.2 NA water-immersion objective (neurons visualized using *GAL4⁸⁻¹²³* and *UAS-mCD8:GFP*) or a Leica SPE microscope using a 40×/1.25 NA oil-immersion objective (neurons visualized using *GAL4⁴⁷⁷* and *UAS-mCD8:GFP* or *ppk-GAL4* and *UAS-mCD8:GFP*). Since the whole dendritic arbor is larger than the 40× objective field, neurons imaged with 40× objectives were imaged in overlapping segments to re-create dendritic fields extending from cell body to dorsal midline. Quantitation of *Dhc*, *egl*, and *BicD* mutant neurons in Figure 2 was performed using a semi-intact larval preparation, which leads to a lower termini number than that of the anesthesia-based preparation.

Branching complexity was quantified in z-series projections of ddaC neurons by counting the total number of terminal branches in a field extending from the cell body to the midline. One neuron from abdominal segment 4, 5, or 6 was analyzed per larva. The two-tailed Student's *t* test was performed to determine significance between datasets.

Nociception assay. Wandering third instar larvae were rinsed with distilled water and placed on apple juice agar egg collection plate and covered with a thin layer of water to keep the larvae moist. A noxious mechanical stimulus was delivered by firmly pressing on the larval cuticle on the dorsal side in abdominal segments 4, 5, or 6 with an insect pin (0.1 mm Minutien pins, Fine Science Tools) held at an angle between a pair of forceps. Each larva was tested once and discarded. A positive score required at least one stereotypic corkscrew rolling response following 1–2 mechanical stimuli. Data were analyzed using the χ^2 test.

Results

nos mRNA particles are transported in da neuron dendrites

Previously, we visualized *nos* mRNA in class IV da neurons *in vivo* by fluorescent labeling with the MS2/MS2 coat protein (MCP) tethering system. MS2-tagged *nos* mRNA labeled with MCP-RFP (*nos^{*}RFP*) forms motile particles that localize to dendritic branches (Brechtel and Gavis, 2008). Here we characterize *nos* particle motility in detail by rapid live-cell imaging under more sensitive microscopy conditions than used previously. Because we had observed that MCP-RFP can form nonspecific particles in da neurons in the absence of MS2-tagged RNA, presumably due to overexpression and self-association (Brechtel and Gavis, 2008), we also analyzed control neurons expressing only MCP-RFP. Three different combinations of da neuron-specific *GAL4* drivers and independent *UAS-MCP-RFP* transgenic lines were tested, with or without MS2-tagged *nos* mRNA, expressed under control of its own promoter.

During the brief imaging periods (see Materials and Methods), the majority of particles detected in dendrites of class IV da neurons expressing either MCP-RFP together with MS2-tagged RNA (referred to hereafter as *nos^{*}RFP* neurons for simplicity) or MCP-RFP alone (referred to hereafter as MCP-RFP-only neurons) appeared to be stationary or jiggling in place, as previously

Figure 1. Visualization of RNP particles in da neuron dendrites. Time-lapse image sequence of a *ddaC* neuron expressing *nos* RFP*. Individual frames are shown at 10 s intervals as noted. Colors are used to highlight examples of motile particles (arrowheads) and static/jiggling particles (dots) within a given branch over the time series. The right-most magenta arrowhead indicates a particle that moves out of the field while the left-most magenta arrowhead indicates a particle that becomes difficult to differentiate from static particles near a branchpoint (see also Movie 1).

Movie 1. Confocal time-lapse movie of a larval class IV da neuron (*ddaC*) expressing *nos* RFP*. Images were captured at 2.3 frames/s and played back at 20 frames/s. Arrows indicate movement of particles.

observed for RNP particles in cultured neurons (Rook et al., 2000; Bianco et al., 2010; Tübing et al., 2010). In addition, we detected particles that exhibited directed movement (defined as motile particles) in both *nos* RFP* and MCP-RFP-only neurons (Fig. 1; Movie 1; data not shown). Unidirectional movement, both retrograde and anterograde, and bidirectional movement, in which particles switched directions, were observed. Similar bidirectional transport of RNP particles has been observed within dendrites from other types of neurons (Doyle and Kiebler, 2011). Motile particles moved through dendritic branch points, from proximal dendrites to distal dendrites and vice versa, but not through branch points from one distal dendrite to another.

Quantification of the total number of dendritic particles normalized to dendrite length shows a trend toward a greater number of particles in dendrites of *nos* RFP* neurons versus MCP-RFP-only neurons (Fig. 2A). Most significantly, the fraction of particles that were motile was consistently higher in *nos* RFP* neurons, with nearly twice as many motile particles as in MCP-RFP-only neurons

(Fig. 2A). In addition, the average velocities of motile particles were significantly greater in *nos* RFP* neurons than in MCP-RFP-only neurons (mean average velocity, 0.55–0.74 $\mu\text{m/s}$ for *nos* RFP* neurons vs 0.35–0.50 $\mu\text{m/s}$ for MCP-RFP-only neurons; Fig. 2B). Analysis of the net displacements of motile particles showed a trend toward higher proportions of motile particles undergoing anterograde transport in *nos* RFP* neurons than in MCP-RFP-only neurons (on average 49 vs 37%; Fig. 2C).

In contrast, when a control MS2-tagged *lacZ* mRNA was expressed in class IV da neurons together with MCP-RFP (*lacZ* RFP*), we observed only a background level of motile particles with average velocities characteristic of MCP-RFP-only particles (Fig. 2D). Moreover, we observed a similar background level of motile particles (0.02 particles/ μm) moving at an average velocity of 0.30 $\mu\text{m/s}$ when RFP tagged with a nuclear localization signal (*UAS-RFP.nls*) was expressed

in da neurons, consistent with self-aggregation of RFP. Finally, when *UAS-nosRNAi* was expressed in *nos* RFP* neurons, both the number and velocities of motile particles were reduced to the levels found in MCP-RFP-only neurons (Fig. 2E).

From these results, we reasoned that there are two populations of particles in the dendrites of *nos* RFP* neurons: nonspecific particles produced by MCP-RFP alone and *nos* RFP* particles. These distinct populations of particles, however, can be readily distinguished based on their behaviors, i.e., motility, velocity, and direction of net displacement. Therefore, in all subsequent studies, we compared *nos* RFP* neurons with MCP-RFP-only neurons.

Motor requirement for *nos* mRNA transport

The linear trajectories and average velocities of *nos* RFP* particles are consistent with motor-dependent movement on cytoskeletal tracks. Previous studies indicated that in contrast to cultured vertebrate neurons, the vast majority of microtubules in the proximal dendrites of *Drosophila* da neurons are oriented with their minus-ends distal to the cell body (Stone et al., 2008; Zheng et al., 2008). Consistent with this organization, accumulation of endosomes in class IV da neuron dendrites is disrupted by mutations in components of the dynein motor complex (Satoh et al., 2008; Zheng et al., 2008). We therefore tested whether dendritic localization of *nos* RNP particles is also dynein-dependent.

We investigated a role for dynein in particle transport using either da neuron-specific RNAi, overexpression of the dynactin subunit dynamitin, or a hypomorphic *Dynein heavy chain* (*Dhc*) allelic combination that allows survival to larval stages. RNAi knockdown of *Dhc* using a *UAS-DhcRNAi* transgene severely compromised da neuron morphogenesis, producing a dramatic dendritic branching defect characteristic of mutations in other dynein motor complex components (Satoh et al., 2008; Zheng et al., 2008; Fig. 3A). Few *nos* RFP* or MCP-RFP-only particles were observed in class IV da neuron dendrites upon *Dhc* RNAi (Fig. 3B; data not shown). Similarly, overexpression of dynamitin, which was previously shown to block dynein function (Zheng et al., 2008), also produced a severe branching defect and dramatic loss of dendritic particles (data not shown). In addition, the axons of *Dhc* RNAi class IV da neurons were thicker than in wild-

type neurons, as previously observed for mutants for other components of the dynein motor complex (Zheng et al., 2008). Moreover, both MCP-RFP-only and *nos**RFP particles accumulated in the axons of *Dhc* RNAi neurons (Fig. 3B; data not shown). This aberrant distribution, which resembles the mislocalization of Golgi outposts to class IV da neuron axons in *Dynein light intermediate chain* mutants (Zheng et al., 2008) and the mislocalization of dendrite-specific vesicle cargos to axons of cultured hippocampal neurons upon dynein inhibition (Kapitein et al., 2010), presumably reflects transport dominated by plus-ended motors such as kinesins in the absence of dynein.

Hypomorphic *Dhc* mutant da neurons exhibited a milder dendrite morphology defect, indicating partially compromised dynein function (Fig. 3A). While particles in MCP-RFP-only neurons were not affected by reduction of *Dhc* function, the number of motile particles in *nos**RFP neurons and their average velocities were reduced to the background level of MCP-RFP-only neurons (Fig. 3C). Moreover, retrograde movement predominated in *Dhc* mutant neurons (Fig. 3C). Thus, while strong dynein knockdown compromised the behavior of both *nos**RFP and MCP-RFP particles, the analysis of hypomorphic *Dhc* mutants indicates that *nos**RFP particles are more sensitive to a reduction in *Dhc* function. This result is consistent with the observation that localizing RNP particles recruit more dynein motors than nonlocalizing RNP particles (Bullock et al., 2006; Amrute-Nayak and Bullock, 2012).

The Bicaudal-D (BicD) protein interacts with dynein and plays a role in dynein-dependent transport of RNP particles in *Drosophila* oocytes and early embryos through its interaction with the RNA-binding protein Egalitarian (Egl; Bullock and Ish-Horowicz, 2001). A role for BicD has recently been identified in class IV da neuron dendrite morphogenesis (Bianco et al., 2010). Therefore, we examined whether Egl also regulates dendrite morphogenesis and whether Egl and BicD are required for dendritic *nos* particle movement. Reduction of *egl* or *BicD* function using either mutant alleles or neuron-specific RNAi resulted in a decrease in the number of dendritic termini in class IV da neurons compared with wild-type controls (Fig. 4A). Live imaging of particles in *egl* and *BicD* RNAi neurons revealed a significant reduction in the number and average velocity of motile particles in *nos**RFP neurons compared

Figure 2. Quantitative analysis of particles in live class IV da neurons. **A**, Quantification of total particle number and the breakdown of static/jiggling and motile particles observed within the first 30 s of each movie, normalized to the length of the dendrites present within the field. Particles were designated as motile if they moved directionally for at least three consecutive frames. Combinations tested: *GAL4*⁴⁷⁷ with *UAS-MCP-RFP*^{12A} (MCP-RFP only, *n* = 13 neurons; *nos**RFP, *n* = 13 neurons); *GAL4*⁸⁻¹²³ with *UAS-MCP-RFP*^{12A} (MCP-RFP only, *n* = 18 neurons; *nos**RFP, *n* = 13 neurons); *GAL4*⁴⁷⁷ with *UAS-MCP-RFP*^{8A} (MCP-RFP only, *n* = 25 neurons; *nos**RFP, *n* = 20 neurons). **B**, Average velocities of motile particles shown in **A**. *GAL4*⁴⁷⁷ with *UAS-MCP-RFP*^{12A} (MCP-RFP only, *n* = 54 particles; *nos**RFP, *n* = 54 particles); *GAL4*⁸⁻¹²³ with *UAS-MCP-RFP*^{12A} (MCP-RFP only, *n* = 66 particles; *nos**RFP, *n* = 55 particles); *GAL4*⁴⁷⁷ with *UAS-MCP-RFP*^{8A} (MCP-RFP only, *n* = 87 particles; *nos**RFP, *n* = 87 particles). **C**, Percentage of motile particles with net anterograde displacement. In all MCP-RFP-only samples, motile particles exhibited significant bias toward retrograde movement (*GAL4*⁴⁷⁷ with *UAS-MCP-RFP*^{12A}, *p* = 0.01; *GAL4*⁸⁻¹²³ with *UAS-MCP-RFP*^{12A}, *p* = 0.006; and *GAL4*⁴⁷⁷ with *UAS-MCP-RFP*^{8A}, *p* = 0.02), whereas no directional bias was observed for all *nos**RFP samples. The three different combinations produced slightly different average values due to different expression levels of the *GAL4* drivers and *UAS-MCP-RFP* transgenes. **D**, Quantification of the number and average velocity of motile particles, as well as total and static particles in neurons expressing *UAS-lacZ-MS2bs* with MCP-RFP (*lacZ**RFP), compared with *nos**RFP and MCP-RFP-only particles. The combination of *GAL4*⁴⁷⁷ with *UAS-MCP-RFP*^{12A} was used in both this and the experiment in **E**. Sample sizes: for particle number, *n* = 11–13 neurons were analyzed; for particle velocity, *n* = 47–54 particles were analyzed. **E**, Quantification of the number and average velocity of motile particles, as well as total and static particles in neurons expressing *UAS-nosRNAi* in addition to *nos**RFP compared with *nos**RFP and MCP-RFP-only neurons. Sample sizes: for particle number, *n* = 9–13 neurons; for particle velocity, *n* = 45–54 particles. Here and in all subsequent figures, values are the mean ± SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Figure 3. Requirements for molecular motors in *nos* RNP particle transport. **A**, Confocal z-series projections of *ddaC* neurons. *ppk-GAL4* was used to express *UAS-DhcRNAi* together with the mCD8-GFP marker and *GAL4⁴⁷⁷* was used to express mCD8-GFP in wild-type (WT) or hypomorphic *Dhc* mutant (*Dhc^{-/-}*) neurons. Quantification of the total number of terminal branches is shown at right (RNAi experiment: *n* = 7–8 neurons per genotype; mutant experiment: *n* = 9–10 neurons per genotype). **B**, Visualization of *nos*RFP* (white) in live wild-type and *Dhc* RNAi-expressing *ddaC* neurons, labeled with mCD8-GFP (green). The combination of *GAL4⁴⁷⁷* with MCP-RFP^{2A} was used. Arrowheads indicate axons. **C**, Quantification of the number, average velocity, and net displacement of motile particles in hypomorphic *Dhc* mutant *da* neurons. The combination of *GAL4⁴⁷⁷* with MCP-RFP^{8A} was used. Sample sizes: for particle number, *n* = 12–25 neurons; for particle velocity and displacement, *n* = 57–87 particles. **D**, Confocal z-series projections of *ddaC* neurons. For unknown reasons, expression of the *UAS-mCD8:GFP* transgene by either *ppk-GAL4* or *GAL4⁴⁷⁷* is suppressed by *Khc* RNAi. Therefore, *GAL4⁴⁷⁷* was used to express *UAS-KhcRNAi* together with *ppk-mCD4-GFP*.

with wild type, whereas particles in MCP-RFP-only neurons were unaffected (Fig. 4B). We observed a similar specific effect on particle behavior in *nos*RFP* neurons in hypomorphic *egl* and *BicD* mutants (data not shown). Together, these results indicate that the minus-end-directed transport machinery is required for *nos* RNP particle transport into dendrites.

The movement of *nos*RFP* particles in both anterograde and retrograde directions and their ability to undergo reversals suggests the involvement of the plus-end motor kinesin as well as dynein. Larvae mutant for even hypomorphic *Kinesin heavy chain* (*Khc*) allelic combinations are generally unhealthy, exhibiting numerous necrotic spots, flaccidity, and limited mobility. Therefore, we used *da* neuron-specific RNAi knockdown of *Khc* to investigate a requirement for kinesin in *nos*RFP* transport. Similarly to *Dhc* RNAi, *Khc* RNAi in class IV *da* neurons produced a strong dendrite morphogenesis defect (Fig. 3D) and re-

sulted in few detectable *nos*RFP* or MCP-RFP-only particles (data not shown). A similar effect was observed for *Drosophila* FMRP particles in cultured brain neurons derived from *Drosophila Khc* mutant larvae (Bianco et al., 2010). Microtubule organization is not overtly altered in dendrites of class IV *da* neurons lacking kinesin or dynein motor activity (Satoh et al., 2008; Zheng et al., 2008; data not shown). A previous study showing that transport of Rab-5-containing endosomes into dendrites is compromised in *Khc* mutant class IV *da* neurons as well as in neurons mutant for dynein motor components suggested that the requirement for kinesin in anterograde transport could reflect a role for this motor in regulating dynein distribution in the dendrites (Satoh et al., 2008). Thus, the lack of *nos*RFP* particles in strong *Khc* RNAi neurons may result from such an effect on dynein. We cannot, however, rule out a role for kinesin in transport of RNP particles into dendrites.

Figure 4. Requirements for motor machinery components in *nos* RNP particle transport. **A**, Class IV da neurons in wild-type (WT) and hypomorphic *egl* and *BicD* mutant (*egl*⁻, *BicD*⁻) larvae visualized by using *GAL4⁸⁻¹²³* to express mCD8-GFP or in larvae expressing *egl* or *BicD* RNAi together with mCD8-GFP using *ppk-GAL4*. Quantification of terminal branch number is shown at right (mutant experiment: *n* = 10 neurons per genotype; RNAi experiment: *n* = 6–8 neurons per genotype). **B**, Quantification of the number of motile particles (*n* = 13–25 neurons) and average velocity (*n* = 36–87 particles) in wild-type, *egl*, and *BicD* RNAi neurons. The combination of *GAL4⁴⁷⁷* with *MCP-RFP^{12A}* was used.

nos mRNA localization pathways share common trans-acting factors

We have previously shown that dendritic localization of *nos* and localization of *nos* to the posterior of the oocyte/embryo are mediated by the same *cis*-acting localization element in the *nos* 3' untranslated region (Brechtel and Gavis, 2008). Rump binds to this element and participates in localization of *nos* to the oocyte posterior via a diffusion and entrapment mechanism (Forrest and Gavis, 2003; Jain and Gavis, 2008; Sinsimer et al., 2011). The entrapment of *nos* at the posterior, which is thought to occur through the association of *nos* RNP particles with germ plasm factors assembled at the posterior by the activity of Osk protein, is required to activate *nos* translation (Becalska and Gavis, 2009). Because dendritic localization and germ plasm localization require the same *cis*-acting *nos* localization element, we tested whether Rump and Osk might also play a role in the dendritic localization process. In both *rump* and *osk* mutant larvae, class IV da neurons showed loss of dendritic terminal branches that could be completely or partially rescued by supplying one copy of a genomic *rump* transgene or an MS2-tagged genomic *osk* transgene, respectively (Fig. 5*A,B*). Similar branching defects were produced by neuron-specific *rump* or *osk* RNAi (Fig. 5*C*), resembling those observed in *nos* mutants or with *nos* RNAi (Brechtel and Gavis, 2008; Olesnick et al., 2012). In addition, both the number of motile particles in *nos**RFP neurons and their average velocities were reduced relative to wild type by mutation or RNAi knockdown of

rump or *osk* (Fig. 5*D*; data not shown). Moreover, particles in MCP-RFP-only neurons were unaffected, indicating that *rump* and *osk* are specifically required for formation and/or transport of *nos* RNP particles (Fig. 5*D*).

Because localization of *osk* mRNA during oogenesis is essential for its function in germ plasm assembly, we investigated whether *osk* might also be localized in da neurons by using the MS2-tagged *osk* transgene. We observed motile *osk**RFP particles significantly in excess of MCP-RFP-only particles. These particles moved bidirectionally with average velocities comparable to *nos**RFP particles (Fig. 5*E*; Movie 2). The transport of *osk**RFP suggests that Osk may function locally in neurons as it does in the oocyte, perhaps through a role in remodeling *nos* RNP particles once they reach the dendrites.

Transport machinery, but not *nos* localization, is required for nociception

Class IV da neurons function as mechanical and thermal nociceptors in *Drosophila* larvae (Tracey et al., 2003; Hwang et al., 2007; Zhong et al., 2010). We therefore tested whether, in addition to its function in dendritic morphogenesis, *nos* is required for neuronal function. Following a noxious mechanical stimulus, ~80% of larvae exhibited the previously described stereotypic nocifensive escape locomotion (NEL) behavior: a corkscrew rolling motion (Fig. 6*A*). To confirm that this behavior depended on class IV da neuron function, we disabled these neurons selectively by using *ppk-GAL4*, a highly spe-

Figure 5. Requirement for *rump* and *osk* in regulating *nos* RNA transport. **A**, Confocal z-series projections of ddaC neurons. *GAL4*⁴⁷⁷ was used to express the mCD8-GFP marker in wild-type (WT), *rump* mutant (*rump*⁻), or *rump* mutant larvae expressing a genomic *rump* rescue transgene (*g-rump; rump*⁻). Quantification of terminal branches is shown at right (*n* = 8–9 neurons per genotype). **B**, ddaC neurons visualized as in **A** from wild-type larva, *osk* mutant larva (*osk*⁻), or *osk* mutant larva with MS2-tagged *osk* transgene [*osk*⁻(*ms2*)₆]. Quantification of terminal branches is shown at right (*n* = 9–10 neurons per genotype). **C**, *ppk-GAL4* was used to express *rump* or *osk* RNAi together with mCD8-GFP. Quantification of terminal branches is shown at right (*n* = 6–8 neurons per genotype). **D**, Quantification of the number (*n* = 10–19 neurons per genotype) and average velocity of motile particles (*n* = 21–63 particles) in *rump* and *osk* RNAi neurons. The combination of *GAL4*⁴⁷⁷ with MCP-RFP^{12A} was used in both this and the experiment in **E**. **E**, Quantification of the number (*n* = 11–13 neurons) and average velocity (*n* = 50–54 particles) of motile particles in *osk*^{*}RFP neurons (Movie 2).

cific class IV da neuron driver, to express tetanus toxin light chain (*UAS-TnTE*). TnTE reduces neurotransmitter release by cleaving the synaptic vesicle protein synaptobrevin, effectively silencing the neuron (Sweeney et al., 1995). Expression of TnTE in class IV da neurons reduced the frequency of NEL behavior to 30% (Fig. 6A). These results are consistent with results from other comparable nociception studies (Hwang et al., 2007).

When tested in the mechanical nociception assay, *nos* mutant larvae exhibited a 40% reduction in NEL behavior relative to control

larvae (Fig. 6B). Because execution of NEL behavior involves both sensory and motor neurons and because *nos* mutants exhibit defects in neuromuscular junction (NMJ) morphogenesis and physiology (Menon et al., 2009), we investigated whether the NEL response requires *nos* function in da neurons, NMJ, or both. RNAi-mediated knockdown of *nos* in class IV da neurons resulted in a 30% reduction in NEL behavior compared with wild-type larvae, similar to that observed for *nos* mutant larvae (Fig. 6B,C). In contrast, presynaptic NMJ expression of *UAS-nos*RNAi using *OK6-GAL4* or postsynaptic

NMJ expression using *Mhc-GAL4*, two drivers that have previously been shown to drive *UAS-nosRNAi* and/or *UAS-nos* leading to NMJ defects (Menon et al., 2009), had no effect on the NEL response (Fig. 6C). Together, these data indicate that the NEL response requires *nos* function specifically in class IV da neurons. *Nos*, a nonspecific RNA-binding protein, functions in a translational repression complex together with *Pum*, a sequence-specific RNA-binding protein. Similarly to *nos* mutant or RNAi larvae, larvae with *pum* mutations or da neuron-specific *pum* RNAi also showed a diminished NEL response (Fig. 6B).

We next tested whether motor machinery components and *trans*-acting factors with roles in *nos* mRNA localization play a role in nociception, using mutants and class IV da neuron-specific RNAi knockdown. Larvae with reduced *Dhc*, *egl*, *BicD*, and *Khc* function all exhibited a deficit in NEL behavior (Fig. 6B). However, the effect on NEL behavior did not clearly correlate with the severity of the morphological defect or the effect on *nos* localization. Thus, defects in trafficking of cargoes distinct from those involved in dendrite morphogenesis per se are likely to be responsible for the NEL deficit in these mutant/RNAi larvae. For example, dynein might be involved in transport of ion channel components important for regulating the physiological functions of the neuron. The specificity of *Egl* for transport of RNP complexes (Dienstbier et al., 2009) suggests that mRNA transport is, however, important for nociceptive function.

Interestingly, *osk* mutants exhibited a slightly elevated response relative to wild-type larvae, whereas neuron-specific *osk* RNAi diminished the response (Fig. 6B). The hypersensitivity of *osk* mutants was confirmed using multiple allelic combinations (data not shown). We hypothesized that *osk* mutations could produce a diver-

Movie 2. Time-lapse movie of a larval class IV da neuron (ddaC) expressing *osk::RFP*. Images were captured at 2.3 frames/s and played back at 20 frames/s. Arrows indicate movement of particles.

gent result from *osk* RNAi if *osk* functions in multiple cell types to regulate different components of nociception. To test this, we compared the effects of *osk* RNAi targeted to da neurons by *ppk-GAL4* with the effects of ubiquitous *osk* RNAi using *tubP-GAL4*. In contrast to da neuron-specific reduction of *osk*, ubiquitous reduction of *osk* by RNAi produced a slight hypersensitive phenotype very similar to

Figure 6. Effects of perturbing RNA localization and transport machinery on nociceptive response. **A**, Nociception assay using an insect pin to apply a mechanical stimulus. The fraction of larvae demonstrating the NEL response upon stimulation was monitored. The NEL response was dramatically decreased in larvae with class IV neurons selectively disabled by *ppk-GAL4*-mediated expression of TnTE ($n = 74$) relative to *ppk-GAL4* control larvae (Ctrl, $n = 117$). **B**, Nociception assays conducted on larvae with the indicated genes disrupted by mutation or by da neuron-specific RNAi. Mutants: WT ($n = 339$), *nos* ($n = 69$), *pum* ($n = 143$), *osk* ($n = 98$), *rump* ($n = 131$), *Dhc* ($n = 123$), *egl* ($n = 62$), *BicD* ($n = 51$), *Khc* ($n = 33$); RNAi: *ppk-GAL4*; *UAS-dicer2* control ($n = 117$), *nos* ($n = 196$), *pum* ($n = 124$), *osk* ($n = 185$), *rump* ($n = 90$), *Dhc* ($n = 139$), *egl* ($n = 155$), *BicD* ($n = 144$), *Khc* ($n = 81$). **C**, Nociception assays performed on larvae expressing *UAS-nosRNAi* in the presynaptic NMJ using *OK6-GAL4* ($n = 121$), the postsynaptic NMJ using *MHC82-GAL4* ($n = 126$), or class IV da neurons using *ppk-GAL4* ($n = 117$) compared with wild-type ($n = 339$) and *nos* mutant ($n = 69$) larvae. **D**, Nociception assay comparing larvae mutant for *osk* with larvae expressing *UAS-oskRNAi* ubiquitously using *tubP-GAL4* ($n = 40$) or specifically in class IV da neurons using *ppk-GAL4* ($n = 185$). *tubP-GAL4* alone ($n = 49$) provides a negative control.

that of *osk* mutations (Fig. 6D). Thus, while *osk* is required in class IV da neurons for nociception, it appears to regulate other components of this neural circuit, either in the peripheral nervous system or the CNS.

Surprisingly, despite the requirement for Rump in *nos* localization, neither mutation of *rump* nor *rump* RNAi had an effect on nociception (Fig. 6B). These results suggest that dendritic localization of *nos* is not required for *nos* function in nociception. Thus, *nos* appears to play distinct roles in da neuron morphogenesis and function, with only the former requiring dendritic localization.

Discussion

We have combined a method that allows live imaging of mRNA in intact *Drosophila* larvae with genetic analysis to investigate the mechanism underlying transport of *nos* mRNA in class IV da neurons. Live imaging over the short time periods allowed has provided a snapshot into the steady-state behavior of *nos**RFP particles in the proximal dendrites of mature da neurons. Our results indicate that anterograde transport of *nos* RNP particles into and within da neuron dendrites is mediated by dynein and is consistent with the minus-end out model for microtubule polarity in the proximal dendrites of da neurons (Zheng et al., 2008). This model predicts that bidirectional trafficking would be mediated by opposite polarity motors and the predominance of retrograde movement of *nos**RFP particles when dynein function is partially compromised is consistent with this. Moreover, Rab-5 endosomes, whose accumulation in class IV da neuron dendrites is dynein-dependent, also exhibit bidirectional movement (Satoh et al., 2008), suggesting that different cargos may use similar dendritic transport strategies. Unfortunately, the severe defects caused by loss of kinesin have thus far hampered us and others from confirming a role for kinesin in these events.

The observed bidirectional movement of *nos* RNP particles resembles the constant bidirectional transport observed for dendritic mRNAs near synapses in hippocampal neurons. In contrast to da neurons, proximal dendrites of mammalian neurons have mixed microtubule polarity (Baas et al., 1988; Stepanova et al., 2003) so that bidirectional trafficking could be mediated by a single motor that switches microtubules or by switching between the activities of plus-end and minus-end motors. The association of kinesin with neuronal RNP granule components and inhibition of *CaMKIIα* RNA transport by dominant-negative inhibition of kinesin has implicated kinesin as the primary motor for dendritic mRNA transport (Doyle and Kiebler, 2011). However, a recent study showed that dynein mediates unidirectional transport of vesicle cargoes into dendrites of cultured hippocampal neurons as well as bidirectional transport within the dendrites (Kapitein et al., 2010). Whether dynein plays a role in RNP particle transport in mammalian dendrites as it does in *Drosophila* neurons remains to be determined.

Despite its prevalence, the role of bidirectional motility is not yet clear. A recently proposed “sushi belt” model suggests that neuronal RNP particles traffic back and forth along the dendrite until they are recruited by an active synapse and disassembled for translation (Doyle and Kiebler, 2011). Although da neuron dendrites do not receive synaptic input, this continual motility may provide a reservoir of *nos* mRNA that can be rapidly mobilized for translation locally in response to external signals that regulate dendrite branching.

Our studies have shown that *nos* mRNA can be adapted for different localization mechanisms depending on cellular context: diffusion/entrapment in late oocytes that lack a requisite polarized microtubule cytoskeleton and microtubule-based transport during germ cell formation in the embryo and in class IV da neurons (For-

rest and Gavis, 2003; Lerit and Gavis, 2011; this article). Surprisingly, Rump and Osk are specifically required for *nos* localization in both oocytes and da neurons, suggesting that they function in the assembly or recognition of a fundamental *nos* RNP that can be adapted to both means of localization. However, because we cannot distinguish individual particles within the cell body, we cannot rule out the possibility that Rump and/or Osk mediate coupling of *nos* RNP particles to dynein motors rather than particle formation. Within the germ plasm, *nos* associates with Vasa (Vas), a DEAD-box helicase, and is transported together with Vas into germ cells (Lerit and Gavis, 2011). Although dendritic branching complexity is reduced in *vas* mutants, we did not detect an effect on dendritic localization of *nos* RNP particles (data not shown), suggesting that only a subset of germ plasm components are shared by neuronal localization machinery. A role for *osk* in learning and memory was proposed based on the isolation of an enhancer trap insertion upstream of *osk* in a screen for mutants with defective long-term memory, but *osk* function in memory formation has not been directly tested (Dubnau et al., 2003). Notably, however, a recent study showed that the *osk* ortholog in the cricket *Gryllus bimaculatus* functions in development of the embryonic nervous system rather than in germ cell formation. Thus, the ancestral function of *osk* appears to be in neural development, whereas its role in germ plasm formation is a later adaptation in higher insects (Ewen-Campen et al., 2012). Our results showing that Osk protein function is not limited to Dipteran germ plasm organization but also plays an important role in neuronal development and function supports this idea.

Our data indicate that the Nos/Pum complex is not only required for da neuron morphogenesis, but also for nociceptive function. However, nociception does not appear to require local function of Nos/Pum in the dendrites and reduced dendritic branching does not necessarily correlate with a deficit in nociception. These results suggest that morphogenesis and function are regulated separately and that Nos/Pum plays a second role in regulating the somatic translation of proteins required for the nociceptive response. Systematic identification of Nos/Pum targets will be essential to further investigate these different roles.

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