Neuroscience 176 (2011) 39–52

SUB-CELLULAR DISTRIBUTION OF UNC-104(KIF1A) UPON BINDING TO ADAPTORS AS UNC-16(JIP3), DNC-1(DCTN1/Glued) AND SYD-2(*LIPRIN-α*) IN *C. ELEGANS* NEURONS

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Abstract—The accumulation of cargo (tau, amyloid precursor protein, neurofilaments etc.) in neurons is a hallmark of various neurodegenerative diseases while we have only little knowledge how axonal transport is regulated. Kinesin-3 UNC-104(KIF1A) is the major transporter of synaptic vesicles and recent reports suggest that a cargo itself can affect the motor's activity. Inspecting an interactome map, we identify three putative UNC-104 interactors, namely UNC-16(JIP3), DNC-1(DCTN1/Glued) and SYD-2(Liprin-α), known to be adaptors in essential neuronal protein complexes. We then employed the novel method bimolecular fluorescence complementation (BiFC) assay to visualize motor-adaptor complexes in the nervous system of living *C. elegans*. Interestingly, the binding of UNC-104 to each adaptor protein results in different sub-cellular distributions and has distinctive effects on the motor's motility. Specifically, if UNC-104 bound to UNC-16, the motor is primarily localized in the soma of neurons while bound to DNC-1, the motor is basically found in axonal termini. On the other hand, if UNC-104 is bound to SYD-2 we identify motor populations mostly along axons. Therefore, these three adaptors inherit different functions in steering the motor to specific sub-cellular locations in the neuron.

Key words: bimolecular fluorescence complementation assay (BiFC), axonal transport, JNK-interacting proteins, kinesin-3, dynein, molecular motors.

Axonal transport is increasingly becoming a spotlight in neurodegenerative disorder research. Proper regulation of cargo transport in lengthy, thin and crowded neuronal extensions is critical while malfunction in molecular-motor based transport might lead to accumulation of proteins (e.g. tau, amyloid precursor protein, neurofilaments) in axons, a hallmark of various neurodegenerative diseases. How molecular motors are regulated and how motors recognize their cargo remains largely unknown, however, increasing evidence suggest that cargo-binding is highly specific and that the same motor can bind various types of cargo. For example, KIF5, a member of the kinesin-1 family, binds amyloid precursor protein (APP) or Apolipoprotein E receptor 2 (ApoER2) containing vesicle through JIPs, on the other hand α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-receptor) containing vesicles are bound via the scaffolding protein glutamate receptor interacting protein 1 (GRIP1). Interestingly, if KIF5 bound to GRIP1, vesicles are steered to dendrites while if bound to JIPs vesicles are directed towards axons (Kaether et al., 2000; Kamal et al., 2000; Verhey et al., 2001; Setou et al., 2002; Inomata et al., 2003; Matsuda et al., 2003). In *C. elegans*, if the conventional kinesin-1 heavy chain homolog UNC-116 binds the JNK-interacting protein UNC-16, vesicles are specifically directed to axons while avoiding dendrites (Byrd et al., 2001). Another example is that upon binding of the active zone protein SYD-2(Liprin-α) to kinesin-3 UNC-104(KIF1A), the motor's anterograde destinations are favored limiting movements backwards to the soma (Wagner et al., 2009). These examples demonstrate that direct and specific binding of cargo to a motor determines the destination (axons vs. dendrites) and/or directionality (anterograde vs. retrograde) of a motor or vesicular cargo (for review; Hirokawa et al., 2009b).

To gain more insight about the specificity of motor-cargo binding and how a cargo (precursor proteins, vesicular structures, adaptors etc.) regulate the destination of a motor, we used an interactome map (Zhong and Sternberg, 2006) to identify putative binding partners for the major synaptic vesicle transporter UNC-104(KIF1A) in *C. elegans*. We identified three proteins UNC-16(JIP3), DNC-1(DCTN1/Glued) and SYD-2(Liprin-α), while all of them have in common to be engaged as adaptors in essential neuronal protein complexes. For example, UNC-16 is known to act as a scaffold protein, binding to the light chain of kinesin-1 (KLC-2) and regulating the transport of synaptic vesicle components by combining kinesin-1 transport and JNK signaling (Byrd et al., 2001; Sakamoto et al., 2005; Brown et al., 2009). It has also been hypothesized that UNC-16 might trigger directionality of cargo by mediating the interaction between kinesin-1 and dynein through its association with $p150^{Glued}$ (Sakamoto et al., 2005; Koushika, 2008; Montagnac et al., 2009). JIP3 facilitates JNK phosphorylation of APP and JIP3 gene disruption in mice leads to defects in axon guidance that may be due to reduced vesicle transport, as observed in *unc-16* mutants in *C. elegans* (Kelkar et al., 2003). There is no study, however, on direct interactions between UNC-16 and UNC-104 and the effect on the motor's neuronal distribu-

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Abbreviation: BiFC assay, bimolecular fluorescence complementation assay; JIP, JNK-interacting protein; JIP3, JNK-interacting protein 3 (or also termed JNK/stress-activated protein kinase-associated protein 1, JSAP1); JNK, c-Jun N-terminal kinase; LAR, leucocyte common antigen-related protein receptor; Liprin, LAR-interacting protein.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2010.12.044

tion. The second identified UNC-104 interactor, DNC-1(DCTN1/Glued), is a homolog of p150^{Glued}, a component of the dynactin complex which is an important adaptor to connect the retrograde motor dynein to both the cargo (vesicle) and the microtubule. It has been suggested that bidirectional movement of synaptic vesicles is a result of opposing molecular motors (bound to one vesicle at the same time) pulling in different directions in a tug-of-war event or as a result of coordinated activity (for review; Holzbaur and Goldman, 2010). Indeed, direct interactions between kinesin-II and p150^{Glued} (Deacon et al., 2003), kinesin-I and dynein (Ligon et al., 2004) and indirect interactions between UNC-104 (Koushika et al., 2004) as well as a *Drosophila* UNC-104 homolog (Barkus et al., 2008) and dynein have been reported. However, direct interactions between UNC-104 and dynactin (or dynein) and the resulting sub-cellular distribution of the motor remains to be unsolved. The third UNC-104 interactor, SYD-2/Liprin-α, is a key player in assembling and regulating active zones (electron dense regions at the synapse at which synaptic vesicle fusion and transmitter release occurs). Through its multifunctional interaction with Rab3-interacting molecule (RIM), LAR and calcium/calmodulin-dependent serine protein kinase 3 (CASK) it mediates targeting the presynaptic transmission machinery to opposite postsynaptic densities (Olsen et al., 2006). Interactions between UNC-104 and SYD-2/Liprin-α have been reported (Wagner et al., 2009), however, it remains unclear how the motor's sub-cellular distribution changes upon direct binding to SYD-2.

UNC-104, a homologue of the mammalian kinesin-3 KIF1A, is a neuron-specific, microtubule plus-end directed motor and is the major transporter of synaptic vesicles in axons. Mutations in the UNC-104 gene in worms result in uncoordinated, slow body motion and a slow growth rate while the concentration of synaptic vesicles increases in cell bodies and decreases at synapses (Hall and Hedgecock, 1991). KIF1A knockout mice are lethal with motor and sensory defects and abnormal distribution of synaptic vesicles (retained in soma and reduced in neuronal terminals) (Yonekawa et al., 1998). In *C. elegans*, fluorescently labeled UNC-104 reveals a bidirectional moving pattern with net transport rates in anterograde directions (Zhou et al., 2001; Wagner et al., 2009).

We use a novel method, bimolecular fluorescence complementation (BiFC) assay, to visualize protein–protein interaction in living animals. The method is based on the principle of protein-fragment complementation using two non-fluorescent fragments derived from fluorescent proteins. When two hybrids of fluorescent proteins are brought together in living cells (by fusing each to one of a pair of interacting proteins) fluorescence is restored (Hu and Kerppola, 2003; Kerppola, 2006a,b; Shyu et al., 2008; Zal, 2008). This not only enables to identify direct and physical interactions between two proteins but also their sub-cellular localization upon interaction.

EXPERIMENTAL PROCEDURES

C. elegans strains and plasmids

C. elegans strains were maintained at 22 °C using standard methods (Brenner, 1974). For BiFC assays, we used the BiFC control vector kit (pCE::bJUN::VN173 and pCE::bFOS::VC155) from Dr. Chang-Deng Hu's Laboratory (Purdue University, USA) and replaced the heat shock promoter hsp16.41 with the pan-neuronal Punc-104 promoter. Positive control vectors for nucleus expression Punc-104::bJUN::VN173 (40 μg/ml) and Punc-104::bFOS::VC155 (30 μg/ml), respectively, were microinjected into N2 hermaphrodites using the pRF4 rol-6(su1006) co-injection marker (employing standard microinjection methods; Mello et al., 1991). Positive control vectors for investigating neuronal expression of two known interacting proteins (UNC-104/UNC-104), Punc-104::UNC-104::VN173 (60 μg/ml) and Punc-104::UNC-104::VC155 (60 μg/ml), were microinjected into CB1265 unc-104(*e1265*) hermaphrodites rescuing the highly uncoordinated and paralytic phenotype (note, that the need of high plasmid dosages to rescue the *unc-104* phenotype has been reported previously; Wagner et al., 2009). To determine (reported) UNC-16/KLC-2 interactions (used as a positive control) we made a worm expressing Punc-104::UNC-16::VN173/Punc-104::KLC-2::VC155 BiFC pairs by microinjecting a Punc-104::UNC-16::VN173 (50 μg/ml) plasmid and a Punc-104::KLC-2::VC155 (50 μg/ml) plasmid into N2 strains (using the co-injection marker pRF4). As a negative control, we deleted UNC-16's KLC-2 binding domain and injected a Punc-104::UNC-16ΔKLCBD::VN173 (50 μg/ml) plasmid and a Punc-104::KLC-2::VC155 (50 μg/ml) plasmid into N2 strains (using the co-injection marker pRF4). To determine reported UNC-16/DNC-1 interactions (also used as a positive control) we designed a worm expressing Punc-104::UNC-16::VN173/Punc-104::DNC-1::VC155 BiFC pairs by microinjecting a Punc-104::UNC-16::VN173 (60 μg/ml) plasmid and a Punc-104::DNC-1::VC155 (60 μg/ml) plasmid into N2 strains (with co-injection marker pRF4).

To evaluate UNC-16/UNC-104 expression, we designed a worm expressing Punc-104::UNC-16::VN173/Punc-104::UNC-104::VC155 BiFC pairs by microinjecting a Punc-104::UNC-16::VN173 (50 μg/ml) and a Punc-104::UNC-104::VC155 (50 μg/ml) plasmid into the strain CB1265 unc-104(*e1265*) rescuing the *unc-104* phenotype. To investigate UNC-104/DNC-1 interactions a strain expressing Punc-104::UNC-104::VN173/Punc-104::DNC-1::VC155 BiFC pairs was generated by microinjecting a Punc-104::UNC-104::VN173 (50 μg/ml) plasmid and a Punc-104::DNC-1::VC155 (50 μg/ml) plasmid into the CB1265 unc-104(*e1265*) line rescuing the *unc-104* phenotype. For SYD-2/UNC-104 interactions, we designed worms expressing a Punc-104::VN173::SYD-2/Punc-104::UNC-104::VC155 BiFC pair by microinjecting a Punc-104::VN173::SYD-2 plasmid (60 μg/ml) and Punc-104::UNC-104::VC155 plasmid (70 μg/ml) into the CB1265 unc-104(*e1265*) strain rescuing the highly uncoordinated phenotype. Strains expressing Punc-104::UNC-104::GFP (to evaluate UNC-104 expression alone) have been described elsewhere (Wagner et al., 2009).

Primary neuronal cell culture

Primary neuronal cells from *C. elegans* were isolated according to the protocols by Christensen et al. (2002) and Strange et al. (2007). In brief, primary neuronal cells are isolated from embryonic eggs collected from L4 worms. To free eggs from a worm, the epidermis of the worm is lysed (in a solution containing high molar NaOH and bleach (NaClO)) whereas the eggs—containing a resistant chorion (chitin-shell)—are not negatively affected by the lysation process. After separating the eggs from the worm debris using sucrose centrifugation, the eggs are subsequently treated with the enzyme chitinase to digest the egg shell. The released embryonic cells are then