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Staufen targets *coracle* mRNA to *Drosophila* neuromuscular junctions and regulates GluRIIA synaptic accumulation and bouton number

Alejandra Gardiol, Daniel St Johnston *

The WellcomeCRUK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, United Kingdom

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ABSTRACT

The post-synaptic translation of localised mRNAs has been postulated to underlie several forms of plasticity at vertebrate synapses, but the mechanisms that target mRNAs to these postsynaptic sites are not well understood. Here we show that the evolutionary conserved dsRNA binding protein, Staufen, localises to the postsynaptic side of the *Drosophila* neuromuscular junction (NMJ), where it is required for the localisation of *coracle* mRNA and protein. Staufen plays a well-characterised role in the localisation of *oskar* mRNA to the oocyte posterior, where Staufen dsRNA-binding domain 5 is specifically required for its translation. Removal of Staufen dsRNA-binding domain 5, disrupts the postsynaptic accumulation of Coracle protein without affecting the localisation of *cora* mRNA, suggesting that Staufen similarly regulates Coracle translation. Tropomyosin II, which functions with Staufen in *oskar* mRNA localisation, is also required for *coracle* mRNA localisation, suggesting that similar mechanisms target mRNAs to the NMJ and the oocyte posterior. Coracle, the orthologue of vertebrate band 4.1, functions in the anchoring of the glutamate receptor IIA subunit (GluRIIA) at the synapse. Consistent with this, *staufen* mutant larvae show reduced accumulation of GluRIIA at synapses. The NMJs of *staufen* mutant larvae have also a reduced number of synaptic boutons. Altogether, this suggests that this novel Staufen-dependent mRNA localisation and local translation pathway may play a role in the developmentally regulated growth of the NMJ.

Introduction

mRNA localisation is a widespread mechanism for targeting proteins to a specific region within a cell, and can be coupled to translational regulation to allow the local control of gene expression (St Johnston, 2005; Holt and Bullock, 2009). This mechanism has been proposed to play an important role in the nervous system, where the translation of dendritically localised mRNAs near synapses is thought to contribute to activity-dependent synaptic remodelling during long-term potentiation or depression. It has been known for many years that polyribosomes are present in dendrites in the vicinity of synapses. In addition, more than 20 mRNAs have been found to be dendritically localised, most of which encode proteins that regulate synaptic structure or function, consistent with the idea that their local translation modifies synaptic strength (Sutton and Schuman, 2006; Zukin et al., 2009; Doyle and Kiebler, 2011; Kindler and Kreienkamp, 2012). This has been most clearly demonstrated in the case of CaMKIIα, where a mutant RNA lacking dendritic targeting signals leads to a reduction of protein levels in distal dendrites and impaired long term potentiation and memory (Miller et al., 2002).

Little is known about the mechanisms that direct the post-synaptic localisation of mRNAs in neurons, but live imaging of CaMKIIα and Arc mRNAs has revealed that they undergo rapid bidirectional movements suggestive of motor-dependent transport along microtubules (Rook et al., 2000; Dynes and Steward, 2007). In support of this view, a number of dendritic mRNAs are found in RNP particles that co-purify with the plus end-directed microtubule motor protein, Kif5 (Kanai et al., 2004). mRNAs are usually targeted to dendrites by localisation elements in their 3′UTRs, which must be recognised by RNA binding proteins (RBPs) that link them to the transport machinery and regulate translation (Kindler and Kreienkamp, 2012).

Amongst the proteins that are suspected to play a direct role in dendritic mRNA transport are the vertebrate Staufen proteins, which contain multiple copies of a conserved dsRNA-binding domain (dsRBD) (St Johnston et al., 1992; Kiebler et al., 1999; Marion et al., 1999; Wickham et al., 1999). In cultured neurons, Staufen forms ribonucleoprotein particles that are transported along microtubules into dendrites, whereas dominant negative versions of the protein remain in the soma and reduce levels of RNA and ribosomes in dendrites (Kiebler et al., 1999; Köhrmann et al., 1999; Tang et al., 2001; Kim and Kim, 2006). Moreover, many dendritically-localised mRNAs

* Corresponding author. Fax: +44 1223 334 089.
E-mail address: d.stjohnston@gurdon.cam.ac.uk (D. St Johnston).

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co-immunoprecipitate with embryonic rat brain Staufen (Heraud-Farlow et al., 2013). Suppressing the expression of either of the two rodent Staufen orthologues affects the morphology of dendritic spines, which are abnormally shaped and immature (Goetze et al., 2006; Vessey et al., 2008). Although *stau 1* mutant mice show no obvious behavioural deficits, RNAi-mediated knock down of Stau1 function in hippocampal slices impairs long term potentiation, whereas knock-down of the second Stau gene disrupts long term depression (Lebeau et al., 2008, 2011; Vessey et al., 2008)

Most of our understanding of the role of Staufen in mRNA localisation comes from *Drosophila*, where it participates three distinct mRNA localisation pathways. First, Staufen forms a complex with *oskar* mRNA and is essential for the kinesin-dependent transport of the mRNA to the posterior of the oocyte and for its local translation at the posterior cortex (Ephrussi et al., 1991; Kim-Ha et al., 1991, 1995). Mutants that disrupt Staufen RNA-binding strongly reduce the localisation of *oskar* mRNA, whereas deletion of the fifth dsRBD, which has the conserved structure of the dsRBD but does not bind dsRNA, prevents the translation of *oskar* mRNA once it has localised (Rongo et al., 1995; Micklem et al., 2000). Second, Staufen is recruited to the *bicoid* 3′UTR by the ESCRT-II complex, and is required for the anchoring of the mRNA at the anterior of the oocyte during late oogenesis (St Johnston et al., 1989; Ferrandon et al., 1994; Weil et al., 2006; Irion and St Johnston, 2007). In addition to its role localising *bicoid* and *oskar* at opposite poles, Staufen is also required for the actin-dependent localisation of *prospero* mRNA to the basal side of asymmetrically dividing neuroblasts (Li et al., 1997; Broadus et al., 1998). This depends on the binding of the fifth dsRBD of Staufen to Miranda, which targets *prospero* RNA/Staufen complexes to the basal cortex (Fuerstenberg et al., 1998; Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1998).

Given its well-characterised role in mRNA localisation, we set out to investigate whether *Drosophila* Staufen plays a role in the targeting of mRNAs to synapses using the neuromuscular junction (NMJ) as a model. Although the post-synaptic cell is a muscle, the NMJ has the advantage of being a well-characterised glutamatergic synapse that displays developmental and activity-dependent synaptic plasticity, and shares some aspects of its cell biology and physiology with vertebrate central nervous system excitatory synapses (Schuster, 2006).

Results

Staufen is localised to the postsynaptic compartment of the NMJ

In the third instar larva, each muscle is a single multinucleated cell that is simultaneously innervated by up to four motorneurons that form synapses en passant after defasciculating from the motor nerve. The NMJ is considered to be the assembly of regularly spaced swellings called boutons that are formed by the axons. Each presynaptic bouton contains in average 20–40 active zones where synaptic vesicles are docked, which are faced by a post-synaptic differentiation (PSD) where neurotransmitter receptors cluster forming junctional excitatory synapses (Budnik, 1996; Schuster, 2006; Thomas and Sigrist, 2012). In double immuno-fluorescent stainings in third instar larva fillets, an antibody against Staufen labelled the periphery of type I boutons, outside the staining for Discs Large (Dlg), a MAGUK protein belonging to the PSD-95, Sap90/97 family that decorates the subsynaptic reticulum (Lahey et al., 1994; Guan et al., 1996) (SSR, Fig. 1A). The Staufen staining was specific, as it was absent from the NMJs of *staufen* null mutant larvae (Fig. 1B). We also performed pre-embedding immune-EM using HRP-conjugated anti-Staufen antibodies and diaminobenzidine (DAB) staining, which precipitates on membranes when oxidised. The electron-dense DAB signal was found around the invaginations of the muscle membrane that form the SSR beneath glutamatergic type I boutons, whereas no signal could be detected in the presynaptic element (Fig. 1C and D). Staufen therefore localises on the postsynaptic side of all type I boutons in third instar larval NMJs.

Staufen mutants have a reduced number of boutons

During larval development, the muscle size increases. In order to maintain efficient innervation, the NMJ expands accordingly and more boutons are added (Budnik, 1996). The NMJs of Staufen mutant larvae appear less developed than their wild type counterparts, and we therefore quantified the number of boutons per NMJ in different *staufen* allelic combinations. The $staufen^{r9}$ allele and a deficiency (*Df*) have molecular lesions that entirely abolish *staufen* expression, whereas $staufen^{HL}$ produces a truncated form of Staufen missing the fifth double-stranded RNA binding domain (see Section 4). In wild type larvae, the NMJ established between muscles 6/7 had as an average of 15 type Ib boutons (Fig. 1E; *wt*: 15.25 ± 0.90, $n=28$). In *staufen* mutant larvae, there was almost a 50% reduction in the number of boutons (Fig. 1E; *HL/Df*: 8.16 ± 0.79, $n=29$; *r9/Df:*, 8.93 ± 0.61, $n=29$). Mutants that fail to incorporate new boutons during the development have deformed NMJs with poorly defined boutons, as if they have been mechanically stretched (Zito et al., 1999). The NMJs in *staufen* mutants have poorly defined boutons of this type with long linear stretches, indicating that Staufen is involved in the process that increases bouton number during the development of the NMJ.

Staufen regulates GluRIIA and GluRIIB levels at the NMJ

The NMJ contains two types of GluRs similar in sequence to vertebrate AMPA and Kainate receptors, GluRIIA and GluRIIB, each of which is a hetero-tetramer of three common subunits, GluRIIC, IID, IIE, and either a GluRIIA or a GluRIIB subunit (Schuster et al., 1991; Chang et al., 1994; Petersen et al., 1997; Marrus and Diantonio, 2004; Featherstone et al., 2005; Qin et al., 2005). It has been reported that in some cases, altered levels of GluRIIA can be related to a reduction in the number of synaptic boutons (Sigrist et al., 2000, 2002, 2003). We therefore investigated by immunocytochemistry whether GluR abundance at the NMJ was affected. A polyclonal antibody directed against the N-terminal region of GluRIIA detected discreet clusters on the postsynaptic side of the NMJ (Saitoe et al., 1997) (Fig. 2A). The antibody, as described previously, was specific for the GluRIIA subunit since no synaptic clusters could be detected in *glurIIa* null mutants (Fig. 2B). The intensity of the synaptic GluRIIA signal was greatly reduced in $staufen^{r9}/Df$ and $staufen^{HL}/Df$ larvae (Fig. 2C–E).

In wild type NMJs, the GluRIIA and GluRIIB subunits compete for their association with the GluRIIC subunit and for their subsequent incorporation into the NMJ, and mutants lacking either GluRIIA or GluRIIB are therefore viable, whereas simultaneous deletion of both genes results in lethality (Petersen et al., 1997; DiAntonio et al., 1999). In contrast, elimination of either GluRIIC, D or E subunits leads to embryonic lethality (Marrus et al., 2004; Featherstone et al., 2005; Qin et al., 2005). Since *staufen* mutant larvae are viable and motile, GluRIIB presumably compensates for the reduction in GluRIIA by associating with the structural subunits (GluRIIC,D or IIE). Indeed, *staufen* mutants showed normal levels of GluRIIC (Fig. 2F and G). GluRIIB synaptic clusters were not affected in *staufen* mutants (Fig. 2H and I). This suggests that, as described before, GluRIIB clusters could have compensated for the reduction in GluRIIA. Our immunostainings indeed suggest that GluRIIB levels are increased. Thus, Staufen modulates the relative