The KIF1A homolog Unc-104 is important for spontaneous release, postsynaptic density maturation and perisynaptic scaffold organization

Yao V. Zhang1,2,3, Shabab B. Hannan1,2,4, Jeannine V. Kern1, Doychin T. Stanchev1, Baran Koç2, Thomas R. Jahn4 & Tobias M. Rasse1,4,†

The kinesin-3 family member KIF1A has been shown to be important for experience dependent neuroplasticity. In Drosophila, amorphic mutations in the KIF1A homolog unc-104 disrupt the formation of mature boutons. Disease associated KIF1A mutations have been associated with motor and sensory dysfunctions as well as non-syndromic intellectual disability in humans. A hypomorphic mutation in the forhead-associated domain of Unc-104, unc-104bris, impairs active zone maturation resulting in an increased fraction of post-synaptic glutamate receptor fields that lack the active zone scaffolding protein Bruchpilot. Here, we show that the unc-104bris mutation causes defects in synaptic transmission as manifested by reduced amplitude of both evoked and miniature excitatory junctional potentials. Structural defects observed in the postsynaptic compartment of mutant NMJs include reduced glutamate receptor field size, and altered glutamate receptor composition. In addition, we observed marked loss of postsynaptic scaffolding proteins and reduced complexity of the sub-synaptic reticulum, which could be rescued by pre- but not postsynaptic expression of unc-104. Our results highlight the importance of kinesin-3 based axonal transport in synaptic transmission and provide novel insights into the role of Unc-104 in synapse maturation.

Kinesins are microtubule based molecular motors that transport various cargos including membranous organelles, protein complexes and messenger RNAs. Thus, they are of fundamental importance for the establishment, plasticity, injury response and survival of neuronal networks. C. elegans Unc-104 and the mammalian kinesin-3 family members KIF1A and KIF1B are the predominant motor proteins for the fast anterograde transport of membranous organelles. KIF1A knockout mice display severe neurological abnormalities including motor and sensory disturbances and die shortly after birth. In Drosophila complete loss of unc-104 function in motor neurons leads to an arrest of synaptogenesis and embryonic lethality.

In humans recessive, autosomal dominant and spontaneous mutations in KIF1A have been associated with hereditary spastic paraplegia (HSP)9–11, and hereditary sensory and autonomic neuropathy type IIC (HSAN2C)14. Moreover, mutations in KIF1B have been implicated in Charcot-Marie-Tooth disease15. These diseases primarily affect nerve cells that have long axons and are thus most dependent on efficient cargo transport, consistent with kinesin-3’s important role for long-range intracellular trafficking. Apart from these symptoms in the peripheral nervous system, KIF1A has also been implicated in brain development and function. In the developing brain, Kif1a expression is restricted to the subventricular zone (SVZ) and the olfactory bulb16. In adult mice, KIF1A expression is highest in the olfactory bulb, hippocampus, and neocortex16. KIF1A knockdown in adult neurons leads to decreased dendritic spine density, reduced excitatory postsynaptic currents, and impaired synaptic plasticity, consistent with its role in maintaining dendritic spine morphology and function.

1 Junior Research Group Synaptic Plasticity, Hertie-Institute for Clinical Brain Research, University of Tübingen, Otfried-Müller-Str. 27, 72076 Tübingen 72076, Germany. 2Graduate School of Cellular and Molecular Neuroscience, University of Tübingen, 72074 Tübingen, Germany. 3The Picower Institute for Learning and Memory, Department of Biology and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. 4CHS Research Group Proteostasis in Neurodegenerative Disease at CellNetworks Heidelberg University and DKFZ Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 581, 69120 Heidelberg, Germany. 5Present address: Advanced Light Microscopy Facility, European Laboratory of Molecular Biology (EMBL), Meyerhofstraße 1, 69117 Heidelberg, Germany. Correspondence and requests for materials should be addressed to T.M.R. (email: tobias.rasse@gmail.com)
nervous system, HSAN2C related mutations in KIF1A were also described to affect the central nervous system causing mental retardation and brain atrophy. While unc-104's mammalian homolog KIF1A is important in facilitating synaptogenesis in an experience dependent manner, Unc-104 has been proposed to be part of the molecular machinery that regulates activity-dependent feedback in *Drosophila* photoreceptors. Thus, defects in plasticity might be of major pathological relevance in the context of disease-related loss of KIF1A function.

We have previously reported a hypomorphic allele, unc-104bris which causes partial loss of kinesin-3 function and thus permits detailed analysis of synapse maturation at the larval stage unlike the unc-104 null mutant that dies at late-embryonic stage. The recessive point mutation unc-104bris at the forkhead associated (FHA) domain causes morphological changes at the neuromuscular junction (NMJ) such as increased NMJ length and synaptic bouton number as well as reduced bouton size. In addition, unc-104bris have increased proportion of postsynaptic glutamate receptor fields that are unapposed by presynaptic active zone (AZ) scaffolding protein Bruchpilot (Brp) and α-Spectrin (α-Spec) as well as reduced folding complexity of the subsynaptic reticulum (SSR). These defects can be rescued by the presynaptic but not postsynaptic expression of unc-104. Collectively, our data reveal new functions of kinesin-3 based transport in synapse development and may have important implications for our understanding of the molecular pathway underlying neurological diseases caused by impaired kinesin-3 function.

Results

Synaptic transmission is impaired in unc-104bris mutants. Unc-104bris mutant larvae demonstrate a distinct synapse maturation defect. At wild-type NMJs only the youngest synapses, in sum approximately 5%, lack the AZ organizing protein Brp. In contrast, at the NMJs of unc-104bris mutant larvae about 25% of the postsynaptic densities (PSDs) are unapposed by Brp. In order to examine the functional consequences of impaired kinesin-3 function, we performed intracellular current clamp recordings at 3rd instar larval NMJs. The amplitude of evoked and miniature excitatory junctional potentials (EJPs and mEJPs) was reduced by 72% and 50% respectively in unc-104bris mutants compared to controls (Fig. 1a–d). The quantal content, which is an estimation of the number of synaptic vesicles (SVs) released per evoked release event, was decreased by 47% at unc-104bris mutant NMJs (Fig. 1e). Furthermore, we observed a 20-fold reduction in mEJP frequency in unc-104bris larvae. All above described functional defects were rescued by ectopic pan-neuronal expression of UAS-unc-104-mcherry (Fig. 1c,f). Expression of the rescue construct in the control background did not affect junctional potentials but resulted in a 42% increase in mEJP frequency (Fig. 1c,f).

Unc-104bris mutants display altered glutamate receptor composition. Multiple presynaptic structural defects have so far been identified in the context of loss of unc-104 function. However, the importance of Unc-104 for postsynaptic development is largely unexplored. We next investigated whether impaired synaptic transmission at unc-104bris mutant NMJs might be caused by altered postsynaptic glutamate receptor composition and/or clustering. To this aim we first performed immunohistochemistry at the NMJ using antibodies against Brp and obligate glutamate receptor subunit IIC (GlurIIC). We observed a 17% reduction of the average GluR field size at the NMJ (Fig. 2a,b). The reduction in size is reflected by plotting all GluR fields based on their size, where unc-104bris mutant NMJs have an increased percentage of small GluR fields (Fig. 2c). To address the role of presynaptic Brp presence on the size of GluR fields, we limited our size analysis only to those GluR fields that were positive for Brp. Intriguingly, we did not observe a difference in GluR field size in the subset of synapses that were positive for Brp (Fig. 2a,d).

Two types of ionotropic glutamate receptors have been identified at the *Drosophila* NMJ synapses. Apart from the three obligatory subunits, they either contain GluRIIA or GluRIIB as a fourth subunit to form a functional heterotetrameric receptor. The *Drosophila* NMJ contains a mixture of IIA- and IIB-type glutamate receptors. We sought to assess whether differential incorporation and stabilization of glutamate receptor complexes could be observed at unc-104bris mutant NMJs. To visualize GluRIIB and GluRIIA, we used a double transgenic line expressing GluRIIB-GFP and GluRIIA-mRFP under the control of their native promotors. The intensity of GluRIIA-mRFP was reduced in unc-104bris mutant synapses (Fig. 2e, arrowheads and I). Localization and abundance of GluRIIB-GFP remained unchanged in unc-104bris mutants (Fig. 2e, arrowheads and g). It has been reported that receptor composition changes during synapse maturation at *Drosophila* NMJ; IIA-type receptors are predominant in immature synapses, whereas the IIA- and IIB-type receptor ratios are balanced as synapses mature. We quantified the IIA- and IIB-type receptors for each PSD and found that consistent with previous reports, small synapses in control group were "IIA-rich" compared with mature ones which had a more balanced glutamate receptor composition (Fig. 2h). In contrast, IIA-receptors are not enriched at small synapses of unc-104bris mutant larvae (Fig. 2h). Irrespective of their size, synapses of unc-104bris mutant larvae contain less IIA-type receptors than control (Fig. 2i), while no changes were observed in the amount of GluRIIB per synapse between control and unc-104bris mutants (Fig. 2j). IIA- and IIB-type glutamate receptors exhibit distinct electrophysiological properties, with the IIA-type receptors showing higher single channel conductance than IIB-type receptors. Therefore, the altered receptor composition may contribute to the decreased EJP and mEJP sizes in unc-104bris mutants.
Impaired abundance of post synaptic markers in *unc-104bris* mutants. Membrane associated guanylate kinase-like proteins (MAGUKs) including PSD protein 93 and 95 (PSD-93, PSD-95), SAP97 and SAP102 are thought to be key organizers of molecular scaffolds at excitatory synapses. We examined the localization of Dlg, the *Drosophila* homolog of PSD-95, at *unc-104bris* mutant synapses to further investigate the impact of reduced Unc-104 function on postsynaptic development. We found that Dlg was severely reduced at *unc-104bris* mutant NMJs. Next, we examined two additional postsynaptic markers, the *Drosophila* NF-κB protein DorB and the SSR enriched, cytoskeletal protein α-Spec. Similar to Dlg, the levels of both DorB and α-Spec were also reduced at the SSR. Reduced levels of synaptic markers observed in *unc-104bris* could be a result of loss of Unc-104 in the presynaptic or postsynaptic compartment or both. Thus, we ectopically expressed UAS-unc-104-mcherry in the *unc-104bris* background either pan-neuronally using elav-Gal4 or in muscles using 24B-Gal4. Presynaptic but not postsynaptic expression of Unc-104 was able to restore normal Dlg abundance in mutant NMJs. Our results suggest that impaired pre-, and not postsynaptic, Unc-104 function is upstream of the partial loss of postsynaptic markers in *unc-104bris* mutants.
Unc-104bris mutants display gross morphological changes in SSR membranes. Type I boutons are surrounded by the SSR, which consists of densely stacked muscle membranes that are thought to be important for the stability of the neuromuscular terminal. Loosening and vacuolization of the SSR has been described in NMJ dismantling during metamorphosis. Given that partial loss of Dlg has been associated with reduced convolution of SSR membranes, we investigated the ultrastructure of the extrasynaptic compartment of larval NMJs using electron microscopy. While wild-type boutons were surrounded by a tight array of muscle membranes, the SSR structure was less dense in Unc-104bris mutant NMJs suggesting that partial loss of Unc-104 function results in gross morphological changes in postsynaptic compartments (Fig. 4a). The ultrastructure of the SSR could be rescued by pre- but not by postsynaptic expression of unc-104 cDNA, suggesting that SSR defects are secondary to loss of presynaptic Unc-104 function (Fig. 4a). Taken together, our results show that partial loss of Unc-104 function resulted in reduced levels of postsynaptic proteins and impaired SSR morphology.

Discussion
Unc-104bris mutant NMJs are characterized by a strong reduction in EJP amplitude (Fig. 1a,b). This is consistent with previous reports that unc-104bris mutant larvae are severely impaired in locomotion; larval lethality is preceded by almost complete paralysis at L3 stage. Notably, the strong reduction in EJP amplitude (Fig. 1a,b) and the number of vesicles released (Fig. 1d) per action potential are not being partially compensated in the postsynaptic compartment by concomitant increase in the mEJP amplitude (Fig. 1c), i.e. the response per vesicle. This is surprising as it has been previously reported that upon decrease in quantal content due to lack of Brp, mEJP amplitudes are scaled up. Loss of Brp from a subset of synapses is a primary defect in unc-104bris larvae.

Figure 2. Impaired glutamate receptor composition at unc-104bris mutant NMJs. (a) Representative confocal images of control and unc-104bris mutant NMJs marked by the presynaptic AZ protein Brp (green) and postsynaptic GluR (grey & magenta). (b) Average PSD size of unc-104bris mutant NMJs is reduced compared to the control (control: 0.395 ± 0.019μm²; unc-104bris: 0.329 ± 0.013μm². P < 0.05). (c) PSD size distribution of control and unc-104bris mutant NMJs. There is an increased proportion of small (<0.3μm²) and decrease of larger (>0.3μm²), likely mature synapses in unc-104bris mutants. (d) The sizes of PSDs apposed by presynaptic Brp are unchanged between control and unc-104bris mutants (control: 0.407 ± 0.020μm²; unc-104bris: 0.412 ± 0.022μm²; P > 0.05). (e) Confocal images of neuromuscular synapses stained with Brp (blue) in control and unc-104bris mutant larvae expressing GluRIIA-mRFP (red) and GluRIIB-GFP (green). GluRIIA was reduced in unc-104bris mutant synapses (arrowheads). (f–h) Quantification of GluRIIA-mRFP intensity, GluRIIB-GFP intensity and GluRIIA/GluRIIB ratio at PSDs in control and unc-104bris mutant NMJs, grouped by synapse size. (f) In control, GluRIIA-mRFP intensity in unc-104bris was significantly lower than control in all size groups. (g) In contrast, GluRIIB intensity in control and unc-104bris mutants PSDs were comparable. (h) As a result, GluRIIA/GluRIIB ratio in all size groups showed stronger impairment. (i) Average size of GluRIIA-mRFP and GluRIIB-GFP fields. (j) Average size of GluRIIB-GFP fields. For f–h and i–j, Number of NMJs analyzed: N ≥ 9 for all size groups. Statistical test: Mann-Whitney test. *P < 0.05; n.s., P > 0.05. Error bars indicate the SEM. Genotypes: control (glurIIA-mrfp, glurIIB-gfp/+), unc-104bris (unc-104bris/unc-104d11024; glurIIA-mrfp, glurIIB-gfp/+).
We observed a strong reduction of mEJP frequency at unc-104bris mutant NMJs (Fig. 1c,f). Kinesin-3 is the major transporter of SVs, and previous reports show several SV markers are severely reduced at unc-104 mutant NMJs. Loss of SVs from the NMJ as well as alterations in the size of the readily releasable pool are possible causes for the decreased mEJP frequency at unc-104bris mutant NMJs. While there is direct ultrastructural evidence for the loss of SVs from boutons in unc-104 null mutant embryos, we did not observe any reduction in the amount of SVs at central synapses in unc-104bris mutant larvae. Interestingly, while presynaptic over-expression of the UAS-unc-104-mcherry transgene in wild-type background did not cause any changes in EJP or mEJP amplitudes, it resulted in an increase in mEJP frequency (Fig. 1c,f). These data suggest that the frequency of spontaneous release is likely particularly sensitive to the abundance of readily releasable pool of SVs at the NMJ which might be regulated by kinesin-3 based axonal transport.

In the nervous system, homeostatic signaling is an essential feedback mechanism to ensure stable synaptic activity in a highly variable environment. Homeostatic signaling has been shown to compensate for perturbation of synaptic excitability through multiple mechanisms including changing the efficacy of SV release, ion channel density and neurotransmitter receptor composition. The conductivity of IIA-type glutamate receptors is much larger than IIB-type receptors, and change in the ratio between these two receptors has been shown to modulate quantal size. Thus, a change in receptor composition is a potential mediator of postsynaptic homeostasis. Synaptic transmission blockage via presynaptic expression of TNT leads for example to higher abundance of the GluRIIA at the PSD. Likewise, the ratio of GluRIIA/GluRIIB containing glutamate receptors changes in dsyd-1 mutants due to a simultaneous increase in GluRIIA and decrease in GluRIIB abundance. However, no such change was observed in liprin-α mutants, that display a similar reduction in glutamate release. Alternatively, this phenotype might thus be caused by a loss of a maturation signal that is present in liprin-α mutants, but lacking in dsyd-1 mutants. Despite the severely impaired presynaptic AZ assembly, no sign of postsynaptic compensation.

**Figure 3. Reduced intensity of postsynaptic markers in unc-104bris mutants.** (a) A stretch of boutons stained with anti-HRP antibody (green) and antibodies against disc large (Dlg, magenta), Dorsal B (DorB, magenta) and α-Spectrin (α-Spec, magenta) showing reduced intensities at the subsynaptic reticulum. (b) Reduced Dlg expression in unc-104bris mutant larvae. Confocal excerpts of NMJ 4 in A2 segment immunostained with antisera to the scaffold protein Dlg. All genotypes were taken with the same laser intensity. The reduced expression of Dlg in unc-104bris mutant larvae can be rescued by the pan-neuronal expression of unc-104-mcherry. Postsynaptic expression of unc-104-mcherry induced by 24B-Gal4 results in no change in the expression of Dlg compared to unc-104bris mutants.
is observed in unc-104bris mutants. Hence, the observed reduction in GlurIIA clustering is likely responsible for the decreased mEJP amplitude, and in turn contributes to the impaired EJP (Fig. 1a–c).

We have previously shown that the unc-104bris mutation causes alteration of the gross NMJ structure, resulting in impaired synapse maturation along with severe reduced anterograde transport of SVs and dense core vesicles20,23. Interestingly, expression of Rab3, an SV-associated protein that is depleted from the nerve terminals of unc-104bris mutants partially ameliorates presynaptic and postsynaptic defects 23. This suggests that some of the phenotypes observed in unc-104bris mutants are likely due to the depletion of Unc-104 cargo at presynaptic terminal. The present study shows a severe loss of mEJP frequency in unc-104bris NMJs (Fig. 1c,f). Given that mEJPs are an instructive signal that guides synaptic development43, some of the observed morphological defects in unc-104bris could be a result of reduced mEJP frequency (Fig. 1c,f). As presynaptic expression of Unc-104 rescues postsynaptic Dlg and SSR phenotypes (Figs 3b and 4a), it would be interestingly to address in a future study, if this rescue is dependent on specific presynaptic Unc-104 cargo.

Dlg, one of the major proteins localized to the SSR, is a known interaction partner of Gtaxin44, the Drosophila homolog of mammalian syntaxin-18, which regulates endoplasmic reticulum (ER) membrane trafficking45. It is intriguing to postulate that Dlg and Gtaxin may cooperatively regulate addition of ER membrane to the SSR46, and via this membrane trafficking pathway ER characteristics might be transferred to the SSR 46. Consistently, ample evidence suggests that local translation might occur at the SSR47–49. This raises the exciting possibility that the reduced SSR complexity observed at unc-104bris mutant NMJs (Fig. 4) might be a result of impaired membrane addition to the SSR. The insufficiently developed SSR at unc-104bris mutant NMJs might be less efficient in local translation of key proteins required for synaptic development. Consistent with this notion, the glutamate receptor subunit IIA, which is strongly reduced at unc-104bris mutant NMJs (Fig. 2f), has been shown to be translated in local, subsynaptic translation aggregates47.

The membrane of insect muscles is invaginated at most synapses to frame a complex array of tubes and folds, the SSR. Although it is morphologically similar to subsynaptic folds that have been proposed to be important to amplify synaptic signals at the vertebrate NMJ, its primary cellular function might be different50. At the Drosophila NMJ, the SSR gradually develops at Type I boutons. It is more pronounced at Type Ib boutons, displaying more fully developed and deeper stacked SSR than Type IIs terminals45,51. Type II and III fibers that often run parallel with Type I innervations might be partially embedded in the SSR emerging from the Type I innervation but do not form a multi-layered complex SSR on their own51. The SSR is also absent from synapses at indirect flight muscles50, suggesting that its role is modulatory rather than essential for neurotransmission. We found that the SSR in unc-104bris mutant larvae is less dense than in control larvae (Fig. 4). While loosening of the SSR has been associated with NMJs dismantling during metamorphosis35, it is not very likely that the reduced SSR complexity at unc-104bris mutant NMJs is due to ongoing neurodegeneration20.

Similar with a previously described HSP Type 10 (SPG10) Drosophila model, larval locomotion was also severely impaired in unc-104bris mutant larvae20,52. However, while robust structural defects were observed upon disturbance of Kinesin-1 based transport43, no signs of synapse dismantling were observed in unc-104bris mutant

![Figure 4. Subsynaptic reticulum development defects in unc-104bris mutants. (a) Electron micrographs of NMJ 4 in A2 segment. In the control group the presynaptic “T-bar” structures are clearly visible and the subsynaptic reticulum (SSR) has a compact shape. The boutons of unc-104bris larvae are considerably smaller and the SSR is less compact. Pan-neuronal expression of unc-104-mcherry rescues this defect. Postsynaptic expression of unc-104-mcherry with 24B-Gal4 does not improve the phenotype.](image-url)
larvae\textsuperscript{20}. Therefore it is more likely that the observed reduction of SSR associated proteins (Fig. 3a–c) and the reduction of SSR density (Fig. 4) at unc-104\textsuperscript{+} mutant NMJs reflects a defect in synapse maturation and/or plasticity rather than being a sign of neurodegeneration.

The degree of behavioral impairment and the lethality of unc-104\textsuperscript{bris} larvae is similar to those described for a Drosophila model for SPG10 caused by dominant negative mutations in kinesin heavy chain (khc), the Drosophila homolog of human KIF5A\textsuperscript{52}. Notably, although both disease models are characterized by a partial loss of kinesin function related to two forms of HSPs, different pathological progressions were observed. SPG10 model larva display a pronounced dystonic posterior paralysis that is reminiscent of the ascending paralysis observed in SPG10 patients\textsuperscript{52}. While SPG30 is also an ascending motoneuron disease that firstly and primarily affects nerve cells that innervate the feet of the patients, unc-104\textsuperscript{bris} larvae suffer from a more generalized paralysis, i.e. NMJs in segments innervated by longer axons seem not to be more severely affected\textsuperscript{50}. This complex phenotype highlights that more detailed studies will be necessary to fully dissect the specific impact of kinesin-3 dysfunction on neural function and human pathology.

While truncation of KIF1A has been associated with another neurological disease HSAN2C\textsuperscript{14}, various mutations of KIF1A have been associated with SPG30 as well as non-syndromic intellectual disability accompanied by variable additional symptoms including progressive encephalopathy and brain atrophy\textsuperscript{16,17,53,54}. Recently, an amino acid exchange (S69L), proposed to be important for the ATP binding of KIF1A, has been shown to underlie SPG30\textsuperscript{52}. A detailed comparison of different disease related mutations may give valuable insight on the specific impact of disrupting the function of different domains of KIF1A.

The amino acid mutated in unc-104\textsuperscript{bris} larvae has been suggested to further stabilize kinesin-3 dimers by electrostatic interaction with the E499 residue\textsuperscript{55}. Defects observed in unc-104\textsuperscript{bris} larvae are not restricted to the axonal compartment, but include defects in dendrite maturation in sensory neurons\textsuperscript{20}, a phenotype most relevant in the context of HSAN2C. We thus suggest that the animal disease model presented herein, rather than being a precise model of any of the human diseases associated with KIF1A dysfunction, might have broad implication for neurological diseases that are associated with impaired stability of kinesin-3 dimer including of HSP, HSAN2C and intellectual disability.

**Methods**

**Fly Stocks.** Flies were cultured on standard soft media seeded with live yeast at 25 °C unless otherwise indicated. w\textsuperscript{1118}, elav-Gal4, 24B-Gal4 and unc-104\textsuperscript{+}\textsuperscript{80} were obtained from the Bloomington Drosophila Stock Center. GluRIIA-mRFP\textsuperscript{24} and GluRIIB-GFP\textsuperscript{26} with endogenous promoters were obtained from Stephan Sigrist (FU Berlin). UAS-unc-104-mcherry was a generous gift from Thomas Schwarz (Harvard University).

**Immunohistochemistry and microscopy.** Middle 3\textsuperscript{rd} instar stage larvae were dissected in Ca\textsuperscript{2+}-free HL3 solution and fixed in 4% formaldehyde in PBS for 3 minutes (for staining with native fluorescent proteins) or for 10 minutes (for staining with only immunofluorescent labeling). Correct NMJs were identified as previously described\textsuperscript{50}. Primary antibody incubation was done overnight at 4 °C in PBS containing 0.05% Triton-X and 5% normal goat serum. Fillets were then washed and incubated with fluorescent-conjugated secondary antibodies at room temperature for 2 hours. Larval fillets were mounted on a glass slide in mounting medium (Vectashield, Vector). Primary antibodies used were: mouse monoclonal anti-Dlg at 1:100, mouse monoclonal anti-Brp (NC82) at 1:100, mouse anti-

**Electrophysiology.** Current clamp intracellular recordings were performed on muscle 6 segment A2 of mid 3\textsuperscript{rd} instar larvae as previously described\textsuperscript{29}. The larvae were pinned and stretched in a Sylvad-coated perfusion chamber and visualized on an Olympus BX51WI microscope. “Bee-stinger” sharp electrodes (10–15 MΩ), made of borosilicate glass (outer diameter 1.5 μm) were filled with 3 M KCl. Only cells with resting potentials between −55 and −80 mV and input resistance higher than 4 MΩ were included in the analysis. Recordings were performed in HL3 Stewart saline\textsuperscript{20} containing (in mM): 70 NaCl, 5 KCl, 20 MgCl\textsubscript{2}, 10 NaHCO\textsubscript{3}, 5 trehalose, 115 sucrose, and 5 HEPES; the concentration of Ca\textsuperscript{2+} was 1, pH adjusted to 7.2. All experiments were performed at 18 °C. Stimulation of the segmental nerve was executed by pulling the cut end of the nerve into a self-made suction electrode (5–6 μm in diameter) filled with HL3 and passing a brief (0.3 ms) bi-polarizing pulse across the nerve. Stimulation was accomplished with an ISO-STIM 01D stimulus modulation unit (NPI electronics GmbH,
Tamm, Germany). The signal was acquired with an Axoclamp 900A amplifier (Axon Instruments), digitized with a Digidata 1440A analog to digital board, and recorded with a PC using pClamp 10.3 (Axon Instruments), and analyzed with AxoGraph X software. The amplitudes of the EJP's were corrected for nonlinear summation. The quantal content was estimated by dividing the averaged corrected EJP by the averaged mEJP amplitude.60

Statistical Analysis. Statistical analysis was performed using the software PRISM 6. Sample errors are given as standard error of the mean (SEM). The following alpha levels were used for all tests: *p < 0.05; **p < 0.01; ***p < 0.001. Data were first tested for normality and then analyzed by either the student's t-test for two groups or by a one-way analysis of variance followed by a Tukey's multiple comparison test. Non-normally distributed data were analyzed by using either a Mann-Whitney test for two groups or a Kruskal-Wallis test for multiple groups.

Use of experimental animals and human subjects. This study did not involve the use of human subjects or samples from human donors. All experiments were performed in accordance with University guidelines and regulations.

References
1. Hirokawa, N., Noda, Y., Tanaka, Y. & Niwa, S. Kinesin superfamily motor proteins and intracellular transport. Nat Rev Mol Cell Biol 10, 682–96 (2009).
2. Hirokawa, N. & Tanaka, Y. Kinesin superfamily proteins (KIFs): Various functions and their relevance for important phenomena in life and diseases. Exp Cell Res 334, 16–23 (2015).
3. Hirokawa, N., Nitta, R. & Okada, Y. The mechanisms of kinesin motor motility: lessons from the monomeric motor KIF1A. Nat Rev Mol Cell Biol 10, 877–84 (2009).
4. Hall, D. H. & Hedgecock, E. M. Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in C. elegans. Cell 65, 837–47 (1991).
5. Otsuka, A. J. et al. The C. elegans unc-104 gene encodes a putative kinesin heavy chain-like protein. Neuron 6, 113–22 (1991).
6. Pack-Chung, E., Kurshan, P. T., Dickman, D. K. & Schwarz, T. A. Drosophila kinesin required for synaptic bouton formation and synaptic vesicle transport. Nat Neurosci 10, 980–9 (2007).
7. Okada, Y., Yamazaki, H., Sekine-Aizawa, Y. & Hirokawa, N. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. Cell 81, 769–80 (1995).
8. Yonekawa, Y. et al. Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice. J Cell Biol 141, 431–41 (1998).
9. Lee, J. R. et al. De novo mutations in the motor domain of KIF1A cause cognitive impairment, spastic paraparesis, axonal neuropathy, and cerebellar atrophy. Hum Mutat 36, 69–78 (2015).
10. Ylikallio, E. et al. Dominant transmission of de novo KIF1A motor domain variant underlying pure spastic paraplegia. Eur J Hum Genet 23, 1427–30 (2015).
11. Klebe, S. et al. KIF1A missense mutations in SPG30, an autosomal recessive spastic paraplegia: distinct phenotypes according to the nature of the mutations. Eur J Hum Genet 20, 645–9 (2012).
12. Hotchkiss, L. et al. Novel De Novo Mutations in KIF1A as a Cause of Hereditary Spastic Paraplegia With Progressive Central Nervous System Involvement. J Child Neurol (2016).
13. Citterio, A. et al. Variants in KIF1A gene in dominant and sporadic forms of hereditary spastic paraparesis. J Neurol 262, 2684–90 (2015).
14. Riviere, J. B. et al. KIF1A, an axonal transporter of synaptic vesicles, is mutated in hereditary sensory and autonomic neuropathy type 2. Am J Hum Genet 89, 219–30 (2011).
15. Zhao, C. et al. Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. Cell 105, 587–97 (2001).
16. Ohba, C. et al. De novo KIF1A mutations cause intellectual deficit, cerebellar atrophy, lower limb spasticity and visual disturbance. J Hum Mutat 60, 739–42 (2015).
17. Esmaeili Nieh, S. et al. De novo mutations in KIF1A cause progressive encephalopathy and brain atrophy. Ann Clin Transl Neurol 2, 623–35 (2015).
18. Kondo, M., Takei, Y. & Hirokawa, N. Motor protein KIF1A is essential for hippocampal synaptogenesis and learning enhancement in an enriched environment. Neuron 73, 743–57 (2012).
19. Sugie, A. et al. Molecular Remodeling of the Presynaptic Active Zone of Drosophila Photoreceptors via Activity-Dependent Feedback. Neuron 86, 711–25 (2015).
20. Kern, J. V., Zhang, Y. V., Kramer, S., Brennan, J. E. & Rasse, T. M. The kinesin-3, unc-104 regulates dendrite morphology and synaptic development in Drosophila. Genetica 195, 59–72 (2013).
21. Kittel, R. J. et al. Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release. Science 312, 1051–4 (2006).
22. Wagh, D. A. et al. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. Neuron 49, 833–44 (2005).
23. Zhang, Y. V. et al. The Drosophila KIF1A Homolog unc-104 Is Important for Site-Specific Synapse Maturation. Front Cell Neurosci 10, 207 (2016).
24. Rasse, T. M. et al. Glutamate receptor dynamics organizing synapse formation in vivo. Nat Neurosci 8, 898–905 (2005).
25. Qin, G. et al. Four different subunits are essential for expressing the synaptic glutamate receptor at neuromuscular junctions of Drosophila. J Neurosci 25, 3209–18 (2005).
26. Schmid, A. et al. Activity-dependent site-specific changes of glutamate receptor composition in vivo. Nat Neurosci 11, 659–66 (2008).
27. Petersen, S. A., Fetter, R. D., Noordermeer, J. N., Goodman, C. S. & DiAntonio, A. Genetic analysis of glutamate receptors in Drosophila reveals a retrograde signal regulating presynaptic transmitter release. Neuron 19, 1237–48 (1997).
28. DiAntonio, A., Petersen, S. A., Heckmann, M. & Goodman, C. S. Glutamate receptor expression regulates quantal size and quantal content at the Drosophila neuromuscular junction. J Neurosci 19, 3023–32 (1999).
29. Marrus, S. B., Portman, S. L., Allen, M. J., Moffat, K. G. & DiAntonio, A. Differential localization of glutamate receptor subunits at the Drosophila neuromuscular junction. J Neurosci 24, 1466–15 (2004).
30. Oliva, C., Escobedo, P., Astorga, C., Molina, C. & Sierra, J. The Role of the MAGUK Protein family in synapse formation and function. Dev Neurobiol 72, 57–72 (2012).
31. Guan, B., Hartmann, B., Kho, Y. H., Gorczyca, M. & Budnik, V. The Drosophila tumor suppressor gene, dgl, is involved in structural plasticity at a glutamatergic synapse. Curr Biol 6, 695–706 (1996).
32. Zhou, B., Lindsay, S. A. & Wasserman, S. A. Alternative NF-kappaB Isoforms in the Drosophila Neuromuscular Junction and Brain. PLoS One 10, e0132793 (2015).
33. Featherstone, D. E., Davis, W. S., Dubreuil, R. R. & Brodie, K. Drosophila alpha- and beta-spectrin mutations disrupt presynaptic neurotransmitter release. J Neurosci 21, 4215–24 (2001).
43. Atwood, H. L., Govind, C. K. & Wu, C. F. Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in Drosophila larvae. J Neurobiol 24, 1008–24 (1993).
45. Liu, Z., Chen, Y., Wang, D., Wang, S. & Zhang, Y. Q. Distinct presynaptic and postsynaptic dismantling processes of Drosophila neuromuscular junctions during metamorphosis. J Neurosci 30, 11624–34 (2010).
47. Budnik, V. et al. Regulation of synapse structure and function by the Drosophila tumor suppressor gene dlg. Neuron 17, 627–40 (1996).
49. Davis, G. W. Homeostatic signaling and the stabilization of neural function. Neuron 80, 718–28 (2013).
51. Murthy, V. N., Schikorski, T., Stevens, C. F. & Zhu, Y. Inactivity produces increases in neurotransmitter release and synapse size. Neuron 32, 673–82 (2001).
53. Thiagarajan, T. C., Piedras-Renteria, E. S. & Tsien, R. W. alpha- and betaCaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. Neuron 36, 1103–14 (2002).
55. Frank, C. A., Kennedy, M. J., Goold, C. P., Marek, K. W. & Davis, G. W. Mechanisms underlying the rapid induction and sustained expression of synaptic homeostasis. Neuron 52, 663–77 (2006).
57. Owald, D. et al. A Syd-1 homologue regulates pre- and postsynaptic maturation in Drosophila. J Cell Biol 188, 565–79 (2010).
59. Zhang, Y. V., B.K., D.T.S., J.V.K., S.B.H. and T.M.R. conceived and supervised the project. Y.V.Z., D.T.S., J.V.K., B.K. and S.B.H. designed the experiments. Y.V.Z., B.K., D.T.S., J.V.K., B.K. and S.B.H. analyzed the data. Y.V.Z., B.K., D.T.S., J.V.K., S.B.H. and T.M.R. wrote the manuscript. All authors revised the manuscript.
Author Contributions

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG, RA 1804/2-1) to T.M.R. We thank Schaller Foundation at the German Cancer Research Institute and University of Heidelberg and Hertie Foundation for supporting this work. We thank Raphael Zinser and Katja Dreissigacker for technical support. We thank Aaron DiAntonio, Hermann Aberle, Stephan Sigrist, Thomas Schwarz and Erich Buchner for providing reagents and fly stocks.

Additional Information

© The Author(s) 2017

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

Competing Interests: The authors declare no competing financial interests.

How to cite this article: Zhang, Y. V. et al. The KIF1A homolog Unc-104 is important for spontaneous release, postsynaptic density maturation and perisynaptic scaffold organization. Sci. Rep. 7, 38172; doi: 10.1038/srep38172 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.