The kinesin-3, Unc-104 regulates dendrite morphogenesis and synaptic development in Drosophila

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Running Head: Unc-104 regulates synaptogenesis

Key words:
Synapse, Kinesin-3, FHA domain, Hereditary spastic paraplegia

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Abstract

Kinesin-based transport is important for synaptogenesis, neuroplasticity, and maintaining synaptic function. In an anatomical screen of neurodevelopmental mutants, we identified the exchange of a conserved residue (R561H) in the forkhead-associated domain of the kinesin-3 family member Unc-104/KIF1A as the genetic cause for defects in synaptic terminal- and dendrite morphogenesis. Previous structure-based analysis suggested that the corresponding residue in KIF1A might be involved in stabilizing the activated state of kinesin-3 dimers. Herein we provide the first in vivo evidence for the functional importance of R561. The R561H allele (unc-104bris) is not embryonic lethal, which allowed us to investigate consequences of disturbed Unc-104 function on post-embryonic synapse development and larval behavior. We demonstrate that Unc-104 regulates the reliable apposition of active zones and postsynaptic densities, possibly by controlling site-specific delivery of its cargo. Next, we identified a role for Unc-104 in restraining neuromuscular junction growth and coordinating dendrite branch morphogenesis, suggesting that Unc-104 is also involved in dendritic transport. Mutations in KIF1A/unc-104 have been associated with hereditary spastic paraplegia and hereditary sensory and autonomic neuropathy type 2. However, we did not observe synapse retraction or dystonic posterior paralysis. Overall, our study demonstrates the specificity of defects caused by selective impairments of distinct molecular motors and highlights the critical importance of Unc-104 for the maturation of neuronal structures during embryonic development, larval synaptic terminal outgrowth and dendrite morphogenesis.

INTRODUCTION

Synapse formation and maturation depend on kinesin-based fast anterograde transport (Hirokawa et al. 2010; Kondo et al. 2012; van den Berg and Hoogenraad 2012). Key synaptic components such as synaptic vesicle precursors (Hall and Hedgecock 1991), mitochondria (Nagakuk et al. 1994), piccolo-bassoon transport vesicles (Cai et al. 2007), and RNA-protein complexes (Ohashi et al. 2002) are transported by members of the kinesin-1 or kinesin-3 families (for review see...
HIROKAWA and NODA 2008). To date, only a limited understanding of the mechanisms underlying cargo specificity and the dynamic regulation of cargo-motor complexes has emerged. In particular, factors that mediate motor activation and cargo binding and release remain to be elucidated (for review see VERHEY and HAMMOND 2009; VERHEY et al. 2011).

In an anatomical screen examining mutants that might be involved in synaptogenesis, we identified a weak hypomorphic mutation (R561H) in the kinesin-3 family member Unc-104. KIF1A/unc-104 has been implicated in hereditary spastic paraplegia (HSP) (ERLICH et al. 2011; KLEBE et al. 2012) and hereditary sensory and autonomic neuropathy type 2 (RIVIERE et al. 2011). The R561H allele (unc-104bris) is not embryonic lethal allowing for detailed larval behavioral analysis. No synapse retraction or dystonic posterior paralysis, as described for the HSP type 10 Drosophila model, in which kinesin-1 based axonal transport is disturbed (FUGER et al. 2012), were observed. Rather, the observed defects were mostly neurodevelopmental.

The affected arginine residue (R561) localizes to the β11-loop of the forkhead-associated (FHA) domain of Unc-104. Structure-based analysis suggests that interaction of the corresponding residue (R583) in human KIF1A with the E499 residue located in the β1/β2-loop might stabilize kinesin-3 dimers (HUO et al. 2012). The functional relevance of the interaction has not yet been elucidated. Here, we employed larvae as a model to investigate the functional importance of Unc-104 during larval development and to characterize the effects of disrupting the proposed electrostatic interaction between the β11- and the β1/β2-loop in vivo.

We show that R561 is important for kinesin-3 function and provide evidence that Unc-104 critically restricts neuromuscular junction (NMJ) growth during larval development. Moreover, Unc-104 is essential for the reliable apposition of active zones (AZs) and postsynaptic densities (PSDs) and controls dendrite branch morphogenesis. In addition to previously described roles (BARKUS et al. 2008; PACK-CHUNG et al. 2007), these novel functions demonstrate that Unc-104 orchestrates
synapse formation and maturation at the NMJ during embryonic and larval development and also regulates dendrite morphogenesis.

MATERIALS AND METHODS

Fly stocks

Flies were raised on standard medium at 25 °C unless otherwise noted. The following fly strains were used in this study: \textit{unc-104}^{d11204} (Thibault et al. 2004), \textit{Df(2R)Exel 7145} (O’Farrell et al. 2008), \textit{Df(2R)Exel 6064} (Parks et al. 2004), \textit{Df(2R)ED 3181}, \textit{elavC155-Gal4}, \textit{D42-Gal4}, and \textit{UAS-actin}^{GFP}. \textit{UAS-unc-104}^{mCherry} was obtained from Thomas Schwarz (Harvard University, USA). Transgenic RNAi stocks were obtained from the Vienna Drosophila RNAi center (Dietzl et al. 2007). \textit{UAS-Brp} and \textit{UAS-Brp-RNAi} stocks were obtained from Stephan Sigrist (FU Berlin, Germany). Fly strains were obtained from Bloomington Stock Center unless otherwise noted.

Immunohistochemistry

For preparation of larval filets, third instar larvae were dissected essentially as previously described (Qin et al. 2005). In brief, larvae were gently stretched and immobilized on a dissection plate with the dorsal midline of the larvae facing upward. Insect pins were positioned at the anterior and posterior end of the larvae. After applying a drop of ice-cold hemolymph-like saline solution, the larvae were cut open along the dorsal midline. The epidermis was stretched and fixed using insect pins. Internal organs were removed using forceps. Care was taken to avoid damaging the body wall muscles and the central nervous system. Next, larvae were fixed (4 % paraformaldehyde in phosphate buffered saline (PBS)), blocked (PBS, 5 % normal goat serum, 0.05 % Triton X100), and stained with primary (4 °C, overnight) and secondary (2 h at room temperature) antibodies essentially as previously described (Qin et al. 2005).
The peptide (PRRSLDKSLDRTPKS) was used to generate and affinity purify the rabbit anti-GluRIII antibody. The purified antibody was used at a 1:1000 dilution. The following primary antibodies, obtained from Developmental Studies Hybridoma Bank, were used at the concentrations indicated: Bruchpilot (nc82) 1:100 and Dlg (4F3) 1:50. VGlut (1:1000) was obtained from Hermann Aberle (MAHR and ABERLE 2006), and Unc-104/Imac (1:1000) was from Thomas Schwarz (PACK-CHUNG et al. 2007). Horseradish peroxidase (HRP)-Cy3 (1:500) and HRP-Cy5 (1:500) were obtained from Dianova. The fluorescence-labeled secondary antibodies were from Invitrogen, Molecular Probes®, or from Fluka/Sigma.

Embryo collection and fixation

Embryos were collected on apple juice plates at 25 °C. At 16.5 h after egg laying (AEL), embryos were prepared for confocal microscopy as previously described (MAHR and ABERLE 2006).

Analysis of sensory neurons

To analyze the dendritic branches in body-wall sensory neurons, larvae were sacrificed by immersing them for 2-4 s in 65 °C water. Next, larvae were mounted in a chamber originally designed for in vivo imaging and imaged within 15 min on a Zeiss LSM 710 Confocal Microscope. Details on the construction of the chamber and larvae mounting have been previously described (FUGER et al. 2007; ZHANG et al. 2010).

Imaging and analysis

Whole larvae or larval filets were imaged on a Zeiss LSM 710 Confocal Microscope, equipped with 405, 445, 488, 514, 561, and 633 laser lines and a ConfoCor 3 scanhead using a 40× Plan-Apochromat 1.3 N.A. or a 63× Plan-Apochromat 1.4 N.A. objective. Unless otherwise specified, the images were obtained using photomultiplier tube (PMT) detection. If fluorescence intensity was low, the sample was imaged using avalanche photodiode (APD) detection. Unless otherwise specified, the voxel
dimensions (x/y/z) for NMJs were 100 x 100 x 500 nm and for ventral nerve cords 300 x 300 x 1000 nm.

Embryonic or larval sensory neurons were recorded with the following voxel sizes: embryonic sensory neurons shown in Figure 3A, A’, B, B’: 346 x 346 x 750 nm; embryonic sensory neurons shown in Figure 3A”, A”’, B”, B”’: 100 x 100 x 500 nm; and larval sensory neurons: 520 x 520 x 750 nm. The cropped images were rescaled by the factor 2, and Gaussian blur filtering was applied (pixel radius = 2). Gamma values were adjusted for illustrational purposes to 0.75. For qualitative comparison of morphology, each genotype was imaged at the maximum level of brightness while avoiding saturation. For quantitative comparisons of intensities, common settings were chosen to avoid oversaturation in any of the genotypes. Image processing was performed using ImageJ v. 1.40g.

The length of NMJs is the sum of the length of all branches. NMJ area was determined by measuring the HRP-positive area. The HRP signal was first rescaled by the factor 2 and then filtered (Gaussian blur, pixel radius: 2) and z-projected (maximum intensity projection). Next, a dynamic threshold was applied to the HRP signal to create a mask that defines the NMJ size. This dynamic threshold is constant within a given genotype, but might differ between genotypes. It was set to 40 % of the mean HRP intensity measured in a given genotype. Bouton numbers were counted manually.

NMJ size and bouton number were normalized to the square of the muscle length, and NMJ length was normalized to the muscle length. Only muscles within the size range: 180 – 350 µm were included in the normalization. Non-normalized NMJ length and size, as well as boutons per NMJ and muscle length are shown for comparison in the supplement (Suppl. Fig. 1A-D).

**Locomotion Analysis**

All solutions used to wash larvae were at room temperature. Behavioral assays were performed at 25 ºC at 70 % humidity.
For qualitative analysis of larval behavior, single larvae were placed on a thin slice of apple juice agar. Dystonic posterior paralysis behavior was scored visually. The animals were recorded at a frame rate of 30 fps for 5 min using a DCM510 camera (ScopeTek, P.R. China) integrated in a custom-built stereomicroscope. Videos were converted using the Prism Video Converter, v. 1.61 (NCH Software Inc., Australia) and further processed using VirtualDub 1.9.10 and ImageJ v. 1.40g. Because size is the main determinant of larval locomotion speed (Fuger et al. 2012), we analyze size-matched, rather than stage- or age-matched larvae that might be slower due to delayed development and smaller size.

For quantitative analysis, up to 200 larvae were recovered from food, dispersed in 15 % sucrose solution, rinsed with tap water, and stored on a temperature- and humidity controlled agar plate for 45 min. Next, the larvae were recorded on a 15 × 15-cm agar plate for 10 min. Locomotion speed and size of larvae were analyzed with the custom-built software Animaltracer (Fuger et al. 2012). Larvae that touched each other were excluded from analysis. Larvae whose velocity was less than 10 % of average velocity of the respective genotype were excluded from analysis. This selection criterion prevented the erroneous inclusion of dead larvae or debris in the data set. Average locomotion speed was separately calculated for each size group. A minimum of six experiments per genotype were analyzed. For all further statistical analysis, “n” was defined as the number of experiments.

**Statistical Analysis**

Statistical tests were performed with PAST software (http://folk.uio.no/ohammer/past/index.html) unless otherwise noted. Sample errors are given as standard deviation (SD) and standard error of the mean (SEM). Statistical tests used in this study are documented in Supplementary Table 1. The following alpha levels were used for all tests: * p<0.05, ** p<0.01, and *** p<0.001.

**Genetic Screen**
To score for defects in synapse formation and maintenance, immunostainings of type I boutons of Drosophila larvae were analyzed. At the investigated NMJ (NMJ 4, Segment A3) typically more than 95% of all PSDs are apposed by mature AZs. We used the cytomatrix of the AZ protein Bruchpilot (Brp) (Kittel et al. 2006) and the glutamate receptor subunit III (GluRIII) (Marrus et al. 2004) as molecular marker for AZs and PSDs, respectively. Mutant strains in which a significant number of unapposed PSDs or AZs were detected upon visual inspection were isolated for further analysis.

**Mapping of bristly**

To map the bris gene mutation, the original stock was outcrossed to a control Drosophila strain (white^1118^) for three generations. Next, we identified three overlapping deficiencies (Ryer et al. 2007; Thibault et al. 2004) (Df(2R): Exel 7145, Exel 6064, and ED 3181) that failed to complement the bristly locus. These deficiencies overlap at position (2R) 12.618.999-12.714.156 of the Drosophila genome. The same area was identified utilizing recombination-based mapping with molecularly defined P-element insertions (Zhai et al. 2003). Next, we tested mutations that affect candidate genes in the identified region.

**RESULTS**

**bristly is an allele of the neuronal kinesin-3 family member unc-104/KIF1A**

Although cytoskeletal structure, membrane protein sorting, and protein targeting mechanisms can be distinct between dendrites and axons, there are a considerable number of proteins important for both axonal and dendritic morphogenesis (Gao et al. 1999). We therefore decided to systematically screen mutants with abnormal dendrite morphogenesis for defects in synapse formation and maintenance. We selected bristly (bris) larvae, in which 17% of all PSDs at NMJs 4 are not apposed by a Brp-positive AZ for further characterization (Fig. 1A).
bris was originally isolated due to enhanced filopodia formation at the tip of the neuron ddaA (Fig. 1B and MEDINA et al. 2006) but did not show obvious defects in dendritic branching (Fig. 1B and MEDINA et al. 2006). The XP insertion unc-104d11204 in the kinesin-3 unc-104 failed to complement the bristly mutation (Fig. 2A, red arrowhead). Sequencing revealed five predicted amino acid changes compared to the referral sequence (Entrez GeneID: 36876, FBgn0034155): R561H, V722L, D1073E, V1170M, and A1450V (Fig. 2B). In contrast to the other four amino acid substitutions, the R561H mutation is absent in the parental strain used to perform the mutagenesis. Except for R561H, the other amino acid substitutions are predicted conservative substitutions. Hence, we concluded that four of the discrepancies from the database sequence (V722L, D1073E, V1170M, A1450V) are naturally occurring polymorphisms (Fig. 2B), while R561H is responsible for the observed phenotype. The original sequences are available in supplemental file S1. R561 is a highly conserved residue in the FHA domain (Fig. 2C,D) that is considered potentially important for dimerization and activation of kinesin-3 motors (Huo et al. 2012).

Previously described unc-104 null mutants (unc-104170/Df(2R)DAlk21) are embryonic lethal (PACK-CHUNG et al. 2007); however, homozygous unc-104bris larvae or transheterozygous (unc-104bris/unc-104d11204 and unc-104bris/unc-104170) larvae die in the third instar larval stage. This new hypomorphic mutation therefore provides a powerful tool to investigate later neuronal processes that might depend on Unc-104/KIF1A. R561H does not adversely affect the stability of Unc-104bris as quantified by immunostaining in ventral nerve cords (Fig. 2E). Pan-neuronal expression of an mCherry-tagged unc-104 cDNA construct is sufficient to rescue transheterozygous unc-104bris/unc-104d11204 larvae (from now on referred to as: unc-104bris/-) to full viability. Pan-neuronal expression of unc-104mCherry using elav-Gal4 is also sufficient to rescue defects in the reliable apposition of AZs and PSDs in unc-104bris/- larvae (Fig. 2F,G). We thus concluded that observed phenotypes are specifically caused by the loss of Unc-104 function.

Dendrite morphogenesis
We next sought to address the cellular basis for the observed dendritic morphology alterations. Dendritic morphology is continuously refined by multiple interrelated pathways controlling outgrowth, branching, guidance, and pruning (Corty et al. 2009). For simplicity, we only differentiated between initial neurite outgrowth in the developing embryo and dendrite maturation, which includes morphological changes during later outgrowth, branching, guidance, and pruning.

*unc-104* expression is first detectable in stage 11 embryos (Pack-Chung et al. 2007). At 14 h AEL, homozygous *unc-104*116 null mutant embryos displayed abnormal axonal growth cone morphology (Pack-Chung et al. 2007). We scored defects in initial sensory neurite outgrowth in mid stage 16 embryos (16.5 h AEL), in which *unc-104*bris has been expressed for roughly 10 h to allow for the manifestation of defects. Sensory neurons were visualized by pan-neuronal expression of UAS-actinGFP. No gross morphological alterations of the peripheral sensory neuron system were observed in *unc-104*bris/- embryos (Fig. 3A,B). Dorsal dendrite extension was nearly completed at 16.5 h AEL both in *unc-104*bris/- and control embryos. No obvious alterations in axonal or dendritic morphology of bipolar neurons (Fig. 3A′, B′, arrowheads) or dendritic arborization neurons (Fig. 3A″,A‴,B″,B‴, arrows) were observed. Hence, we concluded that the morphological defects observed in L3 larvae (Fig. 1B and Medina et al. 2006) were caused by impairments in dendrite maturation.

*unc-104*bris larvae are characterized by impaired larval locomotion

Mutations in the human *unc-104* homolog KIF1A cause HSP (Erlich et al. 2011; Klebe et al. 2012) or hereditary sensory and autonomic neuropathy type 2 (Riviere et al. 2011). We sought to use *unc-104*bris larvae as a model for the cellular consequences of a moderate loss of *unc-104* function that might be relevant in the context of HSP.

HSP is characterized by a progressive spasticity and paralysis of the lower extremities. The selective vulnerability of long motoneuron-axons in the corticospinal tract is an important cellular hallmark of HSP. For HSP Type 10 (HSP10) caused by autosomal dominant mutations in KIF5A/khc, this axon length-dependent vulnerability
of motoneurons could be replicated in Drosophila larvae by ectopic expression of mutant \textit{khc} in the wild-type background (HSP10-model larvae) (Füger et al. 2012). A selective vulnerability of posterior segments was evident by exacerbated morphological defects at synaptic terminals and dystonic posterior paralysis (tail-flip phenotype) (Füger et al. 2012).

Neither homozygous \textit{unc-104}^{bris} larvae nor transheterozygous \textit{unc-104}^{bris/-} larvae displayed dystonic posterior paralysis, indicating that these larvae suffered from impairments distinct from those caused by impaired Khc function (Füger et al. 2012; Hurd and Saxton 1996).

HSP10-model larvae displayed a size-dependent reduction in larval locomotion speed that is most pronounced in large larvae (Füger et al. 2012). Semi-automated analysis using the custom-built algorithm Animaltracer (Füger et al. 2012) revealed that both small and large homozygous \textit{unc-104}^{bris} larvae are slower than control larvae, whereby the degree of impairment in larval locomotion relative to control larvae is independent of larval size (for larval length 1-2.5 mm, wt: $0.42 \pm 0.03$ mm/s, \textit{n}=6; \textit{unc-104}^{bris}: $0.19 \pm 0.01$ mm/s, \textit{n}=7 and for larval length 2.5-4 mm, wt: $0.61 \pm 0.05$ mm/s, \textit{n}=6; \textit{unc-104}^{bris}: $0.24 \pm 0.02$ mm/s, \textit{n}=8). The genetically more impaired transheterozygous \textit{unc-104}^{bris/-} larvae were almost paralyzed (Fig. 4A) and displayed a body posture defect (Fig. 4B).

Comparative behavioral analysis revealed that impairments in anterograde molecular motor function caused by either ectopic overexpression of dominant-negative \textit{khc}-constructs or the hypomorphic \textit{unc-104}^{bris} allele led to distinct behavioral defects. Those caused by the \textit{unc-104}^{bris} allele were likely neurodevelopmental and not neurodegenerative in nature, possibly due to the impaired maturation of a subset of synapses.

\textbf{Synaptic terminals in \textit{unc-104}^{bris} larvae are not subject to neurodegeneration}

KIF1A has been shown to be important for hippocampal synaptogenesis and synaptic bouton formation (Hirokawa et al. 2010). During Drosophila
synaptogenesis, Brp is reliably recruited to nascent AZs within a few hours following new PSD formation (Rasse et al. 2005). This matching of PSDs and AZs is disturbed in unc-104bris/- larvae. Defects in apposition might occur due to (i) presynaptic nerve retraction, (ii) rate limiting abundance of Brp, (iii) delivery of Brp to extra-synaptic sites, or (iv) disturbances in the molecular mechanism that allocate AZ components such that all synapses receive at least the minimal amount necessary to assemble a fully functional AZ.

Firstly, we sought to address whether defects in the apposition of pre- and postsynaptic components are the result of degenerative processes. Distal NMJ regions are preferentially more affected by degenerative processes than proximal regions (Sherwood et al. 2004). In unc-104bris larvae, however, unapposed PSDs were distributed in a “salt-and-pepper” pattern throughout the NMJ without obvious regional preference (Fig. 5A, arrowheads), indicating that synaptic defects are likely caused by impaired synaptogenesis at the level of individual synapses.

Postsynaptic membranes at the Drosophila NMJ are a complex series of membrane folds (subsynaptic reticulum, SSR) that develop only gradually upon presynaptic innervation and remain stable for hours after presynaptic retraction. Thus, terminal boutons positive for SSR marker but negative for the presynaptic membranes or synaptic vesicles (SV) are indicative of synapse retraction (Eaton et al. 2002). We visualized the presynaptic membrane, SSR, and SV with antibodies directed against HRP, the postsynaptic Discs-large protein (Dlg), and the vesicular glutamate transporter VGlut, respectively (Maehr and Aberle 2006). No signs of synapse retraction were observed in unc-104bris larvae (Fig. 5B-E). Immunostaining analyses suggested delayed SSR development, as more synaptic boutons positive for HRP and VGlut but negative for Dlg (Fig. 5C,E, arrowheads) were observed in homozygous unc-104bris larvae (WT: 0.15 ± 0.08, n=10; unc-104bris: 0.85 ± 0.08, n=10; p<0.001, Student’s two-tailed t-test).

Secondly, the reliable apposition of PSDs and AZs might be disturbed by a rate limited supply of Brp. The loss of Unc-104 function has been shown to affect cargo transport as quantified by cargo redistribution in the ventral nerve cord (Barkus et al. 2002).
2008; PACK-CHUNG et al. 2007). In control larvae, Unc-104 cargos are scant in the cortex and enriched in the neuropil. As previously reported for unc-104 null embryos (PACK-CHUNG et al. 2007), this distribution is reversed in unc-104bris/- larvae (Fig. 6A). Furthermore, Brp density and the amount of Brp per NMJ in unc-104bris/- larvae were reduced by 70 % and 74 %, respectively (Fig. 6B-D). Pan-neuronal expression of unc-104mCherry was sufficient to rescue impairments in Brp transport (Fig. 6A-D).

As insufficient supply of Brp is a potential cause of the observed defects in the apposition of AZ and PSDs at unc-104bris mutant synaptic terminals, we next rescued overall Brp abundance at NMJs (Fig. 7A,B) by pan-neuronal overexpression of Brp. Although Brp overexpression was sufficient to restore the overall abundance of Brp at the NMJ and the normal size of individual Brp punctae (Fig. 7A,C), the percentage of presynaptically unapposed PSDs was still four times higher in Brp-overexpressing unc-104bris/- larvae compared to control larvae (Fig. 7A,D). Brp-punctae are generally apposed by PSDs (Fig. 7A), suggesting that the molecular mechanisms that prevent AZ formation in the perisynaptic regions of the NMJ are intact in unc-104bris/- larvae. However, PSDs are not reliably apposed by Brp punctae (Fig. 7A, arrowheads), indicating that Unc-104 might orchestrate the site-specific delivery of AZ components to defined synapses, thereby ensuring the proper allocation of Brp to all AZs.

**Unc-104 is essential to restrict NMJ growth**

Impairments in kinesin-1- and kinesin-3-based anterograde transport cause a reduction in NMJ size and a decrease in the number of synaptic boutons (BARKUS et al. 2008; FUGER et al. 2012; HURD and SAXTON 1996). However, loss of the HSP-associated genes atlastin, spastin, and spichthyin induce NMJ overgrowth (BAYAT et al. 2011).

To test whether impaired Unc-104 function leads to morphological changes at the synaptic terminal the following parameters were determined: NMJ length, NMJ size, PSDs per NMJ, boutons per NMJ, bouton size, and number of synapses per bouton. NMJ size is determined by the area of HRP staining. NMJ length is a measure of synaptic terminal outgrowth, while NMJ size and the number of PSDs per NMJ are a
measure of both outgrowth and functional maturation. NMJs of unc-104bris/- larvae were longer but not larger than NMJs in control larvae (Fig. 8A-C). The number of receptor fields per NMJ was decreased in unc-104bris/- larvae (control: 277 ± 23, n=9; unc-104bris/-: 137 ± 8, n=9; rescue: 226 ±34, n=9; control/unc-104bris/-; p<0.001, Kruskal-Wallis H-test). NMJs of mutant larvae consist of more but smaller synaptic boutons that contain fewer glutamate receptor fields than control boutons, suggesting that terminal outgrowth was not matched by proper functionalization (Fig. 8A,D-G).

The overgrowth of unc-104bris/- NMJs was unexpected because previously described null and hypomorphic alleles cause defects in bouton formation and decreased number of boutons per NMJ, respectively (Barkus et al. 2008; Pack-Chung et al. 2007). We thus sought to (i) further investigate the nature of the putatively hypomorphic unc-104bris mutation and (ii) verify that defects are solely caused by a presynaptic loss of Unc-104 function.

The phenotype of hypomorphic alleles is less severe when homozygous than transheterozygous to a null allele or a deletion. unc-104bris/- larvae have stronger impairments in larval locomotion than homozygous unc-104bris larvae. Generally, morphological defects were more pronounced in the former (Fig. 8A,B,D,E,G-L); however, this difference was only statistically significant for Brp density (Fig. 8I). Defects in bouton size do not follow this general trend (Fig. 8F). Despite a smaller bouton size and less PSDs per bouton, no NMJ morphogenesis defects were observed in heterozygous unc-104bris/+ larvae (Fig. 8E-G).

RNAi-mediated knockdown of Unc-104 in neurons (elav-Gal4, D42-Gal4) but not in muscles (Mhc-Gal4, 24B-Gal4) is sufficient to induce lethality (data not shown). No Unc-104 signal was detectable in non-neuronal tissue (Pack-Chung et al. 2007). Pan-neuronal expression of unc-104mCherry in the unc-104 mutant background was sufficient to rescue defects in NMJ morphogenesis (Fig. 9A-G). Postsynaptic expression of unc-104mCherry did not rescue neuromuscular morphological defects (Fig. 10A-C).
Overall, the morphological and behavioral analyses suggest that (i) \textit{unc-104}^{bris} is a hypomorphic allele and (ii) defects are solely caused by loss of neuronal Unc-104 function.

**NMJ overgrowth is independent of presynaptic Brp abundance**

NMJ overgrowth in \textit{unc-104}^{bris} mutants, as quantified by NMJ length and the number of synaptic boutons, might be attributable to the loss of a specific function of Unc-104. Alternatively, defects in AZ assembly and maturation of synapses caused by the loss of Brp from some synapses might induce compensatory NMJ overgrowth.

We favor the former possibility for the following reasons: firstly, reduction in synaptic Brp levels alone, using transgenic RNAi, do not cause changes in overall NMJ size (\textit{Wagh} \textit{et al.} 2006). Secondly, Brp clusters calcium channels at AZs (\textit{Kittel} \textit{et al.} 2006). Reduced expression of the voltage-dependent calcium channel subunit cacophony (cac) or a point mutation (cac^{NT27}) that disrupts calcium entry and subsequent vesicle fusion limits NMJ growth rather than promoting NMJ overgrowth (\textit{Rieckhof} \textit{et al.} 2003).

If NMJ overgrowth is triggered as a compensatory response to the reduced level of synaptic Brp, further reduction or overexpression of Brp should exacerbate or rescue existing defects, respectively. To evaluate this hypothesis, we utilized previously validated \textit{UAS-Brp} and Brp-RNAi constructs (\textit{Wagh} \textit{et al.} 2006). NMJ overgrowth was neither suppressed by the overexpression of Brp nor exacerbated by further reduction of Brp using RNAi (Fig. 11A-F). Defects in the enlargement and maturation of boutons persisted both after decreasing and increasing Brp abundance (Fig. 11E,F). We thus conclude that neither the overgrowth of NMJs nor the defects in bouton enlargement are secondary to reduced Brp abundance at the NMJ or its loss from a subset of AZs. Rather, they constitute novel functions of the Unc-104 FHA domain in limiting NMJ length and coordinating bouton enlargement.

**DISCUSSION**
Dendrite morphogenesis

Sensory neurons that normally bear actin-rich filopodia display increased filopodial density in \textit{unc}-104\textsuperscript{bris} mutants with no obvious defects in dendritic branching (observed herein and previously MEDINA \textit{et al.} 2006). Interestingly, alterations in the structure of actin-rich dendritic spines were among the first neuroanatomical changes associated with mental retardation (MARIN-PADILLA 1972). More than 100 genes that coordinate dendrite arborization have been identified to date. Among these genes are two molecular motors: KIF5 (HOOGENRAAD \textit{et al.} 2005) and dynein (ZHOU \textit{et al.} 2012). Our results suggest that Unc-104-based dendritic transport is important for maintaining correct dendrite morphology. Because no defects in dendrites were detected 16.5 h AEL, we concluded that impaired Unc-104 function during later outgrowth, branching, guidance, and pruning leads to the defects in larval dendrite morphology described here. Sensory neuron dendritic arbor morphology has been suggested to affect the ability of cells to perceive and transmit external stimuli (HALL and TREININ 2011). The resulting association between form and function might explain the divergence of dendritic morphologies. For example, class I, II, and III multidendritic sensory neurons, which respond to touch and serve as proprioceptors to coordinate larval contraction wave propagation, differ morphologically from class IV multidendritic neurons that sense noxious stimuli, such as heat, high-threshold mechanical stimuli, parasitoid wasps, and noxious light (HALL and TREININ 2011; TSUBOUCHI \textit{et al.} 2012). \textit{unc}-104\textsuperscript{bris} larvae are characterized by enhanced filopodia formation. As the number of dendritic sensory filopodia can correlate with the strength of the gentle touch response in class III multidendritic neurons (TSUBOUCHI \textit{et al.} 2012), a detailed investigation of the behavioral responses to external stimuli in \textit{unc}-104\textsuperscript{bris} larvae might enhance our understanding of the molecular pathways underlying sensory neuron dendrite structure and function.

Synapse apposition

\textit{unc}-104\textsuperscript{bris} larvae are characterized by impairments in the reliable apposition of AZs and PSDs as quantified by staining for Brp (WAGH \textit{et al.} 2006) and glutamate receptors. Brp clusters calcium channels at AZs and stabilizes T-bars, which are
electron-dense presynaptic structures that have been shown to facilitate synaptic release (Kittel et al. 2006). In wild-type larvae primarily very young (<3 h), immature synapses are Brp negative (Rasse et al. 2005). Because Brp-negative synapses have a low vesicle release probability, the accumulation of Brp at nascent AZs is an important step during synapse maturation (Kittel et al. 2006; Rasse et al. 2005; Schmid et al. 2008). The high percentage of Brp-negative synapses in the unc-104bris mutant suggests that synapse maturation is impaired either by rate-limiting axonal transport of Brp, defective delivery of Brp to AZs, or the inability to stabilize synaptic Brp. Restoration of Brp abundance at NMJs in unc-104bris larvae ameliorates but does not rescue defects in the apposition of AZs and PSDs. We thus propose that the R561H mutation in Unc-104’s FHA domain might disrupt a previously unknown function of Unc-104 in orchestrating the site-specific delivery of Brp to defined synapses.

The specificity of defects caused by the R561H mutation is further supported by the analysis of unc-104170/- mutant embryos. The total amount of Brp per NMJ at 21 h AEL was more severely reduced than in unc-104bris larvae (Pack-Chung et al. 2007). Nonetheless, no defects in the apposition of AZ and PSDs were reported; most PSDs were apposed by a weak Brp-positive signal (Pack-Chung et al. 2007).

The precise coordination of synaptic cargo delivery to defined synapses is important to functionalize and facilitate these synapses (for review see Clarke et al. 2012; Vitureira et al. 2011). Intact Unc-104/KIF1A-based cargo trafficking might, reminiscent of kinesin-1-based long-range transport (Puthanveettil et al. 2008), be important for the late phase of long-term potentiation (Bliss and Lomo 1973), which is highly relevant in the context of learning and memory. Indeed, environmental enrichment has been shown to be sufficient to induce brain-derived neurotrophic factor (BDNF)-dependent up-regulation of KIF1A (Kondo et al. 2012). The resulting hippocampal synaptogenesis and learning enhancement is absent in transheterozygous Bdnf+/− and Kif1a+/− mutant mice, highlighting that Unc-104/KIF1A is essential for experience-dependent neuroplasticity (Kondo et al. 2012).
We propose that the presynaptic mechanism underlying neuroplasticity depends on both Unc-104-based long-range axonal transport and the site-specific delivery of synaptic cargo to defined synapses. Both processes might be perturbed by mutation of the R561 residue that localizes to the β11-loop of the FHA domain. The corresponding residue (R583) in KIF1A has been suggested to further stabilize kinesin-3 dimers by electrostatic interaction with the E499 residue (Huo et al. 2012). Further investigation of the effects of R561H on the activation and deactivation of Unc-104 upon cargo loading and release might provide novel insights into the biomechanics of kinesin-3-based cargo trafficking.

Molecular pathways underlying structural defects at NMJs

The herein described overgrowth of NMJs in *unc-104bris* larvae was surprising as both impairments in kinesin-1- and kinesin-3-based anterograde transport were reported to result in reductions in both NMJ size and synaptic bouton number (Barkus et al. 2008; Fuger et al. 2012; Hurd and Saxton 1996). Conversely, phenotypic comparison with other HSP-associated genes revealed that the NMJ overgrowth is a common phenotype in HSP fly models. Null alleles in atlastin, spastin, and spichthyin result in synaptic bouton number increases of 17 %, 60 %, and 100 % respectively (Bayat et al. 2011).

Interestingly, in *unc-104bris/-* larvae, NMJ overgrowth and defects in bouton enlargement and maturation seem to be independent of Unc-104’s role in fast axonal transport of AZ components and might thus represent novel functions of Unc-104.

Defects in bidirectional communication between muscles and motoneurons might induce NMJ overgrowth. The absence of bone morphogenetic protein (BMP)-signaling suppressors, such as daughters against decapentaplegic and the HSP-related gene spichthyin, causes NMJ overgrowth (Sweeney and Davis 2002; Wang et al. 2007). Thus, the NMJ overgrowth observed in *unc-104bris* larvae is consistent with potential over-activation of BMP signaling. Due to the importance of BMP signaling in synapse stabilization (Eaton and Davis 2005), its over-activation might
prevent the elimination of functionally impaired synaptic terminals in \textit{unc-104^{bris}} mutant larvae.

**Conclusions**

Our identification of a novel \textit{unc-104} allele highlights the importance of kinesin activity during later stages of neuronal development. The results described here shed light on three previously unknown roles of Unc-104. First, it is important for maintaining correct dendritic morphology. Second, Unc-104 is required for the reliable apposition of AZs and glutamate receptor fields. Third, Unc-104 is important to limit NMJ outgrowth as measured by NMJ length and bouton number.

Alterations in dendritic and synaptic terminal maturation are independently associated with mental retardation and neurodegeneration. The fact that a single amino-acid substitution of arginine for histidine in the FHA domain of Unc-104 is sufficient to perturb all these processes presents an ideal tool to decipher the biophysical underpinnings of neurodevelopmental disorders.

Further study is needed to decipher the exact role of Unc-104 in orchestrating synaptic terminal outgrowth and functional maturation. Studies of the FHA domain in a biochemical setting may provide novel insights into (i) the biomechanics of kinesin-3 based cargo trafficking, (ii) Unc-104’s roles in synapse development and synaptic plasticity, and (iii) the molecular mechanism underlying HSP and neurodevelopmental diseases in which synapses might fail to mature.

**Acknowledgements**

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG, RA 1804/2-1 to T.M.R.) and NINDS (NS080108 to J.E.B.). Y.V.Z. was supported by a fellowship of the China Scholarship Council. We thank Christian Hünefeld, Roman Beck, Jörg Odenthal, Raphael S. Zinser, Shabab Hannan, Junyi Zhu, and Jutta Bloschies for help and/or advice. We thank Stephan Sigrist, Hermann Aberle, Ann Goldstein, and Thomas Schwarz for providing reagents.
Figure Legends

Figure 1. Bristly (bris) mutants are characterized by defects in synaptogenesis and dendritic filopodia formation. (A) Confocal immunofluorescence images showing several synaptic boutons at NMJ 4 of mid third instar Drosophila larvae. Wild-type and bris larvae were stained for Glutamate receptor III (GluRIII, green) and Bruchpilot (Brp, grey and magenta). Many postsynaptic densities (PSDs) are unapposed by a presynaptic Brp puncta (arrowheads) in bris larvae. Scale bar: 1 µm. (B) While dendritic branching of the neuron ddaA (cell body: blue arrowhead) is unaffected, greatly increased filopodia formation at distal secondary branches is detected in bris third-instar larvae. Scale bar: 20 µm. * For visualization of secondary branches, brightness and contrast were adjusted in both enlargements. Scale bar: 10 µm.

Figure 2. bristly is an allele of the neuronal kinesin-3 family member unc-104/KIF1A. (A) Schematic of the unc-104 gene locus. Numbers indicate the chromosomal base pair position. Three overlapping deficiencies denoted as black boxes (from top to bottom Df(2R): Exel7145, Exel 6064, ED 3181) failed to complement the bris locus. The three deficiencies overlap at position (2R) 12.618.999-12.714.156 (orange) of the Drosophila genome. The XP insertion unc-104<sup>d11204</sup> (red arrowhead) failed to complement bris. Unc-104 is denoted as green box, other genes in the identified region are shown in blue or pink. (B) Five alterations from the unc-104 reference sequence deposited at Flybase (Entrez GeneID: 36876, FBgn0034155) could be identified. Four of these mutations (V722L, D1073E, V1170M, and A1450V) represent likely polymorphisms because they were present in the parental strain used to perform the mutagenesis (left panel: unc-104<sup>bris</sup>, right panel: parental strain). The original sequencing reads are shown. Arrow indicates the sequenced DNA strand. (C) Predicted domain structure of unc-104 and locations of selected mutations. R561H localizes to a conserved residue in the FHA domain of unc-104. Motor domain: purple; Coiled coil rich region: grey; FHA domain: orange, Liprin binding domain (LBD): green, PH-domain: blue. (D) Alignment of Unc-104 in various species (Drosophila melanogaster [D.m.], Tribolium castaneum [T.c.], Caenorhabditis elegans [C. e.], Danio rerio [D.r.], and Homo sapiens [H.s.]). The mutated R561 residue (red
(E) Unc-104 abundance is not significantly reduced in ventral nerve cords of *unc-104*bris* larvae. (F) Percentage of PSDs unapposed by Brp were quantified in NMJ 4, Segment A2 of mid third instar Drosophila larvae of the following genotypes: *elav-Gal4/+;UAS-unc-104mCherry/+* (control), *unc-104*bris*/*;UAS-unc-104mCherry/+ (*unc-104*bris*/*), and *elav-Gal4/+;unc-104*bris*/*;UAS-unc-104mCherry/+ (rescue). The standard error of the mean (SEM) is shown as a box, the standard deviation (SD) as a black line. *** p<0.001. (G) Synaptic boutons at NMJ 4 of mid third instar Drosophila larvae stained for GluRIII (green) and Brp (grey and magenta). Pan-neuronal expression of *unc-104*mCherry rescues defects in the apposition of AZs and PSDs. Arrowheads point at PSDs unapposed by a Brp-positive AZ. Scale bar: 1 µm.

**Figure 3.** Dendrite morphology is unchanged in *unc-104*bris*/*- embryos at 16.5 h AEL. (A,B) Confocal images of sensory neurons in embryos that express UAS-actinGFP pan-neuronally (*elav-Gal4/+;UAS-actinGFP/+ [control] and *unc-104*bris*/*;UAS-actinGFP/+ [unc-104*bris*/-]). (A-B) No morphological alterations were observed in *unc-104*bris*/*- embryos. (A’,B’) The axonal or dendritic morphology of bipolar (arrowheads) or dendritic arborization neurons (A’’,A’’’,B’’,B’’’ arrows) is indistinguishable between *unc-104*bris*/*- and control embryos. Scale bars: A, 20 µm; A’ and A’’, 10 µm; A’’’, 1 µm.

**Figure 4.** Impaired larval locomotor activity in *unc-104*bris*/* larvae. (A) *unc-104*bris*/* larvae are slower than wild-type larvae. *unc-104*bris*/*- larvae are largely immobile and die in the late third instar stage. (B) Larvae in the act of crawling. No dystonic posterior paralysis is observed. *unc-104*bris*/*- larvae display a body posture defect that can be rescued by pan-neuronal expression of *unc-104*mCherry. The SEM is shown as a box, the SD as a black line. *** p<0.001.

**Figure 5.** No synapse retraction observed in *unc-104*bris*/* larvae. (A) Proximal and distal synaptic boutons at NMJ4 of mid third instar Drosophila larvae stained for GluRIII (green) and Brp (grey and magenta). Unapposed PSDs (arrowheads) localize to proximal and distal boutons in *unc-104*bris*/* larvae. Scale bar: 1 µm. (B-E) NMJ 4, segment A2 or A3 of mid third instar Drosophila wild-type (B,D) and *unc-104*bris*/* (C,E) larvae were stained for the membrane marker HRP (B,C; magenta), the postsynaptic
scaffolding protein Discs-large (Dlg) (B-E; green) and the vesicular glutamate transporter (VGlut) (D,E; grey, magenta). The presence of organized postsynaptic Dlg staining unapposed by presynaptic structures is indicative of synapse retraction, which typically occurs at distal boutons (arrowheads). No synapse retraction was observed in unc-104bris larvae. Scale bars: 10 µm and 2 µm (magnified panel).

Figure 6. Axonal transport of Brp is impaired in unc-104bris/- larvae. (A,A’) Ventral nerve cords of mid third instar Drosophila larvae were stained for Brp, which is concentrated in the neuropil region (green N) of wild-type larvae, but is found in the cortex of unc-104bris/- larvae (green C). (A’) Pan-neuronal expression of Unc-104mCherry (rescue) restores Brp neuropil localization in unc-104bris/- larvae. Scale bar: 20 µm. (B) NMJ 4, Segment A2 of mid third instar Drosophila larvae of the following genotypes: elav-Gal4/++;UAS-unc-104mCherry/+ (control), unc-104bris/++;UAS-unc-104mCherry/+ (unc-104bris/), or elav-Gal4/+;unc-104bris/++;UAS-unc-104mCherry/+ (rescue) were stained for Brp. Scale bar: 10 µm. (C) Brp density and (D) Brp abundance per NMJ are reduced in unc-104bris/- larvae compared to control larvae and unc-104bris/- larvae expressing unc-104mCherry. The SEM is shown as a box, the SD as a black line. * p<0.05, ** p<0.01, *** p<0.001.

Figure 7. Brp overexpression does not rescue the AZ and PSD apposition defect in unc-104bris/- larvae. (A) Confocal images of neuromuscular synapses immunostained with Brp (magenta and grey) and GluRIIC (green) in larvae of the following genotypes: elav-Gal4/++;UAS-unc-104bris/ (control), elav-Gal4/++;unc-104bris/ (unc-104bris/-), or elav-Gal4/+;UAS-Brp/ (unc-104bris/,-Brp ↑). Arrows indicate PSDs unapposed by presynaptic Brp punctae. No Brp punctae without an apposed PSD were detected. Scale bar: 1 µm. Quantification of the abundance of Brp per NMJ (B), Brp quantity at a single puncta (C), and the percentage of PSDs unapposed by Brp (D). Experiments were performed at 29 °C. The SEM is shown as a box, the SD as a black line. * p<0.05, ** p<0.01.

Figure 8. Altered NMJ morphology in unc-104bris/- larvae. (A) NMJ 4, Segment A2 of mid third instar Drosophila wild-type, unc-104bris/+, unc-104bris, and unc-104bris/.
larvae were stained with antibodies against HRP (A,E), GluRIII (E,L) and Brp (L).
Scale bars: A: 10 µm, E,L: 1 µm. NMJ length (B), NMJ size (C), bouton number (D),
bouton size (F), and PSD per boutons (G) were determined. The same genotypes
were stained with Brp (H). Brp density (I), Brp per NMJ (J) and percentage of PSDs
unapposed by Brp (K) were quantified. The SEM is shown as a box, the SD as a
black line. * p<0.05, ** p<0.01, *** p<0.001. (L) PSDs unapposed by a presynaptic
Brp puncta (arrowheads) were detected in unc-104bris and unc-104bris/- larvae.

Figure 9. Pan-neuronal expression of unc-104 rescues NMJ morphology defects in
unc-104bris/- larvae. (A) NMJ 4, Segment A2 of mid third instar Drosophila larvae of
the following genotypes elav-Gal4/++;UAS-unc-104mCherry/+ (control), unc-104bris/-;
UAS-unc-104mCherry/+ (unc-104bris/-), and elav-Gal4/++;unc-104bris/-;UAS-unc-
104mCherry/+ (rescue) were stained with antibodies against HRP (A,E) and GluRIII (E).
Enlargements are shown in the lower panel (A1). Scale bars: A: 10 µm, E: 1 µm. NMJ
length (B), NMJ Size (C), bouton number (D), bouton size (F), and PSDs per boutons
(G) were determined. The SEM is shown as a box, the SD as a black line. ** p<0.01,
*** p<0.001.

Figure 10. Postsynaptic expression of unc-104 does not rescue morphological
defects in unc-104bris/- larvae. (A-C) NMJ 4, Segment A2 of mid third instar
Drosophila larvae of the following genotypes wild-type, unc-104bris/-, and unc-104bris/-;
24B-Gal4/UAS-unc-104mCherry (postsynaptic rescue) were stained for the membrane
marker HRP. No rescue was detectable following postsynaptic expression of unc-
104mCherry. Enlargements are shown in the lower panel. Scale bar: 10 µm.

Figure 11. Altering Brp abundance does not overtly alter NMJ morphology. (A-F)
NMJ 4, Segment A2 of mid third instar Drosophila larvae of the following genotypes:
elav-Gal4/+;unc-104bris/- (unc-104bris/-), elav-Gal4/+;unc-104bris/-;UAS-Brp-RNAi/+ (Brp↓),
and elav-Gal4/+;unc-104bris/-;UAS-Brp/+ (Brp↑) were stained for the membrane marker HRP (A).
The following characteristics of NMJs were determined: NMJ length (B), NMJ size (C), bouton number (D), bouton size (E), and PSDs per bouton (F). Scale bar: 10 µm. The SEM is shown as a box, the SD as a black line.
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Figure 1
Figure 3
Figure 6

A

WT  unc-104(n1495)  A'  control  rescue

C

N  N  C  N  C  C  N  C  C  N  C  C  N  C  C

Brp

B

control  unc-104(n1495)  rescue

Brp

C

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<th></th>
<th>control</th>
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<td>50</td>
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D

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<td>Brp per NMJ</td>
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<td>50</td>
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Figure 7

A

control

unc-104^{erm-1}/

unc-104^{erm-1}; Brp†

B

Brl per NMJ

control

unc-104^{erm-1}/

unc-104^{erm-1}; Brp†

C

Brl puncta size

control

unc-104^{erm-1}/

unc-104^{erm-1}; Brp†

D

% PSCs proposed by Brl

control

unc-104^{erm-1}/

unc-104^{erm-1}; Brp†
Figure 9

A

control

unc-104^{1044ts/4.}

rescue

HRP

A'

control

unc-104^{1044ts/4.}

rescue

B

NMJ Length [µm]

control

unc-104^{1044ts/4.}

rescue

C

NMJ Size [µm²]

control

unc-104^{1044ts/4.}

rescue

D

Boutons per NMJ

control

unc-104^{1044ts/4.}

rescue

E

HRP

GluRIII

control

unc-104^{1044ts/4.}

rescue

F

Bouton Size [µm²]

control

unc-104^{1044ts/4.}

rescue

G

PSDs per Bouton

control

unc-104^{1044ts/4.}

rescue
Figure 11

A  
unc-104brk/ 

Brp↓  

Brp↑  

HRP  

B  
NMJ Length [μm]  
unc-104brk/  
unc-104brk/  
Brp↓  
Brp↓  
Brp↓  
Brp↓  

C  
NMJ Size [μm²]  
unc-104brk/  
unc-104brk/  
Brp↓  
Brp↓  
Brp↓  
Brp↓  

D  
Boutons per NMJ  
unc-104brk/  
unc-104brk/  
Brp↓  
Brp↓  
Brp↓  
Brp↓  

E  
Bouton Size [μm²]  
unc-104brk/  
unc-104brk/  
Brp↓  
Brp↓  
Brp↓  
Brp↓  

F  
PSDs per Bouton  
unc-104brk/  
unc-104brk/  
Brp↓  
Brp↓  
Brp↓  
Brp↓  

37