RESEARCH ARTICLE

Loss of the Coffin-Lowry syndrome-associated gene RSK2 alters ERK activity, synaptic function and axonal transport in Drosophila motoneurons

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ABSTRACT
Plastic changes in synaptic properties are considered as fundamental for adaptive behaviors. Extracellular-signal-regulated kinase (ERK)-mediated signaling has been implicated in regulation of synaptic plasticity. Ribosomal S6 kinase 2 (RSK2) acts as a regulator and downstream effector of ERK. In the brain, RSK2 is predominantly expressed in regions required for learning and memory. Loss-of-function mutations in human RSK2 cause Coffin-Lowry syndrome, which is characterized by severe mental retardation and low IQ scores in affected males. Knockout of RSK2 in mice or the RSK ortholog in Drosophila results in a variety of learning and memory defects. However, overall brain structure in these animals is not affected, leaving open the question of the pathophysiological consequences. Using the fly neuromuscular system as a model for excitatory glutamatergic synapses, we show that removal of RSK function causes distinct defects in motoneurons and at the neuromuscular junction. Based on histochemical and electrophysiological analyses, we conclude that RSK is required for normal synaptic morphology and function. Furthermore, loss of RSK function interferes with ERK signaling at different levels. Elevated RSK activity was evident in the somata of motoneurons, whereas decreased ERK activity was observed in axons and the presynapse. In addition, we uncovered a novel function of RSK in anterograde axonal transport. Our results emphasize the importance of fine-tuning ERK activity in neuronal processes underlying higher brain functions. In this context, RSK acts as a modulator of ERK signaling.

KEY WORDS: Drosophila, Motoneuron, Neuromuscular junction, RSK, MAPK signaling, Synapse, Axonal transport

INTRODUCTION
The p90 ribosomal S6 kinases (RSKs) are a family of serine-threonine kinases that act as downstream effectors of the RAS-mitogen-activated protein kinase (MAPK) pathway through direct interaction with the extracellular-signal-regulated kinase (ERK). In this way, RSK proteins link MAPK signaling to a multitude of substrate proteins. Thus, they regulate diverse cellular processes, such as gene expression, cell growth, proliferation and cell survival. The RSK family comprises four isoforms (RSK1-4) in vertebrates, which fulfil partly redundant but also isoform-specific functions (Romeo et al., 2012). In Drosophila melanogaster and in other invertebrates, only a single RSK isoform is expressed. The overall sequence conservation of Drosophila RSK to vertebrate RSK proteins shows no preference for a single isoform and is mainly restricted to the known functional domains.

Common structural features of all RSK proteins are two kinase domains (N-terminal kinase domain and C-terminal kinase domain), which are joined by a regulatory linker region. The C-terminal kinase domain becomes activated by ERK-mediated phosphorylation. Next, ERK and the C-terminal kinase domain phosphorylate several residues in the linker region of RSK. One of these sites is essential for binding 3′-phosphoinositide-dependent kinase-1, which, in turn, then phosphorylates and thereby activates the N-terminal kinase domain as the effector kinase. Finally, autophosphorylation of a serine residue near the ERK docking site by the N-terminal kinase domain promotes dissociation of ERK from RSK (Kim et al., 2006; Tangredi et al., 2012). In addition to its function as a downstream effector of MAPK signaling, RSK acts as a localization determinant of ERK and can negatively feed back to prevent hyperactivation of the MAPK pathway (Romeo et al., 2012). Deregulation of RSK function has been linked to several pathophysiological conditions in humans. Mutations in the human RSK2 gene cause Coffin-Lowry syndrome (CLS), an X-linked disorder characterized by facial and progressive skeletal abnormalities and by severe intellectual disabilities in affected males. More than 140 mutations distributed over the RSK2 gene have been identified in individuals with CLS; most of them are deletions or missense mutations that disrupt RSK function (Pereira et al., 2010). Despite the severity of the neurological defects, the processes regulated by RSK2 in the nervous system remain poorly defined.
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periodicity (Akten et al., 2009; Tangredi et al., 2012). No gross RSK is also required in clock neurons to maintain normal circadian phenotypes were identified. Distinct deficits in different behavioral RSK2 is predominantly expressed in brain regions involved in learning and memory; however, the exact functions of RSK2 remain poorly understood. Behavioral defects are observed in RSK2 knockout mice and in Drosophila upon knockout of RSK, the single fly ortholog of vertebrate RSK proteins. In this study, the authors used the Drosophila neuromuscular system as a well-established model for excitatory glutamatergic synapses to study the physiological consequences of loss of RSK function.

Results

Consistent with previous findings, a general upregulation in activity of the final MAPK component, ERK, was observed in RSK-deficient motoneurons in Drosophila. Intriguingly, the authors observed redistribution of activated ERK from synaptic terminals to the somata, suggesting that local signaling events are altered in the absence of RSK. To determine whether RSK is required for synaptic function, the authors performed immunohistochemical and electrophysiological experiments. They demonstrate that loss of RSK function impairs synaptic transmission and, interestingly, defects in anterograde transport of mitochondria were uncovered using in vivo imaging techniques. Overall, their data indicate a postsynaptic requirement of RSK for efficient synaptic transmission, in line with data from studies in mice, but also uncover a postsynaptic role.

Implications and future directions

This study uncovers a multifaceted requirement of RSK2 for regulation of synaptic function and MAPK-dependent processes in neurons. An emerging common picture from animal models of CLS is a postsynaptic function of RSK2. In addition, the present study is the first to implicate RSK in anterograde axonal transport processes, the distribution of activated ERK and synaptic organization in motoneurons, suggesting an additional role for RSK2 in the presynaptic neuron. Exactly how RSK2 regulates presynaptic processes at the molecular level remains to be discovered. A major challenge will be to distinguish the ambivalent functions of RSK2 both as a downstream mediator but also as a negative regulator of MAPK signaling in these processes, and to explore potential differential effects on local MAPK signaling. Only with this knowledge can our understanding of the pathophysiology of CLS be improved further.

Animal knockout models have been established for mouse RSK2 and Drosophila RSK. In analysis of these mutants, diverse phenotypes were identified. Distinct deficits in different behavioral paradigms, such as spatial learning, long-term spatial memory and consolidation of fear memory in mice (Poirier et al., 2007; Morice et al., 2013) and in olfactory, operant and spatial learning in flies, were observed (Putz et al., 2004; Neuser et al., 2008). Drosophila RSK is also required in clock neurons to maintain normal circadian periodicity (Akten et al., 2009; Tangredi et al., 2012). No gross alterations in brain structure of RSK2 mutants are evident, although a decrease in differentiation of cortical radial progenitors into neurons was observed in mice, which indicated a function of RSK2 in neurogenesis (Dugani et al., 2010). At the cellular level, survival of isolated spinal motoneurons from RSK2-deficient mice was not affected, but axonal outgrowth was increased (Fischer et al., 2009a). In the dentate gyrus, alterations in the morphology of dendritic spines were observed (Morice et al., 2013). Physiological changes include an increase of cortical dopamine levels, which is accompanied by elevated expression of the dopamine receptor DrD2L (Pereira et al., 2008). In the hippocampus, upregulated expression of the GluR2 subunit of AMPA-type glutamate receptors (GluRs) is correlated with changes in channel properties, synaptic transmission and long-term potentiation-induced gene expression (Mehmood et al., 2011; Morice et al., 2013). Increased GluR2 expression is caused by elevated ERK-mediated transcriptional activity, which results from a lack of RSK feedback inhibition of the MAPK pathway (Schneider et al., 2011; Mehmood et al., 2013). Increased ERK activity was also observed in RSK2-deficient motoneurons (Fischer et al., 2009a). In Drosophila, RSK negatively regulates ERK-dependent differentiation processes during eye development by acting as a cytoplasmic localization determinant of ERK (Kim et al., 2006). Altogether, these studies provide evidence for a multilayered, cell-type-specific function of RSK2 in the nervous system.

Owing to its relative simplicity and repetitive organization, the Drosophila larval neuromuscular system is a powerful model for the study of synapse formation, neurotransmission and synaptic plasticity at the structural, physiological and molecular levels (Menon et al., 2013). At each individual muscle, the neuromuscular junction (NMJ) shows a fairly reproducible branching pattern, with terminal structures called boutons, each of which harbors a number of presynaptic active zones as neurotransmitter release sites and opposed postsynaptic densities containing receptor clusters. Unlike the cholinergic NMJ in vertebrates, the Drosophila NMJ is glutamatergic and contains ionotropic GluRs that are homologous to non-NMDA-type GluRs in excitatory synapses of the vertebrate brain. Thus, defects in the fly neuromuscular system caused by loss of RSK function can provide insights into RSK2 functions at excitatory synapses of the vertebrate brain. Based on previous genetic experiments, which indicated a negative regulatory function of RSK in MAPK signaling (Fischer et al., 2009b), we now show that loss of RSK causes several distinct defects in motoneurons. First, we observed pronounced redistribution of activated ERK from synaptic sites to the somata. Second, we observed aberrant accumulations of synaptic components and defects in anterograde axonal transport of mitochondria. Third, changes in the number of active zones and postsynaptic receptor fields are evident, which are correlated with impairment of synaptic transmission. These findings provide new insights on RSK function in synaptic plasticity, which might help in understanding the complex pathophysiology of RSK2 mutants in vertebrates and in human disease.

RESULTS

Presynaptic localization of RSK

Based on previous genetic studies, which implicated a function of Drosophila RSK in motoneurons (Fischer et al., 2009b), we first determined the subcellular localization of RSK. Although available antisera against Drosophila RSK detected the endogenous protein on western blots (Putz et al., 2004), the antisera are not sensitive enough to detect endogenous RSK by immunohistochemistry of larval brain and body-wall preparations. Therefore, transgenic flies expressing green fluorescent protein (GFP)-tagged RSK under control of the UAS-enhancer (UAS-RSK::GFP) were established and crossed with the motoneuron driver line D42-Gal4. NMJs at muscle 6/7 in segment A2 of late third instar larval brain and body-wall preparations were examined with antibodies detecting...
in motoneurons and whether loss of RSK has an impact on ERK activity or its subcellular localization. Immunostaining of Drosophila larval motoneurons using a mouse monoclonal antibody against double-phosphorylated (activated) ERK (pERK) gave inconsistent results, both in our hands (data not shown) and in other studies (Koh et al., 2002; Wairkar et al., 2009). We therefore first re-evaluated localization of pERK by using a rabbit monoclonal antibody (#4370; Cell Signaling) in combination with staining for GFP::RSK expressed in motoneurons by D42-Gal4. At the NMJ, pERK and RSK accumulate at presynaptic sites in a spot-like pattern (Fig. 2A). Colocalization is evident for many but not for all puncta, indicating an interaction of the proteins at selective sites of the presynapse. However, localization of both proteins is not restricted to the presynapse. GFP::RSK was also detected in the perikaryon, but not in the nucleus of motoneurons, as distinguished by staining for the nuclear membrane marker Lamin, whereas weak pERK staining was observed in both the perikaryon and the nucleus (Fig. 2B). Predominant cytoplasmic localization of RSK was also previously shown for ectopically expressed RSK in clock neurons of the fly circadian circuit (Akten et al., 2009).

Loss of RSK affects ERK activity and subcellular localization in motoneurons

Previous studies identified Drosophila RSK as a direct interaction partner of ERK (Kim et al., 2006). Binding of ERK is essential for RSK function in eye and wing development and for circadian behavior, and it stimulates activation of the C-terminal kinase domain by phosphorylation. In contrast to biochemical studies with vertebrate RSK proteins, subsequent activation of the N-terminal kinase domain as the effector kinase for phosphorylation of substrate proteins is dispensable, at least for these processes (Kim et al., 2006; Tangered et al., 2012). Based on genetic studies using different combinations of loss- and gain-of-function mutations, it was concluded that in the case of the developing Drosophila eye, RSK acts as a cytoplasmic anchor for ERK to regulate nuclear entry and thereby ERK-dependent transcription (Kim et al., 2006). By contrast, biochemical studies in vertebrates implicate a function of RSK2 in feedback inhibition of the MAPK pathway (Roméo et al., 2012). Corresponding biochemical evidence for Drosophila RSK is still missing. Given that genetic interaction experiments also indicated a negative regulatory function of RSK in the neuromuscular system (Fischer et al., 2009b), we investigated whether RSK and ERK colocalize

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body-wall preparations from control (n=10) and RSKΔ58/1 animals (n=10) expressing mCD8::GFP in motoneurons. Analysis was done at the level of cell bodies and NMJs in identical conditions. For each preparation, pERK and GFP intensity levels were determined in 10 individual cell bodies and from the NMJ at muscle 6/7 in abdominal segment A2. Compared with control animals, normalized pERK values in RSKΔ58/1 were strongly decreased at the NMJ and significantly increased in cell bodies (Fig. 4D,E; Fig. S2). From this analysis, we conclude that RSK is not only required for regulation of pERK levels, but also determines the distribution of pERK within different motoneuron compartments. This implies that deregulation of motoneuron function in RSKΔ58/1 animals could be caused by altered ERK activity within distinct cellular compartments. One possibility would be enhanced ERK activity in cell bodies, leading to transcriptional activation of target genes. Alternatively, mutants might suffer from a lack of ERK activity at presynaptic sites. Given that ERK has been implicated in regulation of synaptic properties at the Drosophila NMJ (Koh et al., 2002; Wairkar et al., 2009), we focused the following phenotypic and physiological analysis of RSKΔ58/1 on the NMJ.

**Loss of RSK affects NMJ size, synapse numbers and function**

Loss of UNC-51, a negative regulator of ERK, leads to a decrease in the number and density of synapses, apposition defects of GluR fields with presynaptic active zones and impairment of evoked transmitter release (Wairkar et al., 2009). Thus, regulation of ERK activity provides one potential mechanism for synapse-specific control of protein composition and transmission. Taking advantage of the stereotypic branching pattern and terminal-specific number of boutons at muscle 6/7 in abdominal segments A2 and A3, we performed staining for BRP, GluRIID and the presynaptic membrane marker horseradish peroxidase (HRP). We quantified the following parameters in wild-type and RSKΔ58/1 animals: overall size of the NMJs, the number of active zones and the number of GluR fields, and the average areas of single active zones and GluR fields (Fig. 5). For the total NMJ size (Fig. 5A), a significant decrease was observed in RSKΔ58/1 animals compared with the wild-type control (NMJ size wild type=605.5±34.1 µm²; RSKΔ58/1=447.6±20.2 µm², P<0.01). At the presynaptic level, the number (Fig. 5B) and area (Fig. 5C) of active zones per NMJ were significantly reduced in RSKΔ58/1 (active zone number wild type=259.4±13.7 and RSKΔ58/1=205.0±6.1, P<0.01; active zone area wild type=0.72±0.06 µm² and RSKΔ58/1=0.48±0.04 µm², P<0.01). The presynaptic defects correlate with a reduction in the number (Fig. 5D) and area (Fig. 5E) of GluR fields (number wild type=257.0±15.0 and RSKΔ58/1=214.6±10.8, P<0.05; area wild type=1.14±0.06 µm² and RSKΔ58/1=0.81±0.05 µm², P<0.01). Calculation of the ratio of active zones to GluR fields (Fig. 5F) showed no difference between wild type and RSKΔ58/1, indicating that the general assembly and integrity of synaptic connections is not disturbed in the mutant. Indeed, no apposition defects of presynaptic BRP and postsynaptic GluRIID were observed, which distinguishes the RSKΔ58/1 from the UNC-51 loss-of-function phenotype. In the UNC-51 mutant, postsynaptic receptor fields are often unapposed to active zones (Wairkar et al., 2009). To validate the pre- and postsynaptic phenotypes seen in RSKΔ58/1, the independent RSK deletion mutant, RSKΔ5 (Kim et al., 2006), was analyzed. Indeed, the same phenotypes were observed (Fig. S3). In addition, we combined the RSKΔ58/1 mutant with the genomic P/RSKI transgene for rescue experiments. Within these animals, values for NMJ size (Fig. 5A: 550.6±24.1 µm², P<0.05 versus RSKΔ58/1), number (Fig. 5B: 248.4±11.3, P<0.05 versus RSKΔ58/1) and the area of active zones (Fig. 5C: 0.60±0.04 µm², P<0.05 versus RSKΔ58/1) were significantly different from RSKΔ58/1, but not from...
RSK

Compared with wild type, the level of pERK but not of ERK is increased in from at least five independent biological experiments (denoted in the bars).

(B,C) Quantification of ERK (B) and pERK (C) levels normalized to non-phosphorylated (arrowhead) and phosphorylated ERK (open arrowhead).

significantly different from wild type or from however, the number of GluR fields (Fig. 5D: 242.5±9.9) was not be fully rescued with this construct. On the postsynaptic site,

third larval instar ventral ganglia probed with antibodies against total ERK, Fig. 3. Loss of RSK increases ERK activity.

(A) Western blots of lysates from third larval instar ventral ganglia probed with antibodies against total ERK, phosphorylated ERK (pERK) and α-tubulin (α-tub). The ERK antibody detects non-phosphorylated (arrowhead) and phosphorylated ERK (open arrowhead).

(B,C) Quantification of ERK (B) and pERK (C) levels normalized to α-tubulin from at least five independent biological experiments (denoted in the bars). Compared with wild type, the level of pERK but not of ERK is increased in RSKΔ58/+ and returned to wild-type levels in the presence of the P[RSK] transgene. *P≤0.05 and **P≤0.001.

wild-type measurements. Thus, the presynaptic phenotypes could be fully rescued with this construct. On the postsynaptic site, however, the number of GluR fields (Fig. 5D: 242.5±9.9) was not significantly different from wild type or from RSKΔ58/+.

Measurements for the area of GluR fields still corresponded to RSKΔ58/+ mutant values (Fig. 5E: 0.78±0.04 μm²). One potential explanation for rescue of presynaptic but not of postsynaptic phenotypes could be an incomplete expression pattern of transgenic RSK or expression of RSK at non-physiological levels. The lack of a suitable antibody for immunohistochemistry did not allow us to test the first possibility. However, RSK protein levels derived from this transgene did not exactly match endogenous RSK expression level (data not shown). Obviously, RSK levels must be tightly regulated to accomplish normal synapse function (see also Fig. 6).

Furthermore, we investigated how the observed decrease in NMJ size and overall number of active zones and GluR fields in both RSK mutants relate to the previously reported increase in the number of synaptic boutons (Fischer et al., 2009b). Consistent with this previous report, in the present study bouton numbers were significantly increased in RSKΔ58/+, and to the same degree in RSKΔ58/+ (Fig. S4A). Notably, in both mutants, many boutons were much smaller in size (Figs S3A and S4B) and contained correspondingly fewer synaptic sites (Fig. S4C); therefore, the number of synapses per bouton area remained unchanged, as previously reported (Fischer et al., 2009b). In summary, the increase of bouton number at the NMJ of RSKΔ58/+ and RSKΔ58/+ animals is counteracted by a strong decrease in bouton size and the number of synapses per bouton, resulting in an overall reduction of NMJ size and of total active zone and GluR numbers.

The involvement of RSK proteins in feedback inhibition of the MAPK pathway but also as one of many downstream targets of ERK signaling (Romeo et al., 2012) impeded prediction of NMJ phenotypes by interfering with ERK activity. Drosophila ERK is encoded by rolled (rl). Complete loss-of-function mutations in rl cause lethality (Biggs et al., 1994). Impairment of ERK function by the homozygous viable hypomorphic rl allele showed no significant effect on all measured NMJ parameters (Fig. 5A-F), as published previously (Wairkar et al., 2009). On the contrary, a dominant gain-of-function allele of rl, rlSem, resulted in moderately elevated ERK activity levels (Brunner et al., 1994; Oellers and Hafen, 1996). Heterozygous rrlSem/+ and homozygous RSKΔ58/+ animals might display similar NMJ phenotypes as a result of increased ERK activity. However, this only holds true for NMJ size (Fig. 5A: 400.8±29.8 μm², P<0.001 versus wild type), number of active zones (Fig. 5B: 170.9±20.6, P<0.001 versus wild type) and GluR fields (Fig. 5D: 166.5±13.8, P<0.001 versus wild type), whereas active zone and GluR areas in rrlSem/+ were not significantly different from wild-type values (Fig. 5C,E). Given that RSK is unable to bind the RlSem protein (Kim et al., 2006), several interpretations are possible; phenotypes arise because of failure to transmit the signal via RSK or enhanced activation of other substrate proteins by RlSem. However, the presence of a wild-type copy of rl in rrlSem/+ animals raises the additional possibility that some phenotypes are masked because endogenous RSK function is still under control of ERK. Resolving these issues by analysis of RSKΔ58/+rlΔ/rlΔ and RSKΔ58/+rlSem/+ double mutants (data not shown) has provided no coherent picture so far.

To analyze whether the observed morphological changes correlate with altered synaptic transmission, we performed two-electrode voltage-clamp recordings at larval NMJs (Fig. 6). The amplitude of miniature excitatory junctional currents (minis), the postsynaptic response to spontaneous fusions of single glutamate-filled synaptic vesicles, was significantly reduced in RSKΔ58/+ mutants (Fig. 6A; wild type=−1.06±0.04 nA, n=15; RSKΔ58/+−0.88±0.04 nA, n=14, rank sum test P<0.001 versus wild type) and remained so upon RSK re-expression (Fig. 6A; RSKΔ58/+P[RSK]=−0.86±0.03 nA, n=15, rank sum test P<0.001 versus wild type). These data are consistent with the reduced area of glutamate receptor fields, which could not be rescued and indicates diminished postsynaptic sensitivity of RSK mutants. By contrast, we observed no functional change in presynaptic properties. The frequency of minis was similar to wild-type animals (Fig. 6A; wild type=1.21±0.11 Hz, n=15; RSKΔ58/+1.12±0.13 Hz, n=14, rank sum test P=0.83 versus wild type; RSKΔ58/+P[RSK]=1.09±0.15 Hz, n=15, rank sum test P=0.11 versus wild type). The reduction in the amplitude of evoked excitatory junctional currents (eEPSCs; Fig. 6B; wild type=−64.68±5.07 nA, n=15; RSKΔ58/+−49.09±4.35 nA, n=14, rank sum test P=0.064 versus wild type; RSKΔ58/+, P[RSK]=−44.54±2.93 nA, n=15, rank sum test P=0.003) could be ascribed to the smaller minis. Correspondingly, the number of vesicles released per action potential (quantal content) was comparable in all three genotypes (Fig. 6C; 83±8, n=15;
RSKΔ58/1 = 76±7, n=14, rank sum test P=0.527 versus wild type; RSKΔ58/1; P[RSK]Δ58/1 = 70±4, n=15, rank sum test P=0.213 versus wild type). Thus, basal properties of neurotransmitter release were not affected by loss of RSK. Instead, the electrophysiological results support a postsynaptic functional role for RSK at the glutamatergic Drosophila NMJ.

**RSK is required for axonal transport**

The morphological analysis of RSK mutant motoneurons revealed another interesting phenotype. Compared with wild-type animals, an increased number of BRP particles were observed in segmental nerves of RSKΔ58/1 larvae (Fig. 7A). This phenotype was most evident in proximal regions close to the ventral nerve cord but not in distal regions near synaptic termini and could be rescued by the P[RSK]Δ58/1 transgene. Quantification of BRP puncta (Fig. 7B) revealed significant differences between RSKΔ58/1 (0.290±0.022 BRP particles per µm² axon area) and either wild-type (0.179±0.014 BRP particles per µm² axon area; P<0.05) or RSKΔ58/1; P[RSK]Δ58/1 animals (0.125±0.026 BRP particles per µm² axon area; P<0.05).

Presynaptic development and function relies on coordinated assembly, transport and delivery of synaptic vesicle precursors, pre-assembled synaptic cytomatrix proteins and mitochondria.
To investigate whether loss of RSK specifically affects BRP or also affects other synaptic components, analyses with a synaptic vesicle-associated protein, cysteine string protein (CSP; Zinsmaier et al., 1994), were performed (Fig. 7A). CSP is associated with the cytoplasmic surface of synaptic vesicles and transported by synaptic vesicle precursors. Quantifications showed a significant increase of CSP accumulations in motoneuron axons of RSKΔ58/1 larvae (Fig. 7C; 0.381±0.040 CSP particles per µm² axon area) in comparison to wild-type animals (0.159±0.002 CSP particles per µm² axon area; \( P < 0.01 \)) or RSKΔ58/1 animals carrying the P[RSK] rescue construct (0.156±0.005 CSP particles per µm² axon area; \( P < 0.01 \)).

The abnormal enrichment of BRP and CSP in proximal nerve regions could indicate impairment of assembly or transport of cargoes to the synaptic terminals. Microtubule-based transport in the anterograde direction by plus-end-directed Kinesin motors and in the retrograde direction by minus-end-directed Dynein motor proteins are essential for neuronal function and homeostasis. Disturbances of these processes have been implicated in the pathogenesis of several neurological disorders (De Vos et al., 2008; Hirokawa et al., 2010; Maday et al., 2014).

Given that we were unable to follow transport of a fluorescently-tagged variant of BRP in motoneurons reliably by in vivo time-lapse imaging (K.B., data not shown), we monitored bidirectional mitochondrial transport. Motility patterns of mitochondria are complex, with phases of fast movement, abrupt changes in direction of movement and stationary phases, and they rely on physiological changes (Zinsmaier et al., 2009; Sheng and Cai, 2012). Mitochondrial transport in motoneurons of living and intact wild-type or RSKΔ58/1 third instar larvae was measured by expression of the mitochondrial GFP marker construct UAS-mito::GFP with the specific motoneuron driver OK6-Gal4. After photobleaching of an axon stretch, mitochondrial movements were recorded within a recovery period of 12 min and an imaging frequency of 720 ms. Kymographs were analyzed for anterograde, retrograde and stationary mitochondria (Fig. 7D). In axons of RSK-deficient larvae, fewer mitochondria were transported in the anterograde direction compared with control larvae (OK6-Gal4;UAS-mito::GFP = 39.3±2.4%; RSKΔ58/1;OK6-Gal4;UAS-mito::GFP = 34.8±2.5%; \( P < 0.05 \)), and the percentage of stationary mitochondria was significantly increased (OK6-Gal4;UAS-mito::GFP = 30.6±4.2%; RSKΔ58/1;OK6-Gal4;UAS-mito::GFP = 36.2±4.3%; \( P < 0.05 \)). These effects are specific to anterograde transport, because transport of mitochondria in retrograde direction was unaffected (OK6-Gal4;UAS-mito::GFP = 30.6±4.2%; RSKΔ58/1;OK6-Gal4;UAS-mito::GFP = 36.2±4.3%; \( P < 0.05 \)).

Taken together, loss of RSK function affects anterograde transport of mitochondria and leads to a biased accumulation of synaptic vesicle precursors and cytomatrix components in axon regions close to the ventral nerve cord.

**DISCUSSION**

One emerging common picture from studies in animal models for CLS is that loss of RSK2 function in neurons is associated with...
The molecular functions of RSK proteins as an interaction partner of ERK proteins are discussed ambivalently in the literature. On the one hand, RSK2 mediates ERK signaling by phosphorylation of numerous targets; on the other hand, it is described as a negative regulator of ERK (Romeo et al., 2012). This ambivalent picture is also reflected by our genetic interaction experiments between RSK and ERK mutants, which did not provide a conclusive answer about the relationship between RSK and ERK with respect to pre- and postsynaptic functions. Further complexity is added because subcellular localization of pERK was changed in RSKΔ58/1 motoneurons, with elevated pERK levels in the somata and strongly decreased levels at the NMJ. Thus, even in a single cell, opposing effects with respect to ERK targets in different subcellular compartments can be expected.

Our results coincide at several points with findings in the vertebrate nervous system. Elevated pERK levels were observed in the hippocampus of RSK2 knockout mice, resulting in deregulation of ERK-mediated gene transcription (Mehmood et al., 2011; Schneider et al., 2011). For instance, transcription of the Gria2 gene encoding the GLUR2 subunit of the AMPA receptor is upregulated. Nevertheless, electrophysiological, biochemical and ultrastructural analyses carried out with isolated cortical neurons and in the hippocampus revealed impaired AMPA-receptor-mediated synaptic transmission. This can be explained, at least in part, by the requirement of RSK2 for phosphorylation of postsynaptic PDZ [post synaptic density protein-95 (PSD95), discs large 1 (DLG1), zonula occludens-1 (ZO1)] domain-containing proteins to regulate channel properties (Thomas et al., 2005; Morice et al., 2013). Our morphological and electrophysiological data at the NMJ are also consistent with a postsynaptic requirement of RSK for synaptic transmission. In addition, RSK mutants displayed a number of defects in the presynaptic motoneuron, including upregulation and relocation of pERK and a reduction in active zone numbers. How do these phenotypes relate to known functions of ERK in Drosophila motoneurons? First, alterations in ERK activity at the NMJ are inversely correlated with levels of the neural cell adhesion molecule Fasciclin II (Koh et al., 2002). Given that Fasciclin II was found to be excluded from pERK-positive spots at synapses, a direct regulatory mechanism at the protein level seems plausible. Thus, it is conceivable that synaptic RSK contributes to Fasciclin II-mediated cell adhesion either directly, by acting as an upstream kinase, or by feedback inhibition of ERK activity. Second, besides RSK, the serine-threonine kinase UNC-51 also acts as a negative regulator of ERK in motoneurons (Wairkar et al., 2009). It is conceivable that synaptic RSK contributes to Fasciclin II-mediated cell adhesion either directly, by acting as an upstream kinase, or by feedback inhibition of ERK activity. Second, besides RSK, the serine-threonine kinase UNC-51 also acts as a negative regulator of ERK in motoneurons (Wairkar et al., 2009). It could therefore be expected that RSK and UNC-51 mutations display similar synaptic phenotypes. Indeed, NMJ size, number of active zones and eEPSC amplitudes are decreased in both mutants.

Interestingly, transgenic rescue experiments for the electrophysiological defects failed to work in both mutants, emphasizing the importance of fine-tuning ERK activity for maintaining normal synaptic functions. However, there are also significant differences between the two mutants. In general, UNC-51 phenotypes are much more pronounced. In the UNC-51 mutant, many postsynaptic GluRs are unapposed to presynaptic BRP, a phenotype we did not observe in the case of loss of RSK. Both mutants showed a decrease in eEPSC amplitude, but although this was attributable to defective transmitter release at UNC-51 mutant synapses, no such presynaptic defect was observed in RSK mutants (unchanged quantal content; Fig. 6C). Instead, the reduced mini amplitude at RSK mutant synapses indicates impaired postsynaptic

Fig. 6. Electrophysiological characterization of RSK mutant synapses. (A) Example traces and quantification of miniature excitatory junctional currents (minis) recorded in two-electrode voltage-clamp recordings from the larval neuromuscular junction. The average mini amplitude was significantly smaller in RSK58/1 and could not be restored by employing a genomic rescue construct (RSK58/1;pRSK). Mini frequency was not affected by loss of RSK. (B) Representative evoked excitatory postsynaptic currents (eEPSCs; stimulation artifact removed for clarity) during low-frequency nerve stimulation (0.2 Hz) and quantification of amplitudes. (C) Quantal content was comparable stimulation artifact removed for clarity) during low-frequency nerve stimulation (0.2 Hz) and quantification of amplitudes. (C) Quantal content was comparable stimulation artifact removed for clarity) during low-frequency nerve stimulation (0.2 Hz) and quantification of amplitudes. **P<0.01 and ***P<0.001.
sensitivity, which in turn was unaltered in *UNC-51* mutants. Thus, although RSK and UNC-51 act as negative regulators for ERK, their relative contribution to ERK signaling in different cell types appears to be different. At least in the case of the RSK mutant, hyperactivation of ERK is modest and has no effect on development or viability of the fly, which implies a subtle modulatory function of RSK.

Finally, our analyses uncovered aberrant axonal BRP and CSP localization and anterograde transport defects of mitochondria. Transport of presynaptic components and their appropriate delivery at synaptic terminals require a complex interplay between motor proteins, the different transported components and local signaling events. In addition, mechanisms must exist to restrain localization of presynaptic components at the nerve terminals (Goldstein et al., 2008; Maeder et al., 2014). Interfering with these processes in *Drosophila* motoneurons caused distinct phenotypes. For instance, loss of Liprin-α results in ectopic accumulation of synaptic vesicles and presynaptic cytomatrix proteins in distal axon regions close to the synaptic terminals without affecting mitochondria or motor protein localization (Li et al., 2014). SR protein kinase 79D (SRPK79D) is required to prevent formation of large axonal agglomerates of BRP. Given that axonal transport processes and other synaptic proteins are not affected in *SRPK79D* mutants, a function of this kinase for site-specific active zone assembly at presynaptic membranes has been suggested (Johnson et al., 2009; Nieratschker et al., 2009). Large organelle-filled axonal swellings were observed in mutants defective for motor protein components; however, these aggregates do not serve as physical barriers for mitochondrial transport (Pilling et al., 2006). Local effects caused by changes in axonal transport are seen in *dAcsl* mutations. Here, mitochondrial transport is unaffected, but an increased velocity of anterograde transport and reduced velocity of retrograde transport of vesicles results in aggregates in distal axon regions (Liu et al., 2011). Mutation of the human ortholog *ACSL4* (acyl-CoA synthase long chain family member 4) causes non-syndromic X-linked mental retardation.

The axonal phenotypes seen in RSK mutants differ in several respects from these phenotypes. Large axonal swellings are not evident, and the increase in the number of BRP and CSP particles is largely confined to the proximal portion of the nerve (close to the ventral nerve cord). Together with the finding of more stationary mitochondria and fewer mitochondria transported in the anterograde direction, one explanation could be a function of RSK at the level of motor-cargo interaction. Specificity of cargo transport in the anterograde direction is determined at the levels both of individual Kinesins and of cargo-specific adaptor proteins (Hirokawa et al., 2010; Maday et al., 2014). For example, the catalytic subunit Kinesin-1 in *Drosophila* (kinesin heavy chain, KHC) recruits mitochondria via the adapter protein Milton, whereas UNC-76 provides a link to the synaptic vesicle protein Synaptotagmin (Stowers et al., 2002; Gindhart et al., 2003). Motor-cargo interactions are also regulated in a phosphorylation-dependent manner, as exemplified by the UNC-51 kinase-dependent interaction of UNC-76 with Synaptotagmin (Toda et al., 2008). Loss of either UNC-51 or UNC-76 resulted in accumulations of synaptic vesicles along motoneuron axons (Toda et al., 2008). Another example is glycogen synthase kinase 3 (GSK-3), which has been proposed to inhibit anterograde transport by phosphorylating Kinesin light chain and thereby causing dissociation of membrane-bound organelles from KHC (Morfini et al., 2002). Based on genetic analyses in *Drosophila*, an
alternative model proposes a function of GSK-3 in regulating motor protein activity rather than cargo binding (Weaver et al., 2013). Interestingly, RSK2 has been reported to inhibit GSK-3 activity in different cellular contexts and is able to phosphorylate GSK-3, at least in vitro (Romero et al., 2012). Future studies are required to clarify a function of RSK in GSK-3-mediated control of anterograde transport processes. So far, we have no evidence for a direct or an indirect requirement of RSK for phosphorylation of motor protein components and, if so, whether this might have an impact on their in vivo function.

In summary, an emerging common picture from knockout studies in mice and flies as animal models for CLS is a posttranslational loss of function of all RSK isoforms proposed to underlie the phenotype. Whether these phenotypes reflect particular defects in anterograde axonal transport and changes in motor protein components and, if so, whether this might have an impact on the localization of activated ERK, requires to clarify a function of RSK in GSK-3-mediated control of motor protein synthesis.

**MATERIALS AND METHODS**

**Fly strains and genetics**

Flies were reared on standard cornmeal food at 25°C and 60% relative humidity in a 12-hr dark-light cycle. Two viable RSK deletion mutants were used: Df(1)Rik655 (in the following, referred to as RSK<sup>Δ55</sup>; Putz et al., 2004) and RSK<sup>Δ99</sup> (a kind gift from J. Chung, Seoul National University, South Korea; Kim et al., 2006). Other fly stocks used were as follows: P<sup>+</sup>Gal4<sup>UAS-Mito::GFP</sup> (Ranganayakulu et al., 1999) and P<sup>+</sup>Gal4<sup>OK6-Gal4</sup> (Ranganayakulu et al., 1998), OK6-Gal4 (Aberle et al., 2002), UAS-mdCD8::GFP (Lee et al., 1999) and UAS-Mito::GFP (Pilling et al., 2006); w<sup>1118</sup> was used as wild type. For generation of UAS-GFP::RSK transgenic flies, the open reading frame of RSK was amplified by linker PCR from DNA clone SD05277 (Drosophila Genomics Resource Center) and cloned into the Xbal/NotI sites of a modified pUAST-vector (Brand and Perrimon, 1993) 3’ to the coding sequence of GFP. Transgenic lines were established by BestGene Inc. (Chino Hills, CA, USA). For rescue experiments, clone CH322-75N12, which contains a 20 kb genomic fragment encompassing the RSK gene locus, was chosen from a genomic BAC library engineered into the at88-P[acman]-CM<sup>1</sup>-BW vector (Venken et al., 2009). Transgenic flies for this clone (in the following, named P(RSK<sup>Δ55</sup>)<sup>Δ99</sup>) were generated by PhiC31-mediated integration at the at88<sup>P</sup> site located at chromosomal position 65B2 on the third chromosome (BestGene Inc.).

**Western blot**

For quantification of ERK and pERK levels, 50 ventral ganglia from late third instar larvae were dissected in ice-cold fixation solution (4% paraformaldehyde in PBS) to minimize changes in phosphorylation status during handling, fixed for 10 min on ice and washed three times for 20 min in PBS. Following homogenization and sonication, protein lysates were processed in ImageJ 1.48a and analyzed based on kymographs generated in MATLAB (R2010b).

**Immunohistochemistry**

Wandering third instar larvae were dissected in HL-3 medium as described previously (Brent et al., 2009) or directly in fixation solution in the case of anti phospho-ERK staining to minimize dephosphorylation. Depending on the experiment, combined brain and body-wall preparations or only body walls were dissected. Fixation was done for 15 min in 4% (w/v) paraformaldehyde at room temperature. After washing in PB [PBS +0.05% (v/v) Triton X-100] and blocking in PB supplemented with 3% (v/v) normal goat serum for 30 min, samples were incubated overnight at 4°C with combinations of the following antibodies diluted in PB supplemented with 3% (v/v) normal goat serum: chicken anti-GFP (1:1000; Mercil Millipore), rabbit anti-Glutamate receptor subunit GluRiD (Qin et al., 2005; 1:1000; a kind gift from S. Sigrist, University of Berlin, Germany), mouse anti-Bruchpilot (Wagh et al., 2006; anti-BRP<sub>nc82</sub>, 1:100; a kind gift from E. Buchner, University of Würzburg, Germany), mouse anti-Cysteine string protein (Zinsmaier et al., 1994; anti-CSPab49, 1:100; E. Buchner), mouse anti-lamin Dm0 (clones ADL57.10 and ADL195, both at 1:10; Developmental Studies Hybridoma Bank), rabbit anti-Phospho-ERK1/2 (Th202/Tyr204; clone D13.14.4E, 1:200; Cell Signaling) and goat anti-HRP Cy5-conjugated (1:250; Dianova). Secondary antibodies were conjugated with AlexaFluor 488, DyLight488, Cy3 or Cy5 (Dianova) and used at dilutions of 1:100 to 1:200 in PBT. After washing in PBT, preparations were embedded in VectaShield (Vector Laboratories).

**Imaging and analysis of fixed samples**

Confocal images were recorded using either an Olympus Fluoview 1000 IX 81 or a Leica SP5 microscope. Images were processed using ImageJ 1.48a (NIH, Bethesda, MD, USA). To avoid variations within a single experiment, all preparations of larvae from different genotypes were stained simultaneously as described by Viquez et al. (2006). All genotypes in one experiment were imaged with the same gain, avoiding saturation of the signal. For imaging and quantification of NMJ size, areas and numbers of active zones and post synaptic densities at muscle 6/7 in abdominal segment A2 or A3, we exactly followed the step-by-step protocol of Andlauer and Sigrist (2012c) with the following minor modifications: threshold values for detection of active zones and GluR fields were set to equal values in all experiments to allow for semi-automated quantification and comparison of different genotypes. For quantification of BRP and CSP aggregates, images were taken from axons that emerge from the ventral ganglion at segment A3, using the same gain for all genotypes. Numbers of particles for BRP or CSP were normalized to the axon area outlined by HRP staining.

**In vivo imaging**

Examination of anterograde transport in living larvae was carried out in an imaging chamber as described by Andlauer and Sigrist (2012a,b) using an Olympus Fluoview 1000 IX 81 confocal microscope equipped with a 60× oil, NA 1.35 objective. After photo-bleaching of an axon length of 61.44 μm, ∼100 μm distal to the tip of the ventral ganglion, series of 1000 frames were scanned with imaging intervals of 720 ms. The series were processed in ImageJ 1.48a and analyzed based on kymographs generated in MATLAB (R2010b).

**Data analysis**

Statistical analyses of electrophysiological results were performed with the non-parametric rank sum test (Sigma Plot 12.5; Systat Software). For all other data, Mann-Whitney U-tests were used for statistical analyses. For multiple testing within one data set, the level of significance P<0.05 was adjusted according to the Bonferroni correction. Data are reported ±s.e.m., and asterisks depict level of statistical significance as follows: *P<0.05, **P≤0.01 and ***P≤0.001.

**Electrophysiology**

Two-electrode voltage-clamp recordings (Axoclamp 900A amplifier; Molecular Devices) were made from muscle 6, segments A2 and A3 of late third instar male Drosophila larvae essentially as previously reported (Ljaschenko et al., 2013). All measurements were performed at room temperature in extracellular haemolymph-like solution (HL-3; Stewart et al., 1994) containing (in mM): NaCl, 70; KCl, 5; MgCl<sub>2</sub>, 20; HCO_3<sub>2</sub>, 10; trolleyose, 5; sucrose, 115; HEPES, 5; CaCl<sub>2</sub>, 1; pH adjusted to 7.2. Intracellular electrodes with resistances of 10-20 MΩ (filled with 3 M KCl) were used, and only cells with an initial membrane potential of at least...
50 mV and a membrane resistance ≥ 2 MΩ were taken into consideration. During recordings, cells were clamped at a holding potential of ~ 80 mV for minis and ~ 60 mV for eEPSCs. To evoke synaptic currents, nerve stimulation (300 μs pulses, typically at 10 V; Grass S88 stimulator and isolation unit SIU5; Astro-Med) was applied via a suction electrode (diameter ~ 15 μm; filled with extracellular solution). Signals were sampled at 10 kHz, low-pass filtered at 1 kHz and analyzed using Clampfit 10.2 (Molecular Devices). Quantal content was calculated by dividing the average eEPSC amplitude by the average mini amplitude, corrected for the more hyperpolarized holding potential (amplitude reduction to 75%; Hallermann et al., 2010).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
R.J.K., M.F. and T.R. conceived and designed the experiments. K.B., N.E., T.F.M.A., D.L. and K.S. performed the experiments and analyzed data. K.B., N.E., R.J.K. and T.R. wrote the paper.

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