

The human cognition-enhancing **CORD7** mutation increases active zone number and synaptic release

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Abstract

Humans carrying the **CORD7** (cone-rod dystrophy 7) mutation possess increased verbal IQ and working memory. This autosomal dominant syndrome is caused by the single-amino acid R844H exchange (human numbering) located in the 3₁₀ helix of the C₂A domain of RIMS1/RIM1 (Rab3-interacting molecule 1). RIM is an evolutionarily conserved multi-domain protein and essential component of presynaptic active zones, which is centrally involved in fast, Ca²⁺-triggered neurotransmitter release. How the **CORD7** mutation affects synaptic function has remained unclear thus far. Here, we established *Drosophila melanogaster* as a disease model for clarifying the effects of the **CORD7** mutation on RIM function and synaptic vesicle release.

To this end, using protein expression and X-ray crystallography, we solved the molecular structure of the *Drosophila* C₂A domain at 1.92 Å resolution and by comparison to its mammalian homolog ascertained that the location of the **CORD7** mutation is structurally conserved in fly RIM. Further, CRISPR/Cas9-assisted genomic engineering was employed for the generation of *rim* alleles encoding the R915H **CORD7** exchange or R915E,R916E substitutions (fly numbering) to effect local charge reversal at the 3₁₀ helix. Through electrophysiological characterization by two-electrode voltage clamp and focal recordings we determined that the **CORD7** mutation exerts a semi-dominant rather than a dominant effect on synaptic transmission resulting in faster, more efficient synaptic release and increased size of

the readily releasable pool but decreased sensitivity for the fast calcium chelator BAPTA. In addition, the *rim* CORD7 allele increased the number of presynaptic active zones but left their nanoscopic organization unperturbed as revealed by super-resolution microscopy of the presynaptic scaffold protein Bruchpilot/ELKS/CAST.

We conclude that the CORD7 mutation leads to tighter release coupling, an increased readily releasable pool size and more release sites thereby promoting more efficient synaptic transmitter release. These results strongly suggest that similar mechanisms may underlie the CORD7 disease phenotype in patients and that enhanced synaptic transmission may contribute to their increased cognitive abilities.

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Abbreviations: attB = bacterial attachment site; attP = phage attachment site; AZ = active zone; BAPTA-AM = Glycine, N-[2-[(acetyloxy)methoxy]-2-oxoethyl]-N-[4-[[[3',6'-bis(acetyloxy)-2',7'-difluoro-3-oxospiro[isobenzofuran-1(3H),9'-[9H]xanthen]-5-yl]carbonyl]amino]-2-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]phenoxy]ethoxy]phenyl]-, (acetyloxy)methyl ester; Brp = Bruchpilot/ELKS/CAST; CORD7 = cone-rod dystrophy type 7; CRISPR = clustered regularly interspaced short palindromic repeats; *d*STORM = direct stochastic optical reconstruction microscopy; eEPSC = evoked excitatory postsynaptic currents; EGTA = ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; FE = focal electrode; GAL4 = yeast transcription factor; gRNA = guide RNA; NMJ = neuromuscular junction; PPR = paired pulse ratio; RIM = Rab3 interacting molecule; RMSD = root-mean-square deviation; RRP = readily releasable pool; SE = suction electrode; SEM = standard error of the mean; SNARE = Soluble NSF attachment proteins receptor; TEVC = two-electrode voltage clamp; VGCC = voltage-gated calcium channels; UAS = upstream activated sequence

Introduction

Presynaptic active zones (AZs) constitute fundamental building blocks of neuronal connections, and contribute to neuronal computation and memory formation¹⁻⁴. They harbor a well-defined set of proteins, which mediate highly regulated neurotransmitter release occurring with sub-millisecond accuracy². Defects in the orchestrated function of presynaptic proteins typically have devastating consequences. Correspondingly, several neurological diseases have been associated with malfunction of presynaptic proteins⁵. A striking exception of the observation that a protein defect leads unambiguously to functional defects is the *CORD7* syndrome. Affected individuals suffer from an autosomal dominant cone-rod dystrophy, ultimately leading to blindness⁶. However, they also show largely enhanced cognitive abilities characterized by increased verbal IQ and working memory⁷.

The *CORD7* syndrome is caused by a single-amino acid mutation within the 3₁₀-helix of the C₂A domain of RIM (Rab3-interacting molecule)^{2,8}. RIM is a multi-domain AZ protein^{9,10}, encoded by the *RIMS1* locus (human gene name as opposed to the *Drosophila* locus name *rim*), whose interaction with other core AZ proteins such as RIM binding protein (RBP), Munc13, α -Liprin, and ELKS/CAST, as well as with Ca²⁺ channels² is thought to figure centrally in neurotransmitter release by providing a structural lattice that organizes release sites. Co-expression with voltage-gated calcium channels in HEK293 cells revealed that the *CORD7* exchange in RIM1 increased the activation and suppressed the inactivation component of the channel currents¹¹. However, the impact of the *CORD7* mutation on synaptic function has remained largely unclear.

At presynaptic release sites, distance between Ca²⁺ channels, vesicular release sensors and the size of the readily releasable pool (RRP) of vesicles are key determinants of the fidelity of neurotransmitter release^{12,13}. Tight nanodomain coupling (within tens of nanometers)^{12,14,15} promotes fast and efficient release. It can be distinguished from loose coupling¹⁶⁻¹⁹, but how these differences are molecularly implemented and controlled is still subject of intense investigations²⁰⁻²². Given the central role of RIM at AZs²³ we here studied how RIM, its C₂A domain, and the *CORD7* mutation affect synaptic release at the neuromuscular junction of *Drosophila*.

Materials and methods

Fly stocks

Flies and larvae were raised at 25°C on standard cornmeal and molasses medium.

Fly stocks generated in this study

Internal stock IDs given in brackets after the genotype:

$w^{1118}; +; rim^{AC2A} attP DsRed^+/TM3, Sb;$ (RIM98)

$w^{1118}; +; rim^{AC2A} attP DsRed/TM3, Sb;$ (LAT473)

$w^{1118}; +; rim^{AC2A} attP\{C_2A-WT(pLM5) mW^-\}/TM6b, Tb;$ (LAT542/AM28)

$w^{1118}; +; rim^{AC2A} attP\{C_2A-R915H(pLM8) mW^-\}/TM6b, Tb;$ (LAT545/AM29)

$w^{1118}; +; rim^{AC2A} attP\{C_2A-R915E,R916E(pLM9) mW^-\}/TM6b, Tb;$ (LAT549/AM32)

$w^{1118}; P\{UAS-RIM^{wt} w^+\}^{attP40}/Cy0GFP; +;$ (RIM52)

$w^{1118}; P\{UAS-RIM^{R>H} w^+\}^{attP40}/Cy0GFP; +;$ (RIM53)

Other fly stocks

Reference or stock ID given after the genotype:

$w^{1118}; +; rim^{Ex73}; (rim^{KO} \text{ allele})^{24}$

$w^*; ok6-GAL4 mW^+; +;$ ^{25,26}

$y^l M\{GFP[E.3xP3]=vas-Cas9.RFP-\}^{ZH-2A} w^{1118};;;$ (BDSC#55821)

$M\{vas-int.Dm\}^{ZH-2A};;;$ ²⁷

Transgene construction

Transgenes for genomic targeting

All primer sequences used in this study:

| Primer ID | 5'-3' sequence |
|-----------|--|
| lm_30F | ATAGTTTAGCGGCCGCGTTATTCCATGCGCTATATTATAACCACAA |
| lm_31R | AGGCGCGCCTGTGGGTGTTTTTGGTCCTCTCGCTCTTGT |
| mh_25F | CTCCGATAGCGCATGGAATAACCTG |
| mh_26R | AAACCAGGTTATTCCATGCGCTATC |
| mh_27F | CTCCGAGTCTAATGTATACACGTGT |
| mh_28R | AAACACACGTGTATACATTAGACTC |
| mh_46F | CTTCGATAGCGCATGGAATAACCTG |
| mh_47F | CTTCGAGTCTAATGTATACACGTGT |
| mh_65F | GTACGCTCTTCCTATCGTGTATACATTAGACTTCAATCG |
| mh_66R | TAGAGCTCTTCTGACCTTAAATTCGATTTGGGCTCTTAG |
| mh_77F | ATCTCACCTGCAAGCTCGCACTAGTCGGCGCTCGACAACAGGCAG |
| mh_79R | GAATCACCTGCAGAACTACGCTAGCGTCTCGCTGGCGGTGGCTCT |
| mh_82F | TCGCACTAGTCATCCGCAATCCGAACTGTAAACT |
| mh_83R | CTACGCTAGCCTGTGGAAGTTCCTCAGCTTAGGC |
| tl_251F | CAACGGCCGACTGCTCGAGGTGACG |
| tl_252R | AGTCGGCCGTTGAGATCGCAGCGGTGCAGACCCGA |
| tl_379F | CGCGGATCCATCCCCATCGAGGGACGGCTGCAGC |
| tl_380R | ATAGTTTAGCGGCCGCTACTGCAGCTGATACCACTCGGCCTCG |
| tl_849F | TGGAAGAGCGTGAACAAGTGTGAT |
| tl_850R | GCTGAGTGCCACTGGAAGTTGGCA |
| tl_843F | ATTCTACTTCCCGCATAACGATG |
| tl_678R | ATTGAATTAGATCCCGTACGATA |
| tl_679F | TGTGGTTTGTCCAAACTCATCA |
| tl_840R | ATGCAATAGTTATCGTTATCGT |

pU6-gRNA

CRISPR/Cas9 cutting sites 5' and 3' of the genomic region of the *rim/CG33547* gene encoding the C₂A domain were identified by *CRISPR Optimal Target Finder*²⁸. The genomic sequence of all CRISPR/Cas9 cleavage sites were confirmed by DNA sequencing of PCR fragments encompassing the suggested sites prior to cloning. Target-specific sequences for gRNAs (gRNA#1: ATAGCGCATGGAATAAC/CTGTGG; gRNA#2: AGTCTAATGTATACACG/TGTGGG; sequences in 5'-3' order; PAM site in bold, / indicates Cas9 cutting site) were synthesized as 5'-phosphorylated oligonucleotides (gRNA#1: mh_46F/mh_26R; gRNA#2: mh_47F/mh_28R), annealed to produce matching overhangs, and ligated into the *BbsI* sites of the *pU6-BbsI-chiRNA* vector pTL621²⁹ generating vectors pMH4 (gRNA#1) and pMH5 (gRNA#2).

rim^{ΔC2A} HDR plasmid

A 1.1 kb fragment encoding the 3' homology arm was amplified from *w¹¹¹⁸* genomic DNA using primers mh_65F/mh_66R, cut with *SapI* and inserted into de-phosphorylated *SapI*-digested pHD-DsRed-attP (pTL620) generating plasmid pMH15. pMH15 was digested with *AarI* and genomic 0.2 kb PCR product amplified with primers mh_77F/mh_79R, also digested with *AarI*, were ligated generating plasmid pMH25. Finally, pMH25 was digested with *NheI/SpeI* and ligated with a 1.1 kb *NheI/SpeI*-PCR product amplified off genomic DNA with primers mh_82F/mh_83R producing the final HDR vector pMH14.

attB-rim-C₂A^{rescue} plasmid

A 2.5kb fragment of genomic DNA from strain *w¹¹¹⁸* was amplified using primers lm_30F/lm_31R, digested and ligated to a 6.1 kb *NotI/AscI*-fragment of pGE-attB-GMR (pTL370) generating *rim-C₂A^{rescue}* vector pLM5.

attB-rim-C₂A^{R915H} plasmid

A 8.5 kb *HpaI/XhoI* fragment was released from pLM5, and a 0.2 kb *HpaI/XhoI* fragment from pTL823 (insert synthesized by Genscript, NL). Both fragments were ligated generating *rim-C₂A^{R915H}* vector pLM8.

attB-rim-C₂A^{R915E,R916E} plasmid

A 8.5 kb *HpaI/XhoI* fragment was released from pLM5, and a 0.2 kb *HpaI/XhoI* fragment from pTL825 (insert synthesized by Genscript, NL). Both fragments were ligated generating *rim-C₂A^{R915E,R916E}* vector pLM9.

UAS transgenes

Plasmids for *Drosophila* transformation were engineered through the *Drosophila* Gateway system as follows. The *Drosophila* wild type full-length *rim* cDNA (GenBank ID: KF534710.1)²⁴ through a *NotI/XbaI* digest and inserted into the pENTR1A dual plasmid

generating entry vector pJG42 (*RIM*^{wt}). Additional entry vectors for single and double mutated RIM variants were produced by exchanging a DNA fragment of pJG42 encoding the CORD7 site region for matching *rim* fragments containing the mutations. These were produced by subcloning the wt 0.5 kb *HindIII/NcoI*-fragment of pJG42 into pMCS5 (yielding pTL305) to reduce the plasmid size and allow for an outward PCR of the entire vector. The *rim*^{R>H} mutation was introduced via the reverse outward-PCR primer. The PCR-amplicon was cut at an *EagI* site and self-ligated to recircularize the plasmid. From the resulting clone pTL310 (*RIM*^{R>H}) the 0.5 kb *HindIII/NcoI* fragment was used to replace the wt region in pJG42 and generate entry vector pTL431. All entry vector inserts were fully sequenced to ensure no errors were introduced through the cloning procedures. To create the final transformation vectors, pJG42 and pTL431 were recombined with the pTW-attB destination vector²⁷ through a LR reaction according to the manufacturer's instructions (Invitrogen), and correct recombinant clones were identified by suitable restriction digests (*5xUAS-RIM*^{wt}=pTL434; *5xUAS-RIM*^{R>H}=pTL435). All *UAS-RIM* transgenes were inserted into the *attP40* landing site carried on the second chromosome³⁰ using Φ C31 integrase-based transformation³¹ by BestGene Inc. (USA).

Transgenes for protein expression

A PCR-amplified DNA fragment encoding the RIM C₂A domain of *Drosophila* (encoding residues 836-962 of RIM) was subcloned via primer-delivered *BamHI/NotI* sites in a pGEX-6P-2 vector (GE Healthcare) providing a N-terminal GST-tag (pTL423).

CRISPR targeting

All transgenesis steps were performed at Bestgene Inc. (Chino Hills, USA). For CRISPR/Cas9 mediated engineering *gRNA#1* (pMH4) and *gRNA#2* (pMH5), and the matching *rim*^{ΔC2A}-*HDR* plasmid (pMH14) were injected into *w*¹¹¹⁸ flies carrying a germline-expressing *vas-Cas9* source as described before²⁸ producing the *rim*^{ΔC2A}, *DsRed*⁺ allele. Correct gene targeting was confirmed by subsequent sequencing of PCR fragments covering breakpoints between genomic/transgenic DNA amplified off genomic DNA of respective adult transgenic flies using primers tl_843F/tl_687R (covering the 5' breakpoint), tl_679F/tl_840R (covering the 3' breakpoint), and across the deleted fragment using primers tl_849F/tl_850R. The *3xP3-DsRed*

transformation marker was removed from *rim^{ΔC2A}*, *DsRed⁺* flies by expressing a germline Cre source, and confirmed by PCR genotyping. Subsequent insertion of the different *attB-rim-C_{2A}* transgenes into *rim^{ΔC2A}*, *DsRed⁻* flies (LAT473) by Φ C31-mediated transgenesis was performed by Bestgene Inc. (USA).

Protein production

Recombinant protein expression was performed in *Escherichia coli* strain BL21(DE3) in Luria-Bertani medium. Protein expression was induced at 18 °C by addition of 0.3 mM Isopropyl β -D-1-thiogalactopyranoside and allowed to proceed for 16 h. Cell pellets were resuspended in 50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5% v/v Glycerol and 3 mM β -mercaptoethanol (β -ME) buffer, and lysed by lysozyme treatment and sonication. Lysates were incubated with Glutathione-sepharose beads (GE Healthcare), and the beads were washed with Buffer A (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 3 mM β -ME). Proteins were eluted from the beads with Buffer A supplemented with 12 mM reduced glutathione, followed by dialysis in Buffer A and removal of the GST-tag by 3C-protease cleavage. Uncleaved proteins and GST were removed by reverse affinity purification with Glutathione-sepharose beads, followed by size exclusion chromatography into a 30 mM sodium citrate pH 6.0, 150 mM NaCl and 1 mM dithiothreitol buffer.

Crystallization and structure determination

Crystals of the *Drosophila melanogaster* RIM C_{2A} domain were obtained using the sitting drop vapor diffusion technique at 20 °C. A Mosquito robot (TTP LabTech) was used to set up 200 nl-size drops with 1:1 and 1.3:0.7 ratios of protein to mother liquor. Rectangular crystals developed in 2 days when mixing the protein at 4.8 mg/ml concentration with a 0.2 M tri-lithium citrate, 20% w/v polyethylene glycol 3350 solution. Crystals and were cryo-protected with 20% v/v glycerol, flash-cooled in liquid nitrogen and diffracted to 1.93 Å at the Diamond Light Source beamline I04. The space group was determined as P212121 with one molecule per asymmetric unit. Crystallographic data were integrated by XDS and scaled in XSCALE³². The structure was solved by molecular replacement in the programme MrBump³³ using the rat RIM2 C_{2A} domain (PDB 2BWQ) as search model. Iterative model building with COOT³⁴ and

refinement in PHENIX.refine³⁵ using TLS restraints yielded the final model. All figures were prepared in PyMol (PyMOL Molecular Graphics System, Schrödinger LLC). The electrostatics surface potential was calculated with APBS³⁶. The structure was deposited under PDB ID 4TS6.

Two-electrode voltage clamp recordings

Two-electrode voltage clamp recordings (Axo Clamp 2B amplifier, Axon instruments) were performed in extracellular haemolymph-like solution (HL-3)³⁷ that contained (in mM): NaCl 70, KCl 5, MgCl₂ 20, NaHCO₃ 10, trehalose 5, sucrose 115, HEPES 5, CaCl₂ 1.0, pH adjusted to 7.2. Recordings were obtained from NMJs at longitudinal abdominal muscles 6/7 in segments A2 and A3 at 20-22 ± 1 °C room temperature using intracellular electrodes with resistances 10 – 20 MΩ (filled with 3 M KCl). Holding voltage was -60 mV for eEPSCs and -80 mV for mEPSCs. Only muscle cells 6 with an initial membrane potential of ≥ -50 mV and ≥ 4 MΩ input resistance were analyzed. Synaptic responses were generated by pulses of 0.3 ms length and 5-15 V amplitude, applied via a suction electrode (filled with extracellular solution) and filtered by an 10 kHz low-pass filter. We applied a paired-pulse protocol with 0.2 Hz frequency and 30 ms interpulse intervals, averaged 10 EPSCs per muscle cell if not noted otherwise and analyzed the data with Clampfit (Molecular Devices). To estimate RRP size, a train of 50 pulses was applied at 20 Hz. Linear fits to the last 20 pulses were applied to cumulatively plotted eEPSCs and back extrapolated^{38,39}.

Focal recordings

EPSC recordings were performed in extracellular haemolymph-like solution (HL-3)³⁷ that contained (in mM): NaCl 70, KCl 5, MgCl₂ 20, NaHCO₃ 10, trehalose 5, sucrose 115, HEPES 5, pH adjusted to 7.2 with 1 N NaOH. CaCl₂ concentration was 1.0 mM. We only recorded from NMJs at abdominal muscles 6/7 in segments A2 and A3. Room temperature was constantly 20 ± 2 °C and temperature of the bath solution was maintained at 20 ± 1 °C with the help of a constant displacement pump. The focal electrode (resistances 450-550 kΩ, filled with HL-3) was positioned under optical control on proximal boutons (i.e. nearby the access site of the nerve to the muscle) under optical control. Synaptic currents were low-pass filtered with

20 kHz and stored with Patchmaster software using an EPC10 double patch clamp amplifier (HEKA electronics). EPSCs were evoked by stimulating the respective motor neuron via a suction electrode. We applied a paired pulse protocol at 0.2 Hz with 30 ms interpulse intervals, each pulse consisting of 1 ms length and 7 V amplitude. About 60 traces per recording site were averaged and analyzed with Igor Pro 6.05 (Wavemetrics). Rise time was measured from 10-90% of the peak eEPSC amplitude and tau decay was fitted from the 50% eEPSC amplitude. Measurement of the synaptic delay was performed as illustrated in the inset of Figure 1E. BAPTA-AM (Invitrogen) was dissolved in DMSO, which contained 10 % Pluronic (F-127, Invitrogen), to create a 10 mM stock solution. Larvae were dissected and incubated for 20 minutes in 10 μ M BAPTA-AM diluted in HL-3 or in Pluronic in DMSO diluted in HL-3 for controls. After incubation, CNS was removed and focal recordings were performed as described above. For fluctuation analysis, focal electrodes with resistances of 1.4-1.8 M Ω were used to record eEPSCs elicited by the paired pulse stimulation protocol described above at bath temperatures of 20 ± 1 °C with the help of a constant displacement pump. Approximately 20 traces per recording site and concentration were analyzed in consecutive extracellular Ca²⁺ concentrations (0.5, 1.0, 1.5, 3.0, 5.0, 0.5 mM) using Igor Pro 6.37 (Wavemetrics). eEPSC variances and the variance of the variances were calculated according to Neher and colleagues⁴⁰⁻⁴² as described previously^{15,38}. Variance-mean amplitude plots were fitted using the formula $\text{Var}(I) = I/N + qI$, with I meaning the mean eEPSC amplitude, q quantal size and N the number of release-ready vesicles. The release probability p_r for each concentration was calculated as $p_r = I/(Nq)$.

Ca²⁺ imaging

Ca²⁺ imaging was performed in principle as described previously^{43,44}. Male wandering third-instar larvae were dissected in ice-cold, Ca²⁺ free HL-3 and incubated in 5 mM Oregon-Green BAPTA-1 488 (Invitrogen) and 1 mM Alexa Fluor568 (Invitrogen) in HL-3 on ice for 10 min. After incubation, the preparation was washed with HL-3 for 10 min. Synapses were imaged with a Scientifica 2P system. Excitation light (800 nm) from a Tsunami laser was focused onto the NMJ using a 60x dipping objective (Olympus, NA 1.0) and emitted light was detected by 2 PIMS-PMT-20 photomultiplier tubes. Single action-potential evoked spatially averaged Ca²⁺ transients were imaged from proximal type Ib boutons of NMJs on muscles 6/7 in abdominal segments A2 and A3 at 1.0 mM [Ca²⁺]_{Ex}. 1-2 NMJs per preparation and 1-6 boutons per NMJ

were included into the analysis. Line scans (8-12 per bouton) were made at a frequency of 333 Hz. $\Delta F/F$ describes the fluorescence change following an action potential and was quantified as $(F(t) - F_{\text{baseline}}) / (F_{\text{baseline}} - F_{\text{background}})^{43}$. Measurements with $F_{\text{baseline}} < 210$ AD-values were excluded from analysis.

Immunofluorescence imaging

For all imaging experiments we used NMJs formed on abdominal wall muscles 6/7 in segments A2 and A3 from male, wandering third instar larvae. Larvae were dissected in ice-cold Ca^{2+} -free HL-3, fixed with 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 10 minutes and blocked with PBT (PBS containing 0.05 % Triton X-100, Sigma) including 5 % natural goat serum (Dianova) for 30 minutes. Primary antibodies were added for overnight staining at 4 °C. After three washing steps with PBS (20 min each), preparations were incubated with secondary antibodies for 2-4 hours at room temperature followed by three washing steps. For quantification of AZs per NMJ, filets were mounted in Vectashield (Vector Laboratories) and images acquired using an Apotome System (Zeiss, Axiovert 200M Zeiss, objective 63x, NA 1.4, oil). Antibodies were used in the following concentrations: mouse mAb α -Brp^{Nc82} (1:250), goat α -mouse conjugated Alexa Fluor488 (1:250, Invitrogen) and goat α -horseradish peroxidase conjugated Cy3 (α -HRP, 1:250, Jackson Immuno Research). Data were analyzed using ImageJ software (NIH) as described previously⁴⁵. Images were maximum-projected. If more than one image per NMJ was collected, the duplicate signal that was acquired in the first image was used for quantification. Prior to analysis, signals from axons and neighbouring synapses as well as obvious background were removed manually with the 'Rectangle' or 'Freehand selection' tools in corresponding Brp and α -HRP images. If axons or neighbouring synapses crossed or touched the NMJ directly, images were excluded from analysis. NMJ area and number of Brp spots per NMJ were quantified in α -HRP and Brp images, respectively, using the 'Analyze Particles' function in 8-bit converted images applying an intensity threshold that was fixed for data that were compared statistically. If necessary images were background-subtracted using the Rolling ball method (radius = 50 pixels, α -HRP data in Figure 4B). The number of boutons per NMJ was counted manually.

***d*STORM**

Super-resolution imaging of *Drosophila* larval NMJs was performed as previously described⁴⁶. In brief, larvae were dissected in HL-3, fixed, blocked and stained as described above. After staining, muscle preparations were incubated in 100 mM Mercaptoethylamin (MEA, Sigma-Aldrich) buffer in PBS, pH 7.8-7.9 to allow reversible switching of single fluorophores during data acquisition⁴⁷. Images were acquired using an inverted microscope (Olympus IX-71, 60x, NA 1.45, oil immersion) equipped with a nosepiece-stage (IX2-NPS, Olympus). 647 nm (F-04306-113, MBP Communications Inc.), 644 nm (iBEAM-SMART-640-S, Toptica) and 488 nm (iBEAM-SMART-488-S, Toptica) lasers were used for excitation of Alexa Fluor647/Cy5 and Alexa Fluor488 2, respectively. Laser beams were passed through a clean-up filter (Brightline HC 642/10, Semrock, and ZET 488/10, Chroma, respectively) and two dichroic mirrors (Laser-MUX BS 514-543 and HC-quadband BP, Semrock) onto the probe. The emitted fluorescence was filtered with a quadband-filter (HC-quadband 446/523/600/677, Semrock) and divided onto two cameras (iXon Ultra DU-897-U, Andor) using a dichroic mirror (HC-BS 640 imaging, Semrock). In addition, fluorescence was filtered using a longpass- (Edge Basic 635, Semrock) or bandpass-filter (Brightline HC 525/50, Semrock) for red and green channels, respectively. Pixel size for the super-resolved red channel was 126 nm. Single fluorophores were localized and high resolution-images were reconstructed with rapidSTORM⁴⁸⁻⁵¹ (www.super-resolution.de). Only fluorescence spots with more than 12,000 photons were analyzed (10 nm/ pixel sub-pixel binning). Antibodies were used in the following concentrations: mouse mAb α -Brp^{Nc82} (1:2000) and goat α -mouse F(ab')₂ fragments (A10534, Invitrogen) labeled with Cy5-NHS (PA15101, GE Healthcare) at a concentration of 5.2×10^{-8} M in *ok6-GAL4 > UAS-RIM^{wt}* and *> UAS-RIM^{R>H}* larvae, and mouse mAb α -Brp (nc82; 1:100, Antibody Registry ID: AB_2314866, Developmental Studies Hybridoma Bank) and Alexa Fluor647 labeled secondary F(ab')₂ fragments goat α -mouse (1:500, A21237, Thermofisher) in *rim^{rescue}/rim^{rescue}* and *rim^{R>H}/rim^{rescue}* larvae. Presynaptic boutons were visualized with Alexa Fluor488 conjugated goat- α -HRP (1:250, Jackson Immuno Research). All example AZs in Figure 4 are shown in top view (i.e. optical axis perpendicular to the AZ plane).

Localization data were analyzed essentially as described previously⁵² with custom written Python code (<https://www.python.org/>, language version 3.6). RapidSTORM localization tables were directly loaded and analyzed. Regions of interest corresponding to the terminal 6 boutons were masked in the reconstructed, binned images from rapidSTORM using FIJI. For cluster analysis we used the Python implementation of hierarchical density-based spatial

clustering of applications with noise (HDBSCAN)^{53,54}. Brp clusters were extracted with the combination 400 and 100 for ‘minimum cluster size’ and ‘minimum samples’, respectively, in RIM^{wt} and $RIM^{R>H}$ measurements, and 100 and 25 for ‘minimum cluster size’ and ‘minimum samples’ in $rim^{rescue}/rim^{rescue}$ and $rim^{R>H}/rim^{rescue}$ measurements. These differences in data analysis were necessary due to multiple changes within the experimental setup between measurements (i.e. microscope hardware, antibody concentration, type of secondary antibody). The adjustments were necessary due to multiple changes within the experimental setup between measurements (i.e. microscope hardware, antibody concentrations, type of secondary antibody and conjugated dye). Cluster areas were quantified with 2D alpha shapes using CGAL (Computational Geometry Algorithms Library; <https://www.cgal.org>) in Python ($\alpha = 800 \text{ nm}^2$). Exclusion criteria for Brp clusters were area $< 0.01 \mu\text{m}^2$ and $> 0.1 \mu\text{m}^2$, Brp localizations per cluster $> 8,000$ and mean localization density per cluster $> 60,000$ localizations per μm^2 .

Statistical analyses

Datasets in Figure 2 and 6C, D were analyzed with Prism 9 (GraphPad Software) and tested for normal distribution applying a one-sample Kolmogorov-Smirnov test. To compare normally distributed datasets, a one-way ANOVA followed by a post-hoc pairwise comparison with Tukey’s correction was applied. Data that were not distributed normally were compared with a Kruskal-Wallis test followed by pairwise comparison with Dunn’s analysis. Analyses in Figures 3, 4, 5 and Supplementary Figures were performed with Sigma Plot 12 (Systat Software) using a parametric t-test or a non-parametric Mann-Whitney rank sum test depending on data distribution assessed through Kolmogorov-Smirnov testing. For multiple comparisons in Figure 6A and B, a one-way ANOVA was used. Data are reported as mean \pm SEM or median (25th - 75th percentile). In box plots, horizontal lines represent median; boxes quartiles; whiskers 10th and 90th percentiles; scatter plots show individual data points. Whisker plots in Figure 6D show mean \pm SEM.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

Results

RIM and the structure of the C₂A domain are evolutionarily conserved

RIM is a multidomain molecule containing five evolutionarily conserved protein motifs (Figure 1A)⁵⁵ that interact with P/Q- and N-type voltage gated calcium channels (VGCC; through the PDZ and C₂B domains)^{56,57}, Rab3 (through the Zinc finger domain)⁵⁵, other AZ scaffolding partners including ELKS (PDZ domain)^{58,59}, RBP (Zinc finger domain and PxxP-motif)⁶⁰, Synaptotagmin and α -Liprin (C₂B domain)¹⁰, and the presynaptic target membrane⁶¹. This domain architecture is already present in invertebrate organisms including *Caenorhabditis elegans* and *Drosophila melanogaster*. RIM C₂A and C₂B domains show considerable homology across different species (Figure 1B) including over a 3₁₀-helix first observed in structures of the rat homologue that is exposed at the bottom face of the C₂A domain (Figure 1C). This 3₁₀-helix harbors a missense mutation in a human kindred suffering from cone rod dystrophy type 7 (CORD7), a congenital disease hallmarked by progressive vision loss and increased cognitive abilities^{6,7}. The arginine located at the CORD7 site at the N-terminal portion of the 3₁₀-helix is always neighbored by a second arginine forming an RR-motif in all species examined except in the *C. elegans* homolog UNC-10 (Figure 1B). Interestingly, the CORD7 site corresponds to the first arginine of the RR-motif in vertebrate RIMs but assumes the second position in *Drosophila* on the basis of sequence alignment. Both arginines are likely important for RIM function as they define a distinct positively-charged epitope on the bottom domain surface (Figure 1D,E).

In order to determine the effect of the CORD7 mutation on synaptic function it was necessary to confirm that the *Drosophila* RIM C₂A domain also sterically conforms to mammalian homologs. Thus, we recombinantly expressed and crystallized the fly RIM C₂A domain. We obtained a complete diffraction dataset at 1.92 Å resolution (Figure 1C, Supplementary Table 1), which was used to solve the structure of the fly RIM C₂A domain aided by molecular replacement using the C₂A domain of the rat RIM2 homolog as a model (RIMS2, PDBID: 2BWQ)⁸. The final *Drosophila* RIM C₂A model, refined to R/R_{free} of 18.4/21.8 %, accounts for residues 836-962 of the protein (Figure 1C; PDB ID 4TS6). The structure adopts a β -sandwich fold with a small 3₁₀-helix in the loop connecting strands β 5- β 6, and is highly similar to other C₂ domains having a C α root-mean-square deviation (RMSD) of just 0.83 Å compared to the rat RIMS2 C₂A variant⁸. Consistent with the sequence alignments, the side chain of the second arginine (R916) in the fly C₂A domain occupies the same position in space as the CORD7 arginine in rat RIMS2 (R805). Interestingly, the side chain of the fly R915 also projects to the same position in space as the rat R806, the C-terminal neighbor of the CORD7 site. Thus, the surface properties of the 3₁₀-helix are highly similar between fly and rat (and presumably human) RIM despite differences in the alignment of the arginine doublet. We suggest that the RR-motif serves the same role

in fly and man, as both arginines contribute to the same epitope of the 3₁₀-helix at the bottom face of the RIM C₂A domain.

The RIM CORD7 mutation is a semi-dominant enhancer of synaptic release

To address the functional relevance of the CORD7 mutation in the context of the C₂A domain we employed a CRISPR/Cas9-mediated genomic engineering approach utilizing homology directed repair for genome editing. Through a pair of gRNA probes we removed a 2.5 kb fragment of the *rim* locus that contains the coding region for the C₂A domain (Figure 2A) and replaced it with an *attP* landing site generating a *rim*^{ΔC₂A} allele (Figure 2B). Subsequently, this fly strain was used to receive *attB*-flanked transgenes into the *rim* gene through ΦC31 expression (Figure 2B)^{28,62}. The transgenes carried the previously removed *rim* gene fragment with either the wildtype C₂A domain sequence (*rim*^{rescue}), a C₂A domain in which the CORD7 mutation was inserted at position R915 (R915H; *rim*^{R>H}), or in which the arginine doublet was exchanged for glutamate residues (R915E,R916E; *rim*^{RR>EE}). The surface of the 3₁₀-helix of the wild-type RIM C₂A domain appears positively charged (Figure 1E, C₂A^{WT}). We anticipated that insertion of the CORD7 mutation will slightly alter this electrostatic profile (Figure 1D) with the surface of the 3₁₀-helix assuming a neutral charge at the site of the CORD7 mutation (Figure 1E, C₂A^{R>H}). In contrast, insertion of negatively charged glutamate residues would result in a pronounced inversion of charge (Figure 1E, C₂A^{RR>EE}) compared to the wild-type situation. All alleles generated through the genomic engineering approach were confirmed through PCR genotyping and sequencing (Figure 2C). Overt developmental or behavioural abnormalities in animals carrying the different engineered *rim* alleles were not observed.

We first tested the functional consequences of the alleles by two-electrode voltage clamp (TEVC) recordings of postsynaptic currents in response to low-frequency nerve stimulation in 1.0 mM extracellular Ca²⁺ at muscle 6/7 neuromuscular junctions of third instar larvae. Evoked postsynaptic current amplitudes (eEPSCs) of *rim*^{rescue} animals (34.6 ± 1.5 nA mean ± SEM throughout this article; Supplementary Table 2 contain all descriptive statistics and results of group-by-group statistical comparisons of all TEVC and PPR datasets) compared indistinguishably to *rim*⁺ control animals (37.3 ± 1.4 nA) that did not undergo the CRISPR/Cas9 genome manipulations demonstrating that our genomic engineering protocol yields precisely constructed *rim* alleles (Figure 2D,E). We further confirmed that the *rim*^{Ex73} allele²⁴ behaved as a recessive strong loss-of-function mutation in this assay (*rim*^{KO}: 10.7 ± 1.0 nA; *rim*^{rescue}/*rim*^{KO}: 24.2 ± 2.3 nA). In addition, these results also showed that the wildtype *rim*^{rescue} allele is not completely haplo-sufficient for the eEPSC response as *rim*^{rescue}/*rim*^{KO} heterozygotes displayed significantly lower synaptic responses than *rim*^{rescue} homozygotes (Figure

2D,E). This indicates that the amount of *rim* gene product is critical for the physiological level of synaptic release.

Intriguingly, when we assessed eEPSCs of *rim*^{R>H} junctions we noticed that homozygous animals carrying the CORD7 mutation in RIM displayed largely increased currents (54.9 ± 2.5 nA; Figure 2D,E) compared to *rim*^{rescue} homozygotes. In order to further assess the functional consequences of the *rim*^{R>H} mutation for synaptic transmission we reduced the *rim*^{R>H} allele copy number by generating transheterozygous *rim*^{R>H}/*rim*^{rescue} animals reflecting the *RIMS1* genotype of CORD7 patients^{6,7}. Intriguingly, eEPSC amplitudes of the transheterozygotes (*rim*^{R>H}/*rim*^{rescue}: 44.8 ± 3.3 nA) settled between the values of the homozygous parents (*rim*^{rescue}/*rim*^{rescue} and *rim*^{R>H}/*rim*^{R>H}) (Figure 2D,E). In contrast, when *rim*^{R>H} was placed *in trans* to the *rim* null allele eEPSCs of *rim*^{R>H}/*rim*^{KO} animals (26.5 ± 2.5 nA) were indistinguishable from *rim*^{rescue}/*rim*^{KO} heterozygotes (24.2 ± 2.3 nA; Figure 2D,E) showing that CORD7 mutation- carrying *rim*^{R>H} allele exerts a semi-dominant effect over the *rim*^{rescue} but not *rim*^{KO} alleles with regard to eEPSC amplitudes. Collectively, this allelic series uncovered that *rim* gene dosage and the CORD7 change of the C₂A domain of RIM contribute independently to enhanced synaptic release.

Of note, recordings from *rim*^{RR>EE} larvae showed eEPSCs (26.5 ± 2.1 nA mean \pm SEM) that were larger than in *rim*^{KO} animals but not significantly different from *rim*^{rescue} and *rim*⁺ junctions (Figure 2D,E). This indicates that, in contrast to the CORD7 mutation, the charge reversal at the 3₁₀-helix of RIM's C₂A domain did not profoundly affect eEPSC amplitudes.

Paired-pulse ratio (PPR) measurements confirmed that removal of RIM at *rim*^{KO} NMJs resulted in facilitation of synaptic release (Figure 2F; Supplementary Table 2), consistent with previous observations of decreased release probability and strong synaptic facilitation at the NMJ of *rim* hypomorphic *Drosophila* mutants⁴³.

In order to exclude cell non-autonomous effects caused by the germline alteration of *rim* function in engineered *rim*^{R>H} mutants we next sought to genetically isolate larval motoneurons from an otherwise wildtype nervous system by use of the binary GAL4/UAS expression toolkit. We generated *UAS* transgenes containing either the wildtype *rim* cDNA sequence (*RIM*^{wt}; in capital letters to contrast this set of transgenes from *rim* alleles) or a cDNA containing the R915H substitution (*RIM*^{R>H}) and expressed them in larval motoneurons by the *ok6-GAL4* driver. The functional consequences of the *RIM*^{R>H} mutation on synaptic function were tested with TEVC recordings of postsynaptic currents in response to low-frequency nerve stimulation in 1.0 mM extracellular Ca²⁺ (Figure 3A). eEPSCs in *RIM*^{R>H} animals were larger than in *RIM*^{wt} (*RIM*^{R>H}: 59.1 ± 4.5 nA; *RIM*^{wt}: 35.1 ± 6.5 nA; Figure 3B and Supplementary Table 3), while miniature EPSCs (mEPSCs) and short-term plasticity remained unchanged (Figure 3C, Supplementary Fig. 1 and Supplementary Table 3) confirming the semi-dominant effect of the *rim*^{R>H} allele on synaptic release.

While TEVC recordings report the input to an entire muscle cell derived from many boutons, focal electrodes sample from a subset of synaptic boutons with superior temporal resolution (Figure 3D and Supplementary Table 3). Peak current amplitudes are lower in focal recordings than with TEVC, while they permit resolving synaptic delays and eEPSC rise times. Consistent with results from TEVC focal recordings from proximal type Ib boutons in 1.0 mM extracellular Ca^{2+} revealed that the $RIM^{R>H}$ increases synaptic current amplitudes compared to RIM^{wt} ($RIM^{R>H}$: 3.6 ± 0.6 nA; RIM^{wt} : 1.6 ± 0.2 nA; Figure 3E,F and Supplementary Table 3). Interestingly, synaptic delay was decreased in $RIM^{R>H}$ (1.18 ± 0.06 ms and 1.39 ± 0.06 ms; Supplementary Table 3) and in addition, rise and decay time were accelerated compared to RIM^{wt} (rise time: 0.75 ± 0.1 ms and 0.97 ± 0.1 ms; tau decay: 3.3 ± 0.2 ms and 4.2 ± 0.2 ms; Supplementary Table 3). However, at lower extracellular Ca^{2+} concentration (0.5 mM) eEPSC amplitudes and kinetics were not changed in $RIM^{R>H}$ (Figure 3G; amplitude: 2.27 ± 0.74 nA and 1.85 ± 0.81 nA; delay: 1.4 ± 0.07 ms and 1.51 ± 0.25 ms; rise time: 1.06 ± 0.07 ms and 1.06 ± 0.07 ms; tau decay: 4.98 ± 0.59 ms and 4.83 ± 0.64 ms for RIM^{wt} and $RIM^{R>H}$, respectively). This indicates, that the $RIM^{R>H}$ mutation exerts a Ca^{2+} -dependent effect on evoked synaptic release.

Overexpression of $RIM^{R>H}$ leaves synapse and AZ morphology unaltered

To assess whether any of these functional characteristics observed when overexpressing $RIM^{R>H}$ in larval motoneurons are caused by changes of synaptic structure, we performed a morphometric analysis of immunohistochemically processed NMJ preparations. To this end, we focussed on the critical AZ scaffold protein Bruchpilot (Brp) and used the highly specific monoclonal antibody Brp^{Nc82} , which recognizes an epitope in the C-terminal part of Brp^{63-65} .

We quantified the number of Brp puncta per NMJ in RIM^{wt} and $RIM^{R>H}$ using Brp^{Nc82} and found it indistinguishable between the two groups (Figure 4A,B, Supplementary Table 4). In addition, the overall NMJ size measured by the extent of the presynaptic α -HRP area and the number of boutons per NMJ were unchanged. Next, to test the impact of the *CORD7* mutation on the mesoscale architecture (scale level bridging the nanometer scale of atomic structure and the micrometer scale of cellular ultrastructure)⁶⁶ of individual AZs we performed localization microscopy using *d*STORM (*direct stochastic optical reconstruction microscopy*)^{48,50,67} applying a previously approved HDBSCAN analysis⁵². Since Brp is abundantly present at the presynapse and immunolabels by Brp^{Nc82} cover the spatial extent of individual AZs⁴⁶, we equate ‘Brp area’ with ‘AZ area’ (Figure 4C,D). Analysis of AZs in type Ib boutons of the NMJ showed no difference in size or Brp composition of individual AZs between RIM^{wt} and $RIM^{R>H}$ (Figure 4D, Supplementary Table 4). We conclude that AZ number and

synapse morphology as well as the Brp nanoarchitecture are not altered in $RIM^{R>H}$ -overexpressing motoneurons.

The CORD7 mutation tightens release coupling

RIM is required for normal levels of presynaptic Ca^{2+} channels²⁴ and, in addition, the CORD7 mutation was shown to modulate VGCC function¹¹. Therefore, we asked whether the $RIM^{R>H}$ mutation affects Ca^{2+} signals at the *Drosophila* NMJ. To test this, we loaded presynaptic terminals with the Ca^{2+} indicator OGB-1 and imaged spatially-averaged Ca^{2+} transients across type Ib boutons in response to single AP stimulation. We did not observe significant differences in peak amplitudes or decay kinetics of presynaptic Ca^{2+} transients between RIM^{wt} and $RIM^{R>H}$ (Figure 5A,B, Supplementary Table 5). Thus, the $RIM^{R>H}$ mutation does not affect presynaptic spatially-averaged Ca^{2+} transients, implying unaltered Ca^{2+} influx.

Our data show a striking enhancement of evoked synaptic transmission by the $RIM^{R>H}$ mutation, which requires a yet unnoted mechanistic basis of RIM function since the number of AZs was unchanged at $RIM^{R>H}$ NMJs (Figure 4B). Tighter coupling causes the release process to be less susceptible to Ca^{2+} chelators, and through nanodomain coupling²⁰ the slow Ca^{2+} buffer EGTA has little effect at *Drosophila* NMJs⁶⁴. Thus, we loaded presynaptic boutons with the membrane permeable BAPTA-derivative BAPTA-AM (10 μ M for 20 min), which results in an intracellular BAPTA concentration in the range of 200-300 μ M⁶⁸. We observed a substantial reduction of the measured eEPSC amplitudes in RIM^{wt} (dropping to 24 %), whereas the $RIM^{R>H}$ mutation rendered release less sensitive to BAPTA (dropping to 42 %, $p = 0.006$; Figure 5C-E). These results indicate that the $RIM^{R>H}$ mutation tightens the coupling distance of synaptic vesicles to the calcium source at the AZ.

Allelic $rim^{R>H}$ expression increases the number of presynaptic boutons, AZs and RRP size

So far our analysis revealed striking effects of the RIM CORD7 mutation on synaptic function, which could not be linked to structural synapse alterations in this overexpression situation. However, allelic expression of $rim^{R>H}$ also increased eEPSC amplitudes in TEVC recordings. We wondered whether synaptic morphology was influenced by the CORD7 mutation when expressed under endogenous *cis*-regulatory control. To address this question, we performed immunohistochemical analyses of NMJs 6/7 in $rim^{rescue}/rim^{rescue}$, $rim^{rescue}/rim^{R>H}$ and $rim^{R>H}/rim^{R>H}$ animals using Brp^{Nc82} again as a marker for presynaptic AZs and α -HRP to measure the extent of the presynaptic plasma membrane (Figure 6). Surprisingly, we found an increased number of AZs and of presynaptic bouton numbers per NMJ as

well as enlarged NMJ size due to the *rim*^{R>H} mutation in a dose-dependent manner (Figure 6A, Supplementary Table 6). Employing *d*STORM and HDBSCAN algorithms we then analyzed the Brp nanoarchitecture in the three genotypes. We found no effect on AZ area, the number of Brp localizations per AZ and Brp localization density in heterozygous *rim*^{rescue}/*rim*^{R>H} and homozygous *rim*^{R>H}/*rim*^{R>H} variants of the CORD7 mutation (Figure 6B, Supplementary Table 6). These imaging results suggest that allelic expression of *rim*^{R>H} changes the overall NMJ morphology without influencing the Brp organization at the mesoscale level.

Finally, we estimated the size of the RRP of synaptic vesicles in animals carrying the *rim*^{R>H} allele. Through fluctuation analysis of focal recordings we found that *q*, *N* and *p_r* were unchanged (Supplementary Figure 2). However, linear fits of average cumulative eEPSC amplitudes in response to 20 Hz trains were back-extrapolated to allow estimations of the RRP (Figure 6C,D). Applying this approach we determined that the RRP size was increased at *rim*^{R>H} synapses (Figure 6E, Supplementary Table 7).

Discussion

Protein interactions at presynaptic AZs determine fundamental aspects of neuronal communication. Typically, mutations in proteins are deleterious without ambiguity. A significant exception, however, is a specific mutation in the RIM protein that leads to the CORD7 syndrome⁶. The affected individuals suffer from retinal dystrophy leading to blindness but also show enhanced cognitive capabilities characterized by increases in verbal IQ and working memory⁷. As revealed by large-scale exome sequencing efforts the abundance of the CORD7 mutation in the general human population was found with a minor allele frequency of 0.017 % without further indication on putative phenotypes of affected individuals⁶⁹. A thorough analysis of the effects of the CORD7 mutation on neuronal structure or synaptic communication that may ultimately cause the remarkable neurological phenotypes of index family members has thus been warranted.

RIM is a central element of AZs and involved in the configuration of AZ shape, composition and function through multiple protein interactions^{10,11,23,70–72}. These roles are at least partially mediated through RIM's domains, which are structurally and biochemically well defined. The clarification of each domain's contribution to synaptic function is complicated through the genetic redundancy of four different *RIMS* loci in addition to the splicing-dependent heterogeneity of their gene products in mammalian model species⁷³.

Here we studied the function of the sole *rim* homolog CG33547 of *Drosophila melanogaster*^{24,43}. We first employed X-ray crystallography of the fly RIM C₂A domain to demonstrate that the overall domain layout is strikingly similar and has remained highly conserved throughout evolution. We found that the arginine doublet in the 3₁₀-helix, which is affected in CORD7 patients, is also present in the *Drosophila* C₂A domain and sterically matches the mammalian domain layout⁸. We then analyzed the effect of the CORD7 mutation at the *Drosophila* NMJ by genomic engineering of alleles and construction of transgenes with a CORD7-equivalent mutation, and by mimicking the *RIMS1* genotype of CORD7 patients.

Arginine doublets in C₂ domains of AZ proteins, the site at which the CORD7 mutation alters RIMS1's protein structure, have been implicated in different functions and interactions. The arginine doublet of the Synaptotagmin-1 (Syt1) C₂B domain, which is located on the opposite side of the domain compared when to the position of the CORD7 mutation, was previously shown to allow for lipid interaction⁷⁴. This doublet is functionally highly relevant as RR-elimination culminates in release failure and is functionally equivalent to the impairment of calcium binding at the top face of the C₂B domain⁷⁵. In this context the arginine doublet of the C₂B domain appears pertinent for positional priming and site clearance⁷⁶. We found a profound increase of synaptic current amplitudes, and accelerated rise and decay times caused by the CORD7 mutation implying that the *rim*^{R>H} mutation affects synaptic release at AZs in *Drosophila* too, and thus by analogy also in CORD7 patients. In addition, we found NMJ size and the number of presynaptic AZs per NMJ increased by allelic expression of the CORD7 mutation which might be a contributing factor to release potentiation. Interestingly, also release coupling and changes in RRP size are influenced by the mutation.

The molecular mechanism by which the *rim*^{R>H} mutation affects synaptic function remains speculative. It was shown that RIM C₂A and C₂B domains interact with the α - and β -subunits of VGCC, SNARE components and Synaptotagmins^{10,56,57}; one of these interactions may be mediated by the 3₁₀-helix side of the C₂A domain. At the biophysical level *rim*^{R>H} lessens the positive electrostatic charge found at this site of the wildtype protein and would weaken charge-based interaction forces. However, a weaker electrostatic charge effect of C₂A^{R>H} on this putative target binding may be counteracted by higher hydrophobicity of the histidine amino acid compared to arginine, leading to a net strengthening of this interaction. In contrast, the *rim*^{RR>EE} substitution not only inverts the charge of this protein site, but it also offers no compensatory hydrophobic effect causing the weakening or disruption of the putative RIM

complex. Thus, the *rim*^{R>H} and *rim*^{RR>EE} mutations can have opposing molecular effects leading to the observed differences in synaptic response.

Intriguingly, RIM1-mediated modulation of VGCC was altered by the CORD7 mutation *in vitro* displaying impaired function of L-type Cav1.4, the predominant VGCC that controls release from ribbon synapses in the retina, upon co-expression with RIMS1^{R>H}, while calcium conductance through P/Q-type Cav2.1 channels, which partake in central synaptic release, was augmented^{11,77}. These findings offer an explanation for the opposing impact of the CORD7 mutation on the visual and central systems. Our presynaptic calcium indicator measurements argue that the *rim*^{R>H} mutation does not impact presynaptic Ca²⁺ signals *in vivo*. Instead, enhanced synaptic release in the presence of the CORD7 mutation is likely caused by a reduced coupling distance of synaptic vesicles to the calcium source at the AZ as shown by the reduced effect of a calcium chelator on eEPSC amplitudes in the presence of RIM^{R>H}. This is in line with findings showing that RIM C₂A and C₂B domains interact with the α and β -subunits of VGCC, SNARE components and Synaptotagmins^{11,56,78–80}. The RIM-C₂B domain additionally crosstalks with α -Liprin¹⁰. Rescue experiments of RIM-deficient synapses, however, demonstrate that RIM's capacity to recruit calcium channels to the AZ does not rely on the presence of either C₂ domain, while, in contrast, both C₂ domains facilitate synaptic release without modulating presynaptic calcium channel influx *in vivo*^{23,71}. In addition, our investigations revealed an increase of the RRP size at *Drosophila* NMJs. This finding might offer an additional explanation for enhanced synaptic performance in mutants expressing the *rim*^{R>H} CORD7 allele since RRP enlargement - representing a 'pool engram' - was suggested to be suitable for short-term storage of information⁸¹. Remarkably, this effect was Calcium- and dosage-dependent as measurements in the motoneuron-specific overexpression and the allelic expression revealed. One can speculate that the heterogeneity of evoked synaptic release along the NMJ could contribute to these effects too, thus, employing transgenically expressed GCaMP Ca²⁺ sensors imaging to precisely dissect evoked neurotransmission at different release spots may be a fitting approach to better understand this phenomenon^{82,83}. To gain further insight into organization of the synaptic vesicle pool, 3D EM tomographic analyses of vesicle distributions within the presynaptic terminal are attractive⁸⁴. Further investigations are necessary to delineate RIMS1-VGCC interactions and their steric relationship, specifically at retinal and telencephalic mammalian synapses.

A possible cause of the observed release abnormalities at *rim*^{R>H} synapses is the interference of the mutation with direct molecular interactions, which occur between the 3₁₀-helix of the

C₂A domain and yet unknown partners. Alternatively, the CORD7 mutation may impact protein folding and/or stability of the C₂A domain and, thus, the entire RIM molecule. This may result in altered levels of the RIM pool or subcellular localization of RIM at the presynapse and affect neurotransmission. However, the observed gene dosage sensitive gain-of-function effects of *rim*^{R>H} in direct comparison with loss-of-function alleles (*rim*^{RR>EE} or *rim*^{KO}) argue against a reduction or complete depletion of RIM^{R>H} at the synapse. Unfortunately, no α -RIM antibody or transgenically tagged RIM tool is currently available to faithfully assess the amount of RIM protein and its potential change under the influence of the CORD7 mutation.

In sum, the use of the *Drosophila* NMJ as a model system allowed us to uncover the impact of the human CORD7 syndrome mutation of the RIM C₂A domain on speed and efficacy of synaptic release. Future investigations have to detail how the striking cognitive enhancement of CORD7 patients and the functional augmentation of synaptic transmission at *Drosophila* synapses are accomplished through this discrete change in the RIM C₂A domain.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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Figure legends

Figure 1 The structure of the RIM C₂A domain is conserved from fly to man.

(A) RIM protein layout for the human RIMS1 and the *Drosophila* homolog showing the position of its Rab3/Zinc-finger, PDZ, PxxP, C₂A and C₂B domains, known interaction

partners are displayed above (the interaction with Munc13 [in brackets] is observed only in mammals but not *Drosophila*). Position of the CORD7 mutation site in the C₂A domain (red) is indicated. (B) Protein alignments of wild-type RIM C₂A and C₂B domains (truncated for display purpose) from different species (HS, *Homo sapiens*; MM, *Mus musculus*; RN, *Rattus norvegicus*; DM, *Drosophila melanogaster*; CE; *Caenorhabditis elegans*). Alignments for C₂A and C₂B domains were calculated separately. Blue residues correspond to positions that are fully conserved between C₂A and C₂B domains in all displayed RIM homologs. Positions of the 3₁₀-helix (yellow), β -strands 5 and 6 (grey) of the *Drosophila* domain and the CORD7 mutation site in human (red) as part of an RR-motif are denoted. Note that the 3₁₀-helix with the arginine doublet is absent from RIM C₂B domains. GenBank/Uniprot entries: Q86UR5 (RIMS1-HS), Q99NE5 (RIMS1-MM), Q9EQZ7 (RIMS2-MM), Q80U57 (RIMS3-MM), P60191 (RIMS4-MM), AF199329 (RIM2-RN), V5M054 (RIM-DM), Q22366 (UNC-10-CE). (C) Schematic representation of the *Drosophila* RIM C₂A domain structure. β -strands (β 1- β 8) are colored in magenta and the 3₁₀-helix connecting β 5 to β 6 in yellow. The arginine doublet linked to CORD7 (R915, R916) is shown as sticks. The insert shows the superposition of the 3₁₀-helix region of *Drosophila* RIM C₂A (magenta) with rat RIM2 C₂A (green; PDB ID 2BWQ). (D) Electrostatic potential of accessible surfaces for the *Drosophila* RIM C₂A domain. Blue indicates positively charged regions, red negatively charged and white regions without charge. (E) Magnification of the domain bottom face in the wild-type, the CORD7 mutation and the RR>EE variants.

See also Supplementary Table 1.

Figure 2 Construction and effect of the *rim*^{R>H} CORD7 allele. (A) Overview, gene structure and genomic targeting strategy of the *rim* locus on chromosome III. The position of the *gRNA* probes are marked by #1 and #2, the grey bar denotes the extent of the *rim*^{Ex73} excision²⁴ used as a *rim*^{KO} null allele in this study. (B) Location and size of the *rim* gene fragment excised by CRISPR/Cas9 and replaced by phiC31 integration to generate *rim*^{rescue} (blue), *rim*^{R>H} (red) and *rim*^{RR>EE} (light grey) alleles. (C) PCR genotyping confirming the generation of *rim*^{AC2A} allele that allowed for subsequent C₂A domain engineering. (D) Representative eEPSC recordings of indicated *rim* genotypes showing the semi-dominant character of CORD7 mutation. (E, F) Summary graphs and mean values for peak synaptic current amplitudes (E) and paired-pulse ratios (F) for indicated *rim* genotypes (*rim*^{KO}/*rim*^{KO}: n = 17 NMJs from 8 animals;

rim^{KO}/*rim*^{rescue}: n = 14 NMJs from 5 animals; *rim*^{RR>EE}/*rim*^{RR>EE}: n = 16 NMJs from 8 animals; *rim*^{KO}/*rim*^{R>H}: n = 13 NMJs from 6 animals; *rim*^{rescue}/*rim*^{rescue}: n = 20 NMJs from 13 animals; *rim*⁺/*rim*⁺: n = 37 NMJs from 22 animals; *rim*^{R>H}/*rim*^{rescue}: n = 12 NMJs from 6 animals; *rim*^{R>H}/*rim*^{R>H}: n = 24 NMJs from 17 animals).

See also Supplementary Table 2 for all descriptive statistics and group-by-group statistical comparisons of TEVC and PPR datasets.

Figure 3 Motoneuron-specific expression of *RIM*^{R>H} increases and accelerates evoked synaptic currents. (A) Two-electrode voltage clamp (TEVC) recording configuration from *Drosophila* abdominal wall muscle 6 (blue). Action potentials were elicited via motoneuron stimulation using a suction electrode (SE). (B) Averaged evoked excitatory postsynaptic currents ($n_{\text{eEPSC}} = 20$ per experiment) measured with TEVC at NMJs expressing *RIM*^{wt} (grey) and *RIM*^{R>H} (red) under *ok6-GAL4* control and summary graph for eEPSC amplitudes ($n = 11$ NMJs from 11 animals, respectively). (C) Example spontaneous miniature excitatory postsynaptic currents (mEPSCs) and summary graphs for mEPSC amplitude, rise time and frequency ($n_{\text{mEPSC}} > 100$ per experiment) in *RIM*^{wt} (grey) and *RIM*^{R>H} (red; $n = 14$ NMJs from 14 animals, respectively). (D) Focal recording configuration from the *Drosophila* NMJ formed on abdominal wall muscles 6/7. As in A, action potentials were elicited via motoneuron stimulation using a suction electrode (SE), but postsynaptic currents were recorded using a focal electrode (FE) placed extracellularly on type Ib boutons (larger boutons in A and D). (E) Averaged focally recorded eEPSCs in *RIM*^{wt} (grey) and *RIM*^{R>H} (red; $n_{\text{eEPSC}} = 60$ per experiment) and rightmost, overlay of normalized traces. Inset illustrates determination of synaptic delay measured from the end of the stimulation artifact to 10 % of the eEPSC rise time. (F) Summary graphs for peak synaptic current amplitude, synaptic delay, rise time and tau decay for *RIM*^{wt} (grey; $n = 11$ NMJs from 11 animals) and *RIM*^{R>H} (red; 10 NMJs from 10 animals). Throughout this manuscript, horizontal lines in box plots represent median; boxes quartiles; whiskers 10th and 90th percentiles; scatter plots show individual data points. p-values are indicated above summary graphs. (G) Summary graphs for peak synaptic current amplitude, synaptic delay, rise time and tau decay measured in 0.5 mM extracellular Ca²⁺ concentration in *RIM*^{wt} (grey; $n = 6$ NMJs from 3 animals) and *RIM*^{R>H} (red; $n = 6$ NMJs from 3 animals). Throughout this manuscript, horizontal lines in box plots represent median; boxes quartiles; whiskers 10th and 90th percentiles; scatter plots show individual data points. p-values

are indicated above summary graphs. All displayed measurements made with L3 larvae expressing indicated *RIM* variant as *UAS-RIM^X* transgene under control of the motoneuron-specific *ok6-GAL4* enhancer.

See also Supplementary Table 3.

Figure 4 Unchanged AZ number and Brp mesoscale arrangement at *RIM^{R>H}* NMJs. (A) Exemplary *Drosophila RIM^{R>H}* NMJ on abdominal wall muscles 6/7 stained for presynaptic plasma membranes with α -HRP (magenta) and the presynaptic scaffold protein Bruchpilot (Brp^{Nc82} , green). (B) Summary graphs for the number of Brp puncta (i.e. considered as ‘AZ’) per NMJ 6/7 in abdominal segments A2 and A3, NMJ size and the number of boutons per NMJ in *RIM^{wt}* (grey) and *RIM^{R>H}* (red; $n = 17$ NMJs from 5 animals, respectively). (C) Scatter plot of *d*STORM localizations of individual AZs recognized by Brp^{Nc82} labeled with Cy5 conjugated F(ab’)₂ fragments in *RIM^{wt}* and *RIM^{R>H}*. (D) Summary graphs for AZ area, the number of Brp localizations (locs.) per individual AZ and Brp localization (loc.) density measured by *d*STORM in *RIM^{wt}* (grey) and *RIM^{R>H}* (red; $n = 591$ and 712 AZs from 15 NMJs and 4 animals, respectively). All displayed measurements made with L3 larvae expressing indicated *RIM* variant as *UAS-RIM^X* transgene under control of the motoneuron-specific *ok6-GAL4* enhancer.

See also Supplementary Tables 4 and 5.

Figure 5 The *RIM^{R>H}* mutation tightens influx-release coupling while leaving spatially averaged presynaptic Ca^{2+} transients unaffected. (A) Representative traces of spatially averaged Ca^{2+} transients in type Ib boutons of *RIM^{wt}* (grey) and *RIM^{R>H}* (red) NMJs (average of 8-12 traces per bouton). (B) Summary graphs for peak amplitude ($\Delta F/F$), average baseline fluorescence (F baseline) and tau decay in *RIM^{wt}* ($n = 28$ boutons, 10 NMJs, 9 animals) and *RIM^{R>H}* ($n = 36$ boutons, 10 NMJs, 7 animals). (C) Averaged focally recorded eEPSCs in *RIM^{wt}* (grey) and *RIM^{R>H}* (red; $n_{\text{eEPSC}} = 60$ per experiment) before (continuous lines) and after application of BAPTA-AM (dashed lines). (D and E) Summary graphs for normalized eEPSC amplitude reduction and normalized eEPSC PPR increase induced by BAPTA in *RIM^{wt}* (grey, $n = 22$ experiments from 12 NMJs in 6 animals for control and BAPTA, respectively) and *RIM^{R>H}* (red, $n = 21$ experiments from 14 and 16 NMJs in 7 and 6 animals for control and

BAPTA, respectively). All displayed measurements made with L3 larvae expressing indicated *RIM* variant as *UAS-RIM^X* transgene under control of the motoneuron-specific *ok6-GAL4* enhancer.

See also Supplementary Table 5.

Figure 6 The *rim^{R>H}* CORD7 allele changes NMJ morphology and increases the RRP. (A) Summary graphs for the number of Brp puncta per NMJ, NMJ size and the number of boutons per NMJ in *rim^{rescue}/rim^{rescue}* (grey), *rim^{R>H}/rim^{rescue}* (red) and *rim^{R>H}/rim^{R>H}* (dark red; n = 19, 16 and 18 NMJs from 7, 7 and 6 animals, respectively). (B) Summary graphs for AZ area, the number of Brp localizations (locs.) per individual AZ and Brp localization (loc.) density measured by *dSTORM* *rim^{rescue}/rim^{rescue}*, *rim^{R>H}/rim^{rescue}* and *rim^{R>H}/rim^{R>H}* (n = 1088, 898 and 855 AZs from 17, 17 and 16 NMJs and 6, 6 and 6 animals, respectively). (C) Example eEPSCs in response to 20 Hz trains (50 pulses) in the three genotypes. Scale bars 200 ms and 20 nA. (D) Cumulative eEPSC amplitudes in *rim^{rescue}/rim^{rescue}*, *rim^{R>H}/rim^{rescue}* and *rim^{R>H}/rim^{R>H}* (n = 9, 7 and 10 NMJs and animals, respectively). Back-extrapolation of linear fits to the average cumulative eEPSC amplitude of the last 20 pulses (straight lines) yielded estimates for the RRP of 174, 226 and 248 for the three genotypes. (E) Summary graphs for the estimates of back-extrapolations of the cumulative eEPSC amplitudes in *rim^{rescue}/rim^{rescue}*, *rim^{R>H}/rim^{rescue}* and *rim^{R>H}/rim^{R>H}*. Whisker plots show mean \pm SEM, scatter plots individual data points. All displayed measurements were performed with L3 larvae carrying indicated *rim* alleles (*rim^x/rim^x*).

See also Supplementary Tables 6 and 7.

Figures

Figure 1

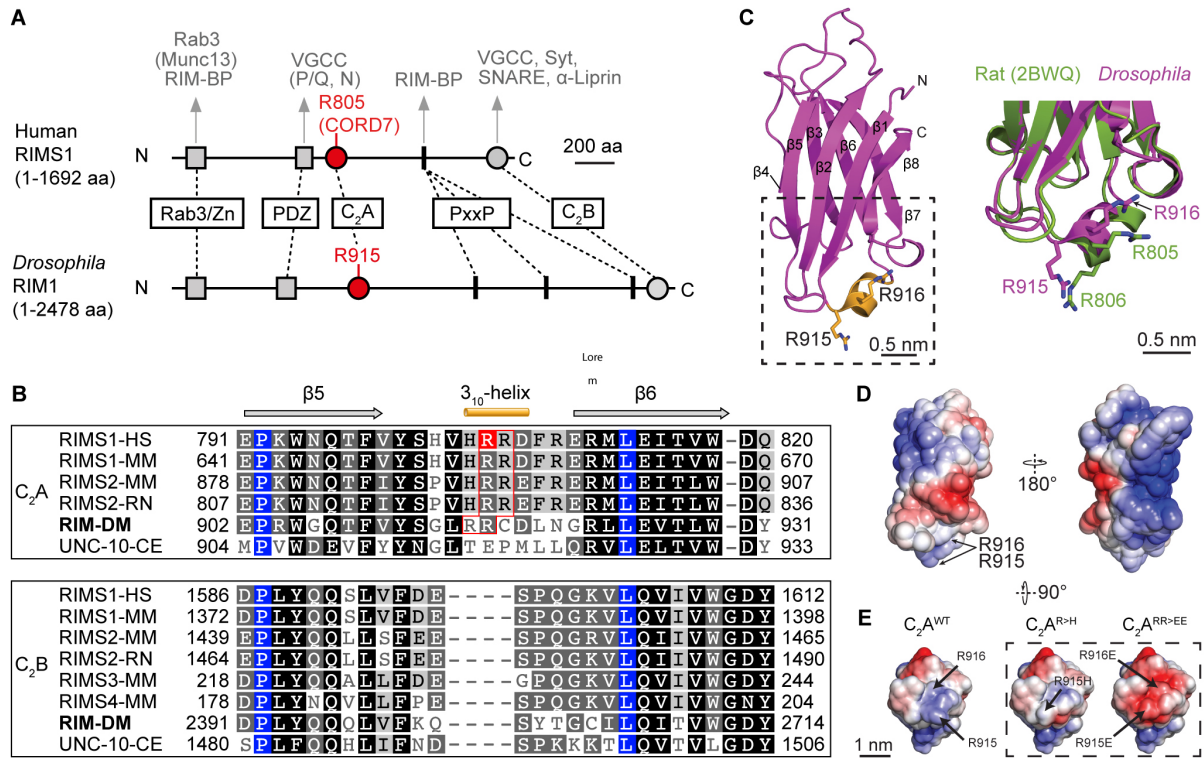


Figure 2

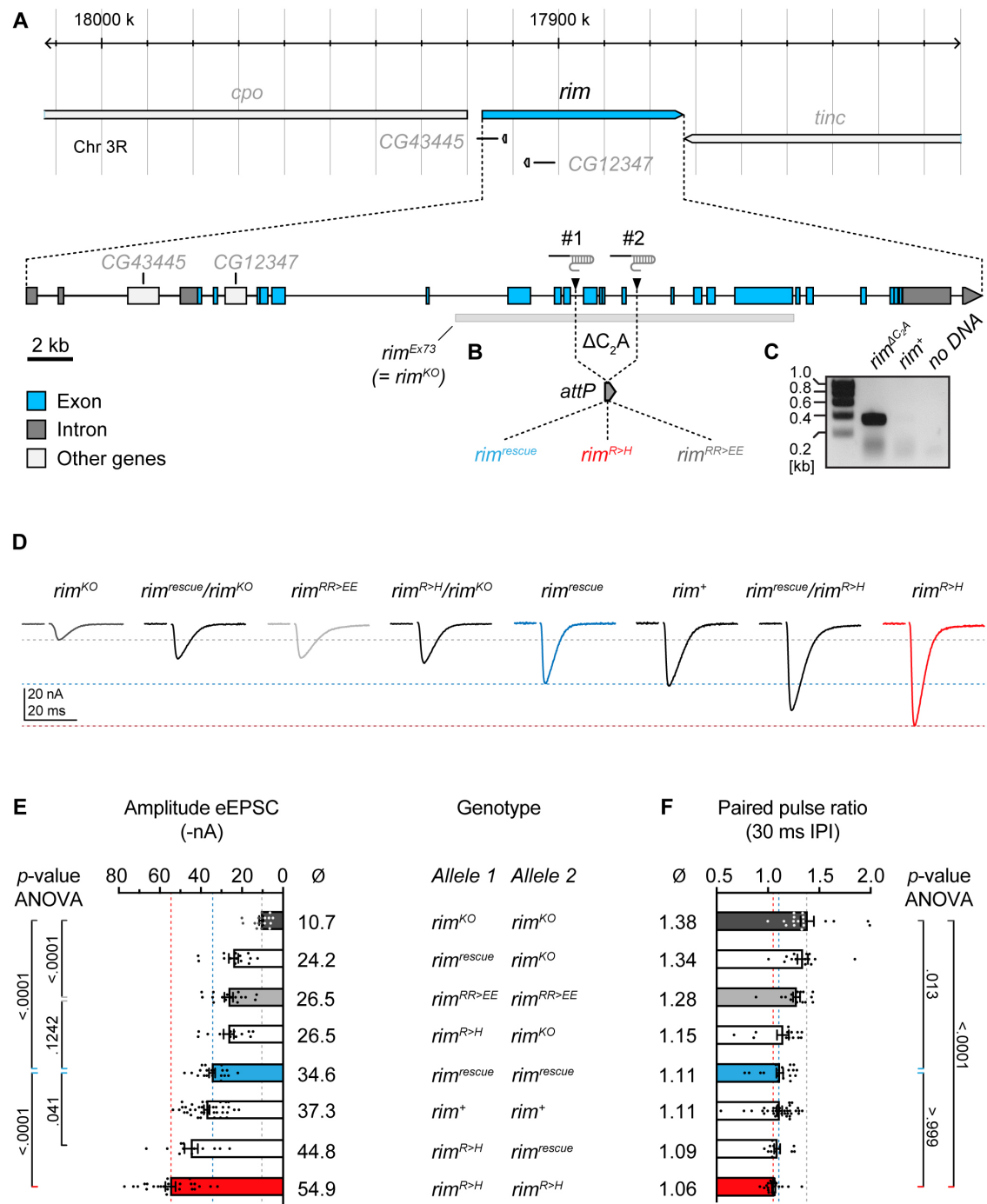


Figure 3

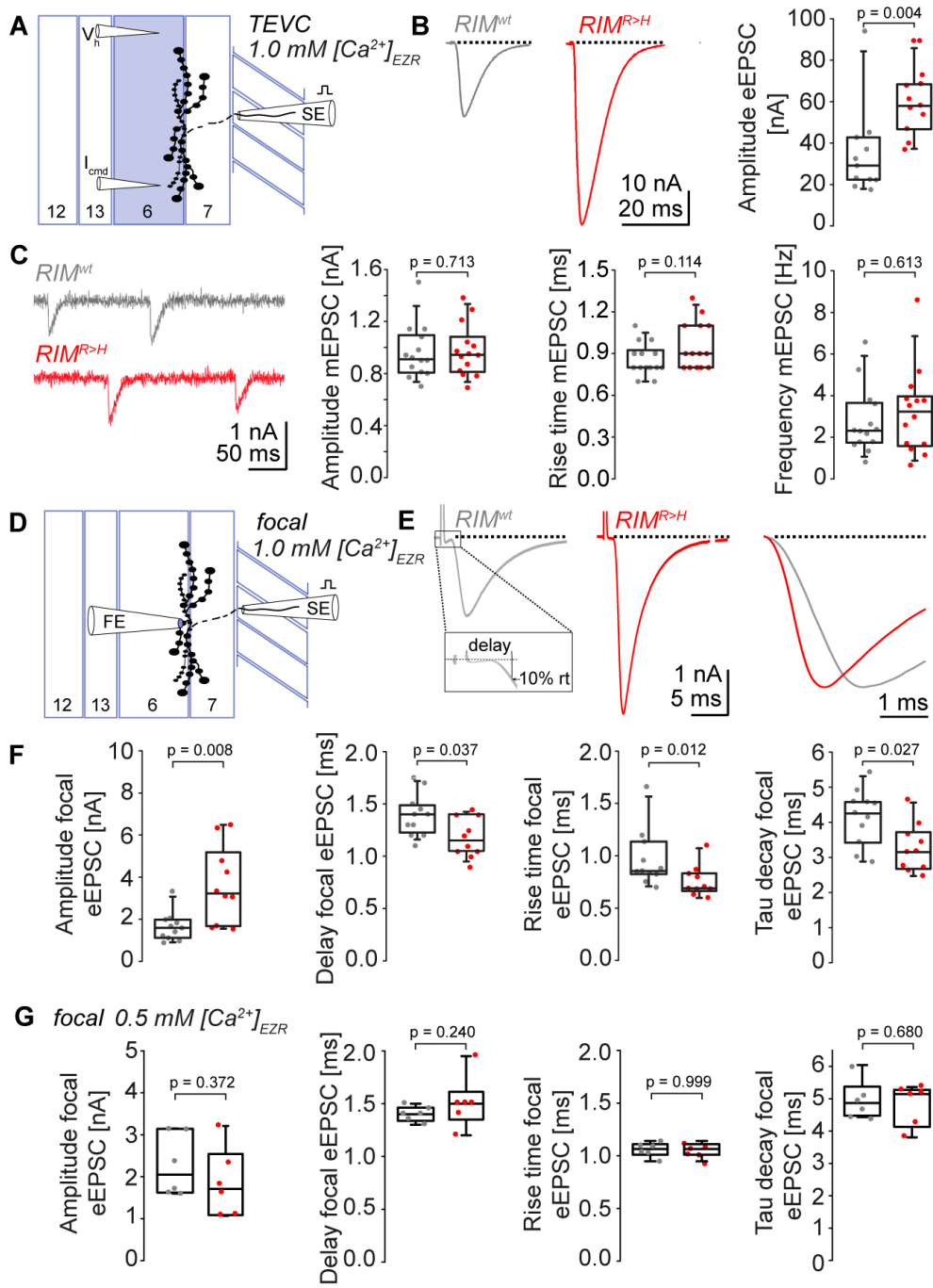


Figure 4

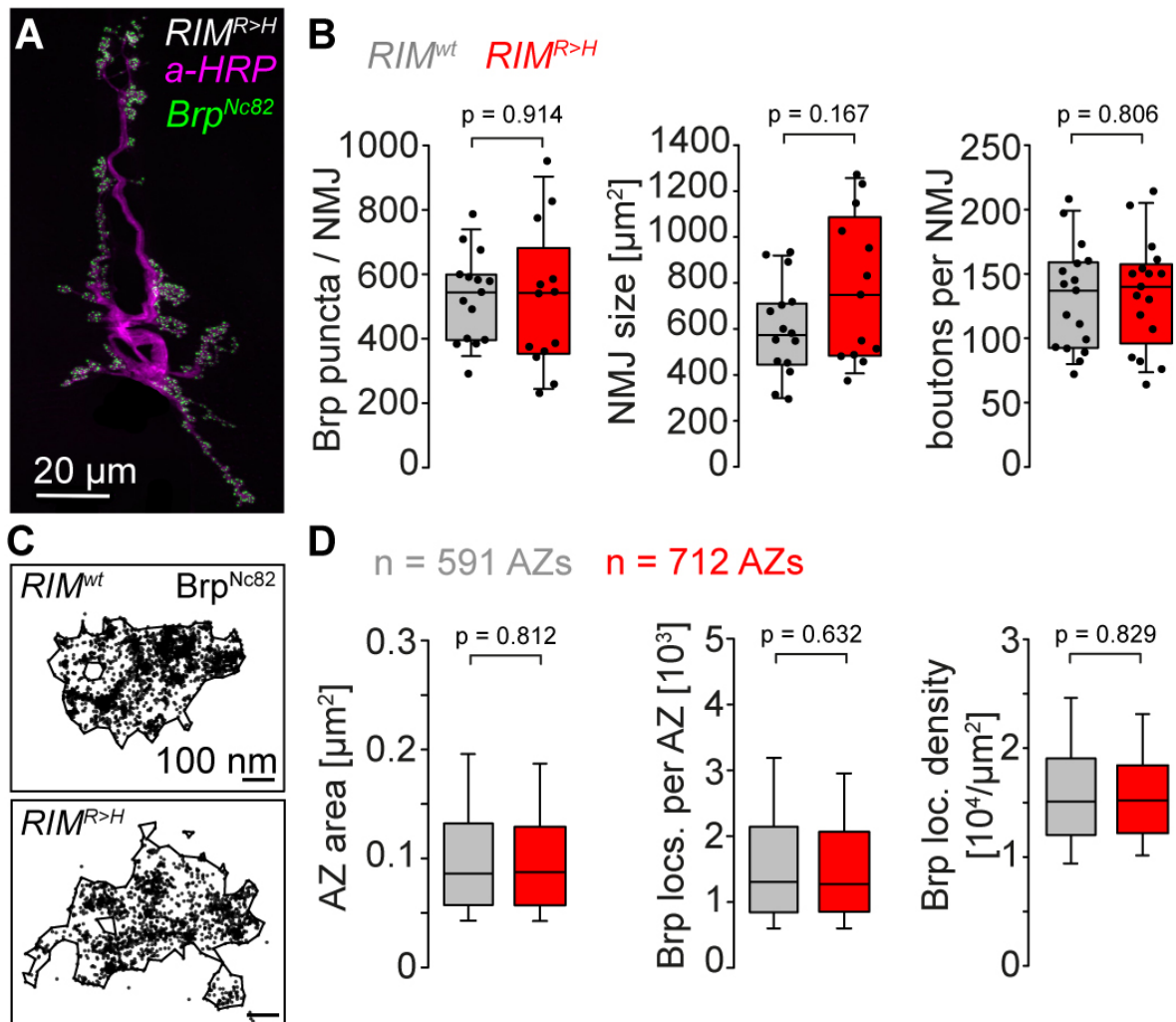


Figure 5

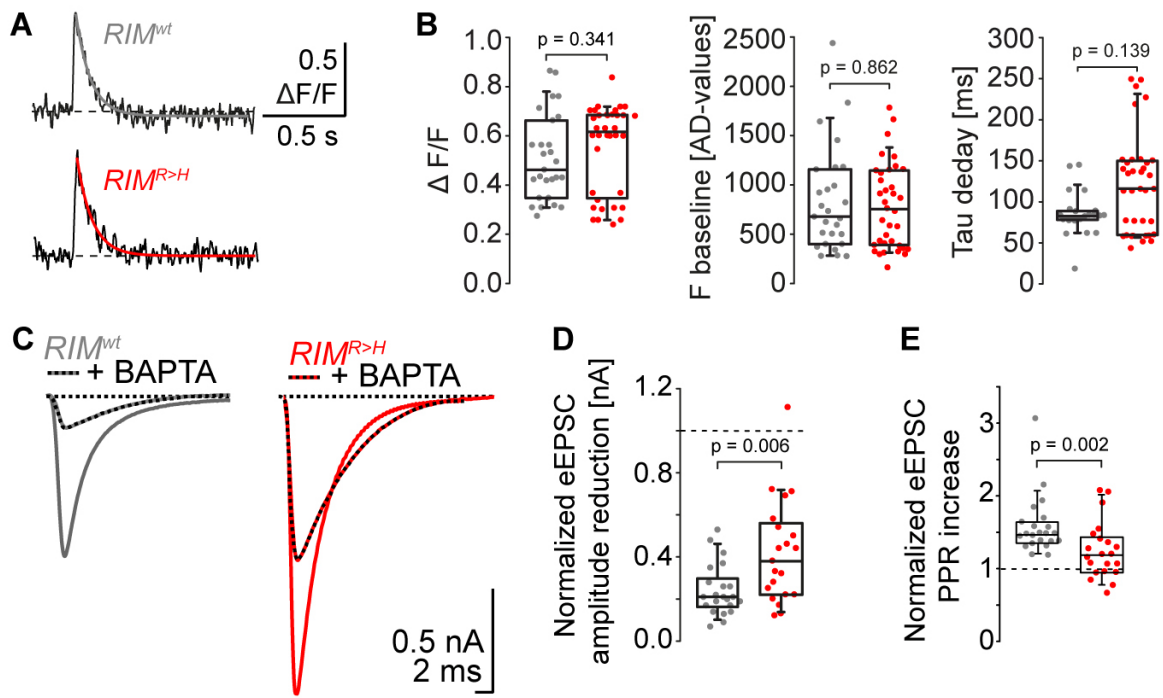
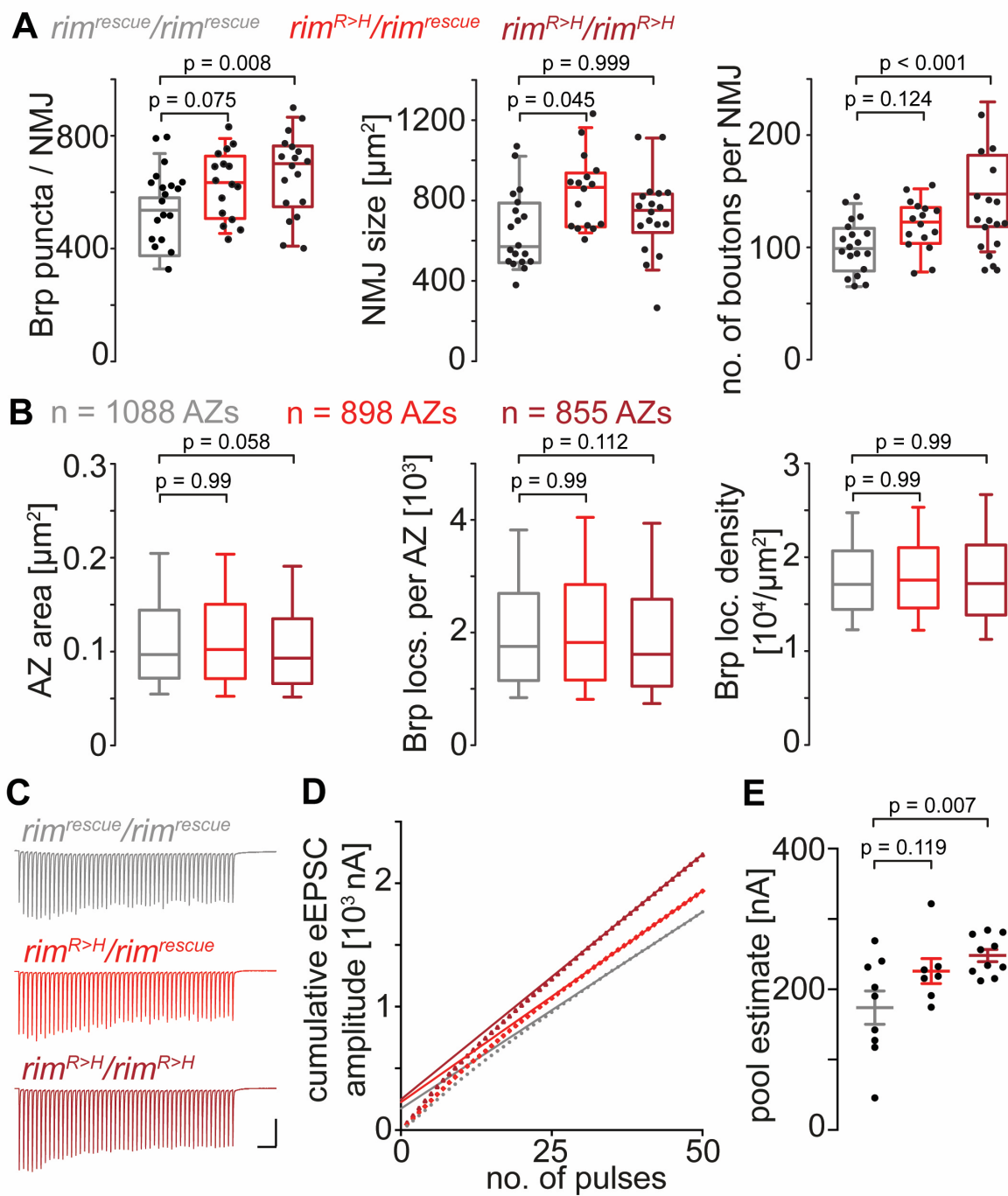
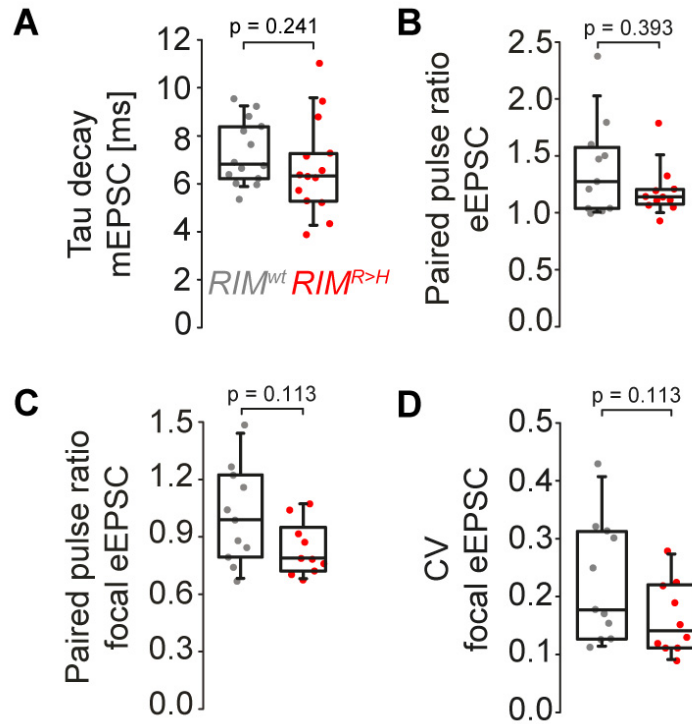


Figure 6



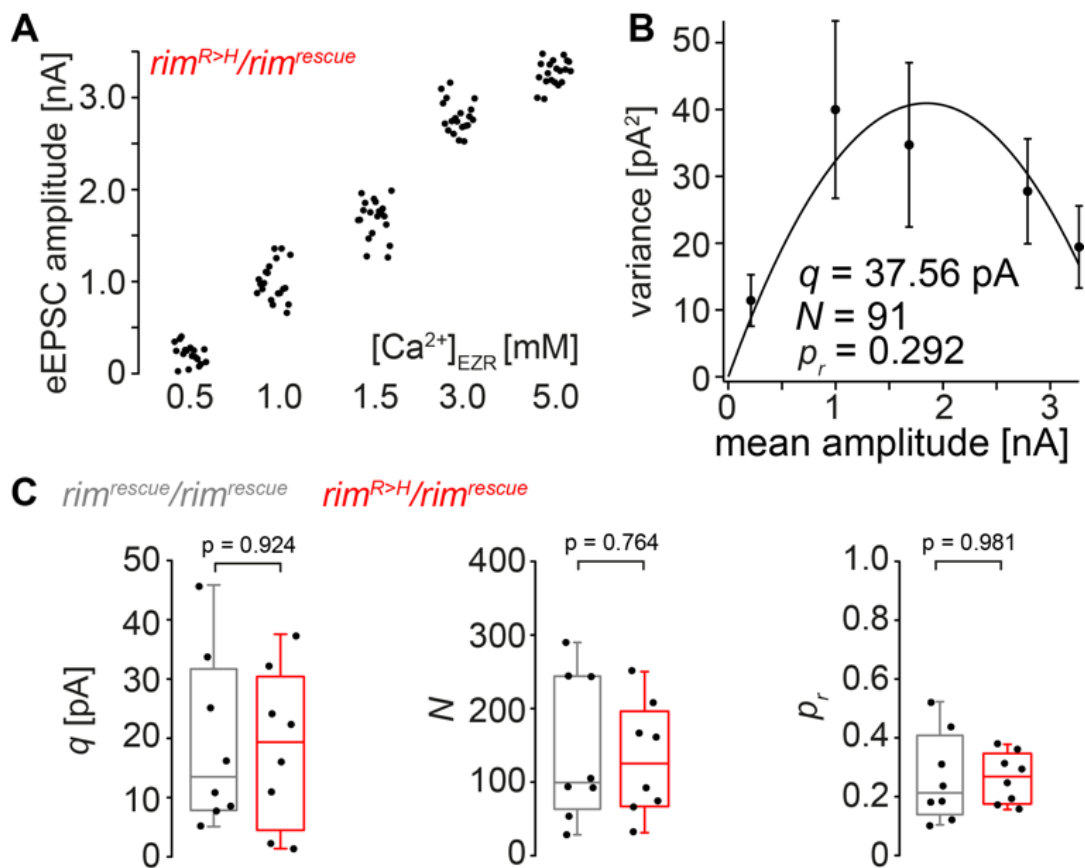
Supplementary Fig. 1 Short-term plasticity is unchanged by the CORD7 mutation.
Related to Figure 3.



(A) Summary graph for mEPSC tau decay ($n_{\text{mEPSC}} > 100$ per experiment) in TEVC recording configuration in *RIM^{wt}* (grey) and *RIM^{R>H}* (red; $n = 14$ experiments and animals, respectively). (B) Summary graph for paired pulse ratio measured with TEVC in *RIM^{wt}* (grey) and *RIM^{R>H}* (red; $n = 11$ measurements and animals for each genotype). (C) Summary graph for paired pulse ratio and (D) coefficient of variation (CV) in focal recordings in *RIM^{wt}* (grey) and *RIM^{R>H}* (red; $n = 11$ and 10 experiments and animals, respectively). Horizontal lines in box plots represent median; boxes quartiles; whiskers 10th and 90th percentiles; scatter plots show individual data points.

All displayed measurements made with L3 larvae expressing indicated *RIM* variant as *UAS-RIM^X* transgene under control of the motoneuron-specific *ok6-GAL4* enhancer.

Supplementary Fig. 2 Unchanged N and p_r in $rim^{R>H}/rim^{rescue}$. Related to Figure 6.



(A) Fluctuation analysis of eEPSC amplitudes recorded focally at the indicated extracellular Ca²⁺ concentrations at NMJs 6/7. Example amplitude distribution from a *rim^{R>H}/rim^{rescue}* animal. (B) Variance of the eEPSC amplitudes from (A) plotted against the corresponding amplitude means, error bars indicate variances of the variance. The parabolic fit yields quantal size q , the binominal parameter N and the release probability p_r . q , N and p_r at 1 mM extracellular Ca²⁺ for the recording in (A) are indicated. (C) Summary graphs for q , N and p_r in *rim^{rescue}/rim^{rescue}* (grey) and *rim^{R>H}/rim^{rescue}* (red; $n = 8$ NMJs from 8 animals, respectively).

All displayed measurements were performed with L3 larvae carrying indicated *rim* alleles (*rim^x/rim^x*).

Supplementary Table 1 Crystallographic data collection and refinement statistics. Related to Figure 1.

| Protein | DmRIM C₂A |
|--|--|
| PDB entry ID | 4TS6 |
| Space group | P 2 ₁ 2 ₁ 2 ₁ |
| Unit cell (Å) | a = 32.08; b = 38.70; c = 131.37 |
| Beamline | DLS/I04 |
| Wavelength (Å) | 0.9999 |
| Resolution range (Å) | 65.68-1.92 |
| High resolution shell (Å) | 1.97-1.92 |
| R _{Merge} ^a | 0.057 (0.643) |
| R _{Pim} ^a | 0.025 (0.281) |
| Completeness ^a (%) | 99.9 (99.7) |
| Multiplicity ^a | 6.9 (7.1) |
| I/σ (I) ^a | 17.5 (2.8) |
| Refinement statistics | |
| R _{work} (reflections) | 0.184 (12492) |
| R _{free} (reflections) | 0.218 (648) |
| Number of atoms | |
| Protein atoms | 1046 |
| Water | 89 |
| Average B factors (Å²) | |
| Protein atoms | 35.4 |
| Water | 48.5 |
| RMSD from ideal values | |
| Bonds / angles (Å/°) | 0.008 / 1.22 |
| MolProbity statistics ⁷³ | |
| Ramachandran favored (%) | 98.39 |
| Ramachandran disallowed (%) | 0.0 |
| Clashscore (percentile) | 1.93 (100%) |
| MolProbity score (percentile) | 0.96 (100%) |

^a Values in parentheses correspond to the high resolution shell

Supplementary Table 2 Data summary and statistical information of TEVC recordings of L3 larvae carrying indicated *rim* alleles. Related to Figure 2.

| Genotype | eEPSC amplitude [nA] | | PPR (30 ms IPI) | |
|--|----------------------|---------|-----------------|---------|
| | mean ± SEM | P-value | mean ± SEM | P-value |
| <i>rim</i> ^{KO} / <i>rim</i> ^{KO} (n = 17 NMJs from 8 larvae) | 10.67 ± 1.043 | | 1.383 ± 0.065 | |
| vs. <i>rim</i> ^{KO} / <i>rim</i> ^{rescue} | | 0.0011 | | >0.9999 |
| vs. <i>rim</i> ^{R915E.R916E} / <i>rim</i> ^{R915E.R916E} | | <0.0001 | | >0.9999 |
| vs. <i>rim</i> ^{KO} / <i>rim</i> ^{R915H} | | <0.0001 | | 0.6604 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{rescue} | | <0.0001 | | 0.013 |
| vs. <i>rim</i> ⁺ / <i>rim</i> ⁺ | | <0.0001 | | 0.0018 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{R915H} | | <0.0001 | | 0.0031 |
| vs. <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} | | <0.0001 | | <0.0001 |
| <i>rim</i> ^{KO} / <i>rim</i> ^{rescue} (n = 14 NMJs from 5 larvae) | 24.17 ± 2.312 | | 1.338 ± 0.051 | |
| vs. <i>rim</i> ^{R915E.R916E} / <i>rim</i> ^{R915E.R916E} | | 0.9964 | | >0.9999 |
| vs. <i>rim</i> ^{KO} / <i>rim</i> ^{R915H} | | 0.9972 | | 0.809 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{rescue} | | 0.0209 | | 0.0238 |
| vs. <i>rim</i> ⁺ / <i>rim</i> ⁺ | | 0.0002 | | 0.0049 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{R915H} | | <0.0001 | | 0.0054 |
| vs. <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} | | <0.0001 | | <0.0001 |
| <i>rim</i> ^{R915E.R916E} / <i>rim</i> ^{R915E.R916E} (n = 16 NMJs from 8 larvae) | 26.49 ± 2.072 | | 1.278 ± 0.034 | |
| vs. <i>rim</i> ^{KO} / <i>rim</i> ^{R915H} | | >0.9999 | | >0.9999 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{rescue} | | 0.1242 | | 0.0585 |
| vs. <i>rim</i> ⁺ / <i>rim</i> ⁺ | | 0.0021 | | 0.0131 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{R915H} | | <0.0001 | | 0.0131 |
| vs. <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} | | <0.0001 | | <0.0001 |
| <i>rim</i> ^{KO} / <i>rim</i> ^{R915H} (n = 13 NMJs from 6 larvae) | 26.52 ± 2.500 | | 1.146 ± 0.057 | |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{rescue} | | 0.1816 | | >0.9999 |
| vs. <i>rim</i> ⁺ / <i>rim</i> ⁺ | | 0.0061 | | >0.9999 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{R915H} | | <0.0001 | | >0.9999 |
| vs. <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} | | <0.0001 | | 0.2986 |
| <i>rim</i> ^{rescue} / <i>rim</i> ^{rescue} (n = 20 NMJs from 13 larvae) | 34.60 ± 1.472 | | 1.114 ± 0.032 | |
| vs. <i>rim</i> ⁺ / <i>rim</i> ⁺ | | 0.9607 | | >0.9999 |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{R915H} | | 0.041 | | >0.9999 |
| vs. <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} | | <0.0001 | | >0.9999 |
| <i>rim</i> ⁺ / <i>rim</i> ⁺ (n = 37 NMJs from 22 larvae) | 37.25 ± 1.383 | | 1.112 ± 0.026 | |
| vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{R915H} | | 0.18 | | >0.9999 |
| vs. <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} | | <0.0001 | | >0.9999 |
| <i>rim</i> ^{rescue} / <i>rim</i> ^{R915H} (n = 12 NMJs from 6 larvae) | 44.80 ± 3.269 | | 1.089 ± 0.029 | |
| vs. <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} | | 0.0333 | | >0.9999 |
| <i>rim</i> ^{R915H} / <i>rim</i> ^{R915H} (n = 24 NMJs from 17 larvae) | 54.90 ± 2.472 | | 1.056 ± 0.017 | |

Numerical values not stated in text or figure legends including p-values and sample sizes for measurements shown in Figure 2. PPR = paired pulse ratio, IPI = interpulse interval.

Supplementary Table 3 Data summary and statistical information of TEVC and focal recordings of RIM-overexpression constructs. Related to Figure 3.

| TEVC parameter median (25 th – 75 th percentile) | <i>ok6-GAL4 > UAS- RIM^{wt}</i> | <i>ok6-GAL4 > UAS- RIM^{R>H}</i> | P-value |
|---|--|--|---------|
| 1.0 mM [Ca²⁺]_{EZR} | | | |
| eEPSC amplitude [nA] | 29.10 (22.30-42.75) | 57.94 (46.68-68.34) | 0.004 |
| n (NMJs, animals) | 11, 11 | 11, 11 | |
| mEPSC amplitude [nA] | 0.910 (0.808-1.095) | 0.945 (0.813-1.083) | 0.713 |
| mEPSC rise time [ms] | 0.800 (0.800-0.925) | 0.900 (0.800-1.100) | 0.114 |
| mEPSC frequency [ms] | 2.310 (1.738-3.645) | 3.225 (1.583-3.968) | 0.613 |
| n (NMJs, animals) | 14, 14 | 14, 14 | |

| Focal recordings parameter median (25 th – 75 th percentile) | <i>ok6-GAL4 > UAS- RIM^{wt}</i> | <i>ok6-GAL4 > UAS- RIM^{R>H}</i> | P-value |
|---|--|--|---------|
| 1.0 mM [Ca²⁺]_{EZR} | | | |
| eEPSC amplitude [nA] | 1.59 (1.14-1.95) | 3.23 (1.70-4.79) | 0.008 |
| eEPSC delay time [ms] | 1.400 (1.225-1.488) | 1.150 (1.050-1.400) | 0.037 |
| eEPSC rise time [ms] | 0.856 (0.828-1.090) | 0.690 (0.676-0.823) | 0.012 |
| eEPSC tau decay time [ms] | 4.33 (3.60-4.66) | 3.21 (2.75-3.71) | 0.027 |
| n (NMJs, animals) | 11, 11 | 10, 10 | |

| Focal recordings parameter median (25 th – 75 th percentile) | <i>ok6-GAL4 > UAS- RIM^{wt}</i> | <i>ok6-GAL4 > UAS- RIM^{R>H}</i> | P-value |
|---|--|--|---------|
| 0.5 mM [Ca²⁺]_{EZR} | | | |
| eEPSC amplitude [nA] | 2.05 (1.62-3.14) | 1.71 (1.09-2.54) | 0.372 |
| eEPSC delay time [ms] | 1.4 (1.337-1.462) | 1.5 (1.350-1.613) | 0.240 |
| eEPSC rise time [ms] | 1.07 (1.01-1.11) | 1.07 (1.01-1.11) | 0.999 |
| eEPSC tau decay time [ms] | 4.87 (4.48-5.38) | 5.15 (4.13-5.28) | 0.860 |
| n (NMJs, animals) | 6, 3 | 6, 3 | |

Numerical values not stated in text or figure legends including p-values and sample sizes for measurements with motoneuron-specific expression of *RIM^{wt}* (*ok6-GAL4 > UAS-RIM^{wt}*) and *RIM^{R>H}* (*ok6-GAL4 > UAS-RIM^{R>H}*) shown in Figure 3.

Supplementary Table 4 Data summary and statistical information of AZ morphology in larvae overexpressing RIM variants. Related to Figure 4.

| Parameter median (25 th – 75 th percentile) | <i>ok6-GAL4 > UAS-RIM^{wt}</i> | <i>ok6-GAL4 > UAS-RIM^{R>H}</i> | P-value |
|--|---|---|---------------------|
| confocal data | | | |
| Brp puncta / NMJ | 541 (393-596) | 539 (351-679) | 0.914 ^t |
| NMJ size [μm^2] | 573.812 (444.969-710.532) | 748.684 (483.197-1086.828) | 0.167 ^t |
| no. of boutons per NMJ | 137 (93-159) | 140 (96-158) | 0.806 ^t |
| <i>n</i> (NMJs, animals) | 17, 5 | 17, 5 | |
| dSTORM data | | | |
| AZ area [μm^2] | 0.086 (0.057-0.132) | 0.087 (0.057-0.129) | 0.812 ^{rs} |
| Brp locs. per AZ | 1305 (844-2144) | 1270 (852-2066) | 0.632 ^{rs} |
| Brp loc. density [$10^4/\mu\text{m}^2$] | 1.508 (1.201-1.907) | 1.519 (1.218-1.843) | 0.829 ^{rs} |
| <i>n</i> (AZs, NMJs, animals) | 591, 15, 4 | 712, 15, 4 | |

Numerical values not stated in text or figure legends including p-values and sample sizes for measurements with motoneuron-specific expression of *RIM^{wt}* (*ok6-GAL4 > UAS-RIM^{wt}*) and *RIM^{R>H}* (*ok6-GAL4 > UAS-RIM^{R>H}*) shown in Figure 4. Statistical test used for comparison is indicated for each p-value with ^t = parametric t-test and ^{rs} = non-parametric rank sum test.

Supplementary Table 5 Data summary and statistical information of Ca²⁺ transients and BAPTA measurements in *RIM*^{wt} and *RIM*^{R>H}. Related to Figure 5.

| Parameter median (25 th – 75 th percentile) | <i>ok6-GAL4 > UAS-RIM</i> ^{wt} | <i>ok6-GAL4 > UAS-RIM</i> ^{R>H} | P-value |
|--|--|--|---------|
| $\Delta F/F$ | 0.462 (0.347-0.663) | 0.616 (0.346-0.685) | 0.341 |
| F baseline [AD-values] | 693.334 (416.509-1168.890) | 767.715 (407.559-1157.262) | 0.862 |
| Tau decay [ms] | 0.083 (0.078-0.089) | 0.116 (0.060-0.150) | 0.139 |
| <i>n</i> (boutons, NMJs, animals) | 28, 10, 9 | 36, 10, 7 | |
| normalized eEPSC amplitude reduction [nA] | 0.210 (0.163-0.298) | 0.380 (0.22-0.560) | 0.006 |
| <i>n</i> (boutons, NMJs, animals) | 22, 12, 6 | 21, 14, 7 | |
| normalized eEPSC PPR increase | 1.464 (1.351-1.639) | 1.188 (0.946-1.434) | 0.002 |
| <i>n</i> (boutons, NMJs, animals) | 22, 12, 6 | 21, 16, 6 | |

Numerical values not stated in text or figure legends including p-values and sample sizes for measurements with motoneuron-specific expression of *RIM*^{wt} (*ok6-GAL4 > UAS-RIM*^{wt}) and *RIM*^{R>H} (*ok6-GAL4 > UAS-RIM*^{R>H}) shown in Figure 5.

Supplementary Table 6. Data summary and statistical information of AZ morphology in RIM-CRISPR/Cas9 constructs. Related to Figure 6.

| Parameter median (25 th – 75 th percentile) | <i>rim^{rescue}/rim^{rescue}</i> | <i>rim^{R>H}/rim^{rescue}</i> | <i>rim^{R>H}/rim^{R>H}</i> |
|--|--|--|--|
| confocal data | | | |
| Brp puncta / NMJ | 536 (375-580) | 634 (507-728) | 701 (548-764) |
| p vs. <i>rim^{rescue}/rim^{rescue}</i> | | 0.075 | 0.008 |
| NMJ size [μm^2] | 570.873 (490.488-787.649) | 865.870 (668.837-937.458) | 751.125 (640.979-832.716) |
| p vs. <i>rim^{rescue}/rim^{rescue}</i> | | 0.045 | 0.999 |
| no. of boutons per NMJ | 99 (79-117) | 123 (104-136) | 148 (119-182) |
| p vs. <i>rim^{rescue}/rim^{rescue}</i> | | 0.124 | < 0.001 |
| n (NMJs, animals) | 19, 7 | 16, 7 | 18, 6 |
| dSTORM data | | | |
| dSTORM AZ area [μm^2] | 0.097 (0.072-0.144) | 0.102 (0.071-0.150) | 0.0930 (0.066-0.135) |
| p vs. <i>rim^{rescue}/rim^{rescue}</i> | | 0.990 | 0.058 |
| Brp locs. per AZ | 1755 (1148-2695) | 1824 (1156-2854) | 1613 (1049-2591) |
| p vs. <i>rim^{rescue}/rim^{rescue}</i> | | 0.990 | 0.112 |
| Brp loc. density [$10^4/\mu\text{m}^2$] | 1.709 (1.444-2.067) | 1.756 (1.459-2.101) | 1.719 (1.384-2.129) |
| p vs. <i>rim^{rescue}/rim^{rescue}</i> | | 0.990 | 0.990 |
| n (AZs, NMJs, animals) | 1088, 17, 6 | 898, 17, 6 | 855, 16, 6 |

Numerical values including p-values and sample sizes for confocal and dSTORM measurements in flies carrying *rim* alleles (*rim^{rescue}/rim^{rescue}*, *rim^{R>H}/rim^{rescue}* and *rim^{R>H}/rim^{R>H}*) generated with CRISPR/Cas9 as shown in Figure 6. Anova on Ranks was used for statistical testing.

Supplementary Table 7. Data summary and statistical information of release-ready vesicle pool estimates in flies carrying the *rim*^{R>H} CORD7 allele. Related to Figure 6.

| Parameter (mean ± SEM) | <i>rim</i> ^{rescue} / <i>rim</i> ^{rescue} | <i>rim</i> ^{R>H} / <i>rim</i> ^{rescue} | <i>rim</i> ^{R>H} / <i>rim</i> ^{R>H} |
|---|---|---|---|
| pool estimate [nA] | 174 ± 24 | 226 ± 18 | 248 ± 9 |
| p vs. <i>rim</i> ^{rescue} / <i>rim</i> ^{rescue} | | 0.119 | 0.007 |
| n (NMJs, animals) | 9, 5 | 7, 4 | 10, 4 |

Numerical values including p-values and sample sizes for pool measurements in *rim*^{rescue}/*rim*^{rescue}, *rim*^{R>H}/*rim*^{rescue} and *rim*^{R>H}/*rim*^{R>H} generated with CRISPR/Cas9 as shown in Figure 6. All displayed measurements were performed with L3 larvae carrying indicated *rim* alleles (*rim*^x/*rim*^x).