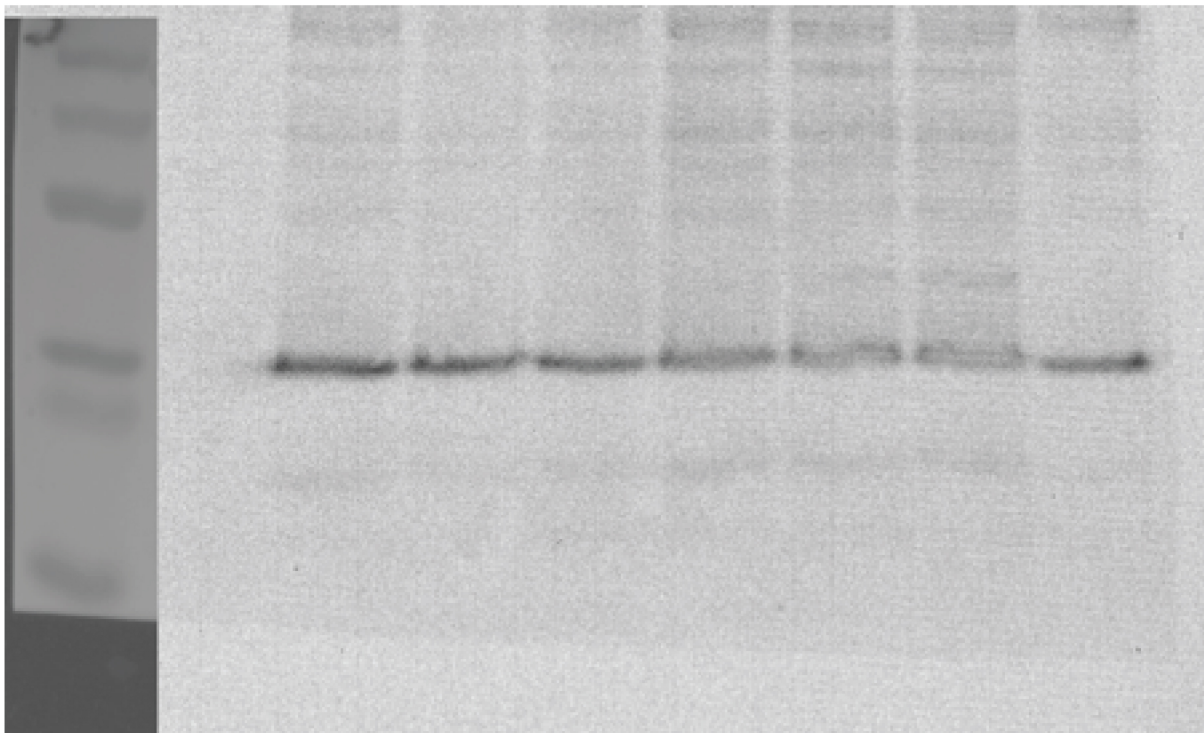
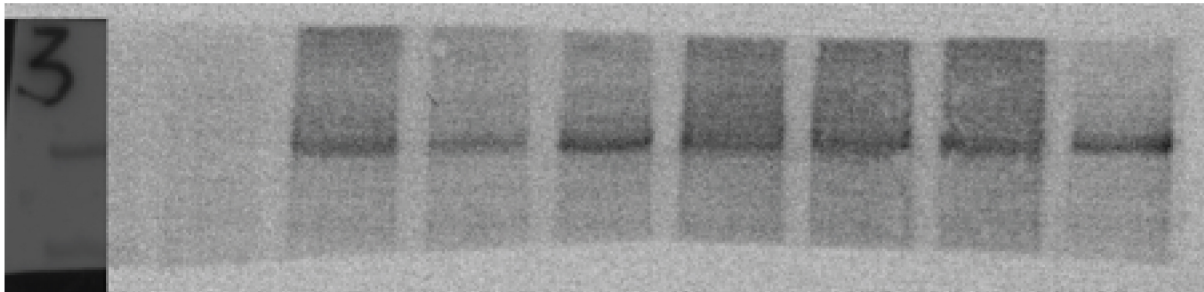


**Uncropped blots (For Extended data figure 6;
high contrast to illustrate membrane boundaries)**



Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a Confirmed
- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
 - ☒ ☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
 - ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
 - ☒ ☐ A description of all covariates tested
 - ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
 - ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
 - ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
 - ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
 - ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
 - ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection pCLAMP 10 software (Molecular Devices)

Data analysis Electrophysiological recordings were analyzed using IgorPro (v6) and AxoGraph (v X) and organized using Graph Pad Prism 9.0.0. Imaging data was analyzed by Imaris (9.10.0), and organized with the KNIME Analytics Platform 4.7.1. Statistical comparisons were performed by Graph Pad Prism 9.0.0 or R 4.4.0. For illustration, representative images were processed with the FIJI software package version 2.14.0/1.54f. MD simulations: the following software packages were used: VMD 1.93, Gromacs 2021.2, AlphaFold2 (01 JUL 21), Matplotlib 3.1.3, MDAnalysis 2.4.2.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Data availability statement: All data supporting the findings of this study are available within the paper and in Source Data Tables 1-4. MD simulation data are available in <https://zenodo.org/uploads/14554776>. All other primary data will be made available on request from the corresponding authors. Publicly available databases used in this study include GeneMatcher (<https://genematcher.org>), MetaDome web server (<https://stuart.radboundumc.nl/metadome/>), ClinVAR (<https://www.ncbi.nlm.nih.gov/clinvar>), Genome Aggregation Database (gnomAD; <http://gnomad.broadinstitute.org>), OMIM (<http://www.omim.org>), UCSC Genome Browser (<https://genome.ucsc.edu>), Ensembl (<https://www.ensembl.org>), and UniProt (<https://www.uniprot.org>).

Code availability statement: Code has not been developed in this manuscript.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender	The sex of identified patients is indicated in supplemental table 1, no further sex or gender-related analysis was made.
Reporting on race, ethnicity, or other socially relevant groupings	We do not report on the race, ethnic affiliation, or other socially relevant groupings in our patient cohort.
Population characteristics	Our study population includes patients with neurodevelopmental features, ranging in age from 11 months to 32 years, who underwent exome or genome sequencing on a clinical or research basis at academic institutes or diagnostic labs worldwide. Informed consent was obtained from parents or guardians of the affected individuals, with approval from local institutional review boards. De-identified genetic variants, clinical data, and facial photographs (shown in Figures 3-5 and Supplementary Figures 1 and 2) were obtained from collaborating institutions based on these informed consents.
Recruitment	Individuals included in this study underwent exome or genome sequencing on diverse sequencing platforms on a clinical or research basis in academic institutes or diagnostic labs worldwide. Using GeneMatcher1 and personal communication with colleagues enabled us to assemble clinical and genetic details on a total of 48 individuals with de novo or inherited heterozygous or biallelic variants in UNC13A (NM_001080421.3), including two previously published cases harboring c.154G>A, p.(E52K) [case 74 of Lionel et al., 20182], and c.4379C>T, p.(A1460V) [case UPN-0740 of 3] variants with further follow up information.
Ethics oversight	This study was performed as part of a research study approved by the ethics commission of the Canton of Zurich (ID PB_2016-02520 [SIV 11/09]). In addition, for each patient, ethical approvals and informed consent forms from parents or guardians were obtained by the respective research teams and institutions for data use and publication, including photographs or videos where applicable. Participants did not receive compensation.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size calculations for each of the recorded parameters were not performed prior to these experiments. In general, for the electrophysiological experiments presented, we were interested in detecting large and therefore likely biologically significant effects, i.e. differences in sample means of 0.8 to 1 times SD, corresponding to a 'Large effect' according to Cohen. Sample sizes (n) to resolve such effects are $\geq 17-26$ for each group for a statistical power of 0.8 and a significance level of 0.05. For all experiments, n was ≥ 19 .
Data exclusions	In electrophysiological experiments, all data which fulfilled the quality criteria (e.g. leak current etc) was included in the analysis. No 'outliers detection' or related procedures were applied.
Replication	Data was collected from two or more cultures. In the vast majority of experiments, data was independently obtained by two experimenters

Replication	(Figure 3 - in different laboratories, in Figures 4-7 by different experimenters), to ensure reproducibility of the observations. Approximately equal numbers of WT and Munc13-1-variant recordings were obtained during each measurement day.
Randomization	Randomization was not used in this study, as it is not relevant in the experiments described here.
Blinding	Blinding was not used in this study. Instead, for each of the variants, recordings were performed by two or three independent experimenters and across two or three different labs, and all phenotypes were confirmed.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
<input type="checkbox"/>	<input checked="" type="checkbox"/> Antibodies
<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input type="checkbox"/>	<input checked="" type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern
<input checked="" type="checkbox"/>	<input type="checkbox"/> Plants

Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used

Antibody; Source; Dilution used; RRID; Identifier; clone ID (when relevant)
 Ab 1. Mouse monoclonal GFP; Merck Millipore; 1:250; AB_94936; MAB3580, Clone ID: N/A
 Ab 2. Rabbit polyclonal VGLUT1; Synaptic Systems; 1:1000; AB_887877; 135 302
 Ab 3. Guinea pig polyclonal Shank 2; Synaptic Systems; 1:250; AB_2619861; 162 204
 Ab 4. Chicken polyclonal MAP2; Novus Biologicals; 1:1000; AB_2138178; NB300-213
 Secondary Abs for immunostaining
 Ab 5. Goat anti-Mouse Alexa 488; Thermo Fisher; 1:2000; AB_2534088; A11029
 Ab 6. Goat anti-Rabbit Alexa 633; Thermo Fisher; 1:2000; AB_141419; A21071
 Ab 7. Goat anti-Guinea Pig Alexa 568; Abeam; 1:2000; AB_2864763; Ab1 75714
 Ab 8. Goat anti-Chicken Alexa 405; Abeam; 1:1000; AB_2890171; Ab175674
 Primary antibodies for Western Blot analysis
 Ab 9. Polyclonal rabbit anti-FLAG; Sigma-Aldrich; 1:2000; AB_439687; F7425
 Ab 10. Monoclonal mouse anti- Green Fluorescent Protein (1E4); Enzo Life Sciences; 1:1000; ADI-SAB-500-E; Clone ID: 1E4
 Secondary antibodies for Western Blot analysis
 Ab 12. Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L), Jackson immunoResearch; 1:5000; AB_2307392; 115-035-146
 Ab 13. Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L), Jackson immunoResearch; 1:30000 AB_2307391; 111-035-144

Validation

Ab 1. Mouse monoclonal GFP; Merck Millipore; 1:250; AB_94936; MAB3580
 Recognizes a protein tag
 Website information: Antibody validated for use in ELISA, IC, IH & WB.
 In this study: immunostaining signal was absent in a non-transfected control (Figure 2c, 2e)
 Ab 2. Rabbit polyclonal VGLUT1; Synaptic Systems; 1:1000; AB_887877; 135 302
 Website information: Antibody was validated using KO samples, citations can be found in <https://sysy.com/product/135302#list>
 Ab 3. Guinea pig polyclonal Shank 2; Synaptic Systems; 1:250; AB_2619861; 162 204
 Website information: Antibody was validated using KO samples (PMID: 2997098)
 Ab 4. Chicken polyclonal MAP2; Novus Biologicals; 1:1000; AB_2138178; NB300-213
 Website information: Knockdown Validated (PMID: 32294442). More publications in https://www.novusbio.com/products/map2-antibody_nb300-213?srsltid=AfmBOorEtC2sqRWpEaMtjX36c2y8L3tkHsKuvStTIAstIKx6DkunwKBB#reviews-publications
 Ab 9. Polyclonal rabbit anti-FLAG; Sigma-Aldrich; 1:2000; AB_439687; F7425
 Recognizes a protein tag
 In this study: validated in this study by targeting a non-transfected control using western blot (Supplementary figure 4b)
 Ab 10. Monoclonal mouse anti- Green Fluorescent Protein (1E4); Enzo Life Sciences; 1:1000; ADI-SAB-500-E
 Recognizes a protein tag
 In this study: validated in this study by targeting a non-transfected control using western blot (Supplementary figure 4b)

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	HEK293T (ATCC-CRL-3216), human embryonic kidney, commercially available
Authentication	The cell line was not authenticated.
Mycoplasma contamination	Mycoplasma contamination was not detected.
Commonly misidentified lines (See ICLAC register)	Not relevant

Animals and other research organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	<p>Mice: We used mice at embryonic day 18 (E18) to prepare primary hippocampal neuronal cultures</p> <p>Strain: Unc13a/b double knock-out mice (MGI Unc13atm1Bros and Unc13btm2Bros) - made in the lab of co-authors of this study (Dr. Nils Brose). Adult mice were kept under IVC/SPF conditions, at 12h/12h light/dark cycle, at room temperature of 22 +/-2°C, and humidity levels of 55 +/-10%.</p> <p>C. elegans: Control strains used: N2, unc-13(nu641). unc-13(nu641) harbors a C-terminal mScarlet in the unc-13 locus. CRISPRmodified strains were outcrossed at least four times and the relevant genomic region was sequenced to confirm the target mutation.</p>
Wild animals	No wild animals were used
Reporting on sex	<p>Mice: Sex was not considered and both female and male embryonic mice were used to make cultures</p> <p>C. elegans: hermaphrodite worms were used</p>
Field-collected samples	The study did not involve samples collected from the field.
Ethics oversight	The use of the Unc13a/b knockout mice were approved by the responsible local government organizations in Germany (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit; 33.19-42502-04-15/1817 and 33.19-42502-04-20/3589, and Landesamt für Gesundheit und Soziales; G106/20)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	<i>Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.</i>
Novel plant genotypes	<i>Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.</i>
Authentication	<i>Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.</i>

Pathogenic UNC13A variants cause a neurodevelopmental syndrome by impairing synaptic function

Corresponding Author: Dr Noa Lipstein

Version 0:

Decision Letter:

25th Jul 2024

Dear Noa,

Your Article, "A Novel Neurodevelopmental Syndrome Caused by Pathogenic UNC13A Variants that Impair Synaptic Function" has now been seen by 3 referees. You will see from their comments below that while they find your work of interest, some important points are raised. We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

In brief, the three referees are all very supportive of this work and strike notably positive tones throughout. There are a few requests to improve a revision, but in our reading they are not asking for any major further expansion of the work and the requests seem by and large easily addressable. We therefore hope you and your co-authors will be motivated to respond to them in full.

To guide the scope of the revisions, the editors discuss the referee reports in detail within the team, including with the chief editor, with a view to identifying key priorities that should be addressed in revision and sometimes overruling referee requests that are deemed beyond the scope of the current study. We hope that you will find the prioritized set of referee points to be useful when revising your study. Please do not hesitate to get in touch if you would like to discuss these issues further.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

*1) Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available

[here](http://www.nature.com/ng/authors/article_types/index.html).

Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: <https://www.nature.com/documents/nr-reporting-summary.pdf>

It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.

A revised checklist is essential for re-review of the paper.

Please be aware of our [guidelines on](https://www.nature.com/nature-research/editorial-policies/image-integrity)

digital image standards.

Please use the link below to submit your revised manuscript and related files:

Link Redacted

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Genetics is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Michael Fletcher, PhD
Senior Editor, Nature Genetics
ORCID: 0000-0003-1589-7087

Referee expertise:

Referee #1: functional genomics; neurogenetics

Referee #2: neuronal biology, including synapses

Referee #3: ALS/FTD, clinical neurology

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The study by Asadollahi et al describes a series of genetic variants in UNC13A in 48 individuals that lead to neurodevelopmental disorders. The authors systematically characterize the consequences of these variants in an allelic series of 7 representative mutations on synapse formation and function in cultured mouse hippocampal neurons and at the nematode neuromuscular junction. The authors demonstrate that UNC13A variants affect synaptic expression levels and synaptic transmission in a variant-specific manner, with different underlying molecular mechanisms: reduction of UNC13A expression levels, gain of function in synaptic transmission, altered synaptic plasticity and protein regulation. Based on these functional analyses, the authors propose three pathogenic mechanisms and classify patients accordingly.

This study uncovers a fascinating complexity in disease mechanisms for genetic variants in this gene, with classical heterozygous loss of function mutations, both de novo and inherited, but also biallelic recessive mutations, that cause haploinsufficiency, but also gain of function phenotypes and neomorphs. The functional analyses are state of the art, using primary neurons and in vivo models (nematodes). Consequently, the precision of the functional phenotyping is exceptional with well-defined and selective effects on expression levels, synaptic transmission, -plasticity and probably transport to the synapse. The proposed discrimination of variants into three classes of mutations is very convincing. This new classification is important for future patient stratification. The manuscript is very well written. The study also delivers new information on the physiological functions of this protein (e.g. the critical role of the 'hinge' domain, with interesting new gain of function phenotype). Taken together, this study contains all the ingredients for a hallmark paper: a detailed genetic characterization, excellent functional analysis and a classification of three subtypes that will guide stratification of future cases and therapy design.

On the other hand, the translation of findings in mouse neurons to the human situation is underdeveloped, the genotype-phenotype relations can be defined more precisely, the organization of the manuscript can be improved and the Discussion is a bit limited in scope.

(1) Translation: The manuscript contains little guidance how to extrapolate findings made in prototypical mouse synapses towards the human phenotypes. Previous studies in mouse neurons show that UNC13B is redundant with UNC13A function in some neurons, but not others, giving rise to selective deficits in excitatory neurons. Hence, phenotypes observed in neurons where both UNC13A and B have been inactivated, are expected to translate in a different manner in human

neurons that do or do not express UNC13B and (often) one normal UNC13A allele. The aspect of selective deficits in excitatory neurons is probably important to better understand human symptoms (excitation/inhibition balance) and should be discussed. The known expression pattern of both genes in the human brain can help to further predict disease mechanisms at the systems level. Finally, it would be good to emphasize it is not known how phenotypes observed in double null mutant mouse neurons impact human neurons that express two healthy UNC13B and (in most cases) one healthy UNC13A allele.

(2) Genotype-phenotype relations: The first part of the Discussion (line 509-534) contains valuable leads on how different classes of mutations give rise to distinct symptoms. However, there is no clear synthesis on what are the emerging differences in the symptom spectrum among the three classes. This is a key issue that will interest the majority of the readership and should be defined in the Discussion and probably also the Abstract and end of the Introduction.

(3) Data presentation can be improved. It is hard to compare different mutants without memorizing the amino acids. Why are similar mutants kept apart in Fig 2? Even if they were tested in separate experiments and have their own control groups, they can still be together. This makes it easier to conclude how similar they behave. At the very least make the scales the same. Organization of Fig 5-6 is not great. Now Fig 5 combines a family tree of one mutation with synaptic plasticity data from three and only in Fig 6 we find the basic transmission data of the mutation of the family tree in Fig 5. Why not present these two together in Fig 5 and move synaptic plasticity data from three mutations to Fig 6? It would be great to add some summary data at the end of the manuscript, bringing (normalized?) key data of all mutations studied together so the readership can evaluate the defining differences in synaptic parameters in a single figure/panel.

(4) The discussion is currently oriented towards molecular mechanisms and delivers relatively little scope (except the comparison between effects of UNC13A mutations early and later in life, which is great but also long). It would be great to touch upon a few more general topics, e.g. (i) genotype-phenotype relations (see above), (ii) is the classification into three groups definitive or are more classes predicted to emerge based on what we know about UNC13A function and the fact that currently known mutations are both in tolerant and intolerant regions? (iii) a comparison to developmental disorders caused by mutations in genes that operate in the same molecular machine as UNC13A and their fascinating genetic complexity.

Reviewer #2:

Remarks to the Author:

The (many) authors of an international collaboration went through a concerted effort which resulted in describing a novel neurodevelopmental syndrome(s) linked to germline coding or splice site variants in the UNC13A gene, which can follow autosomal dominant (de novo or inherited heterozygous pathogenic variants) or autosomal recessive (biallelic pathogenic variants) inheritance patterns. The syndrome presents with a spectrum of symptoms, including developmental delay or intellectual disability, epilepsy, tremor, dyskinetic movements, and, in severe cases, early childhood mortality.

They use a broad array of assays to evaluate UNC13A protein stability and abundance at synapses, the strength and plasticity of neurotransmitter release using electrophysiological recordings in mouse hippocampal excitatory neurons, movement in *C. elegans* knock-in worms, synaptic responses to second messenger signaling, and structural changes through molecular dynamic modeling. Thus, they describe three distinct pathogenic mechanisms:

- Reduction in synaptic strength due to decreased UNC13A protein levels
- Increased neurotransmission resulting from a gain of function in UNC13A
- Impaired synaptic response to second messenger signaling

Based on this analysis (genotype, phenotype, physiological phenotype), they further classify UNC13A syndrome subtypes.

I am in the rare situation of having little to nothing to criticize here. The identified mutations significantly advance our understanding of UNC13A biology in humans and might potentially lay a foundation for developing therapeutic interventions. Clearly, this material apart from its clinical implications will also be of importance concerning our mechanistic understanding of Unc13 function in a generic manner. This also given that a bulk of the physiological information is retrieved from autaptic recordings, and much remains to be learned concerning the truly in vivo impact of these mutations (e.g. in the transmission across frequency space). Equally, it will (in the future) be important to address at which synapses these mutations ultimately execute their pathologically relevant dysfunctions. Still, given the major progress this paper marks, I do unreservedly suggest publication of the manuscript in its current form.

Reviewer #3:

Remarks to the Author:

This is very interesting study showing that pathogenic variants in UNC13A are (1) either biallelic or monoallelic with de novo or familial inheritance and (2) that pathogenic variants affect different UNC13A protein domains that participate in defining different properties of synaptic transmission, with evidence for at least three independent mechanisms: loss of function, gain-of-function (increased synaptic transmission) and dysregulation of transmission.

In OMIM two early onset syndromes are linked to UNC13A variants, albeit not uncontroversial: a congenital myasthenic syndrome and a dyskinetic movement disorder associated with delayed development and behavioral abnormalities. Could the authors discuss how their reported syndromes relate to these prior reported syndromes, and if not, how we should interpret these prior reports?

The authors frequently refer to the recent ALS/FTD literature on UNC13A cryptic exon splicing induced by TDP-43 mislocalisation. Indeed, the question is, if this study helps to understand the mechanisms at play in ALS/FTD, since this has not yet been resolved. The cryptic exon is located between exon 20 and 21, so sort of right in the "UNC13A Hinge" as the authors call it. Still, we "think" the effect in ALS/FTD is "loss of function" as UNC13A is misspliced and the aberrant mRNA species are degraded by non-sense mediated decay. Nevertheless, we know that in ALS/FTD glutamate excitotoxicity is one of many pathogenic mechanisms, so I wondered if the authors could reflect on which of their presented mechanisms could be at play in ALS/FTD? It is very intriguing to see that coding mutations in UNC13A could lead to a toxic gain of increased synaptic transmission, but how can one this reconcile with the presumed loss of function following missplicing of UNC13A? It would be great if the authors could reflect on this, and dedicate a paragraph to this.

I have one methodological question: how exactly did the authors quantify synapse numbers and in which (non)human systems? This is a bit unclear from the methods section. Is it done using antibodies against SHANK2 and VGLUT1? In *C. elegans*?

Textual:

The introduction has a strange start with "In a ground-breaking screen for genes controlling *C. elegans* behaviour¹, uncoordinated (UNC) strain number 13 (unc-13) was scored as severely affected." I would start with a combination of the second sentence, e.g. In a groundbreaking.... worms with a"

Version 1:

Decision Letter:

Our ref: NG-A65794R

6th Dec 2024

Dear Noa,

Thank you for submitting your revised manuscript "A Novel Neurodevelopmental Syndrome Caused by Pathogenic UNC13A Variants that Impair Synaptic Function" (NG-A65794R). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Genetics, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements soon. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Genetics Please do not hesitate to contact me if you have any questions.

Sincerely,

Michael Fletcher, PhD
Senior Editor, Nature Genetics
ORCID: 0000-0003-1589-7087

Reviewer #1 (Remarks to the Author):

the authors provide adequate responses to all my prior concerns and made adequate adjustment to the manuscript. The authors deserve a big complement on this excellent study

Reviewer #2 (Remarks to the Author):

I am impressed by the revision and suggest publication unreservedly.

Reviewer #3 (Remarks to the Author):

The authors have addressed all reviewers comments more than adequately, I appreciate the additions in the Discussion further placing the findings into context.
I only noticed on typo: Extended data Figure 8: the table says "Inheritance", needs to be "Inheritance". Very well done all in all.

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hippocampal inhibitory neurons, according to the Allen Brain Atlas Transcriptomics explorer (Human M1 10x), UNC13B transcript expression levels appear lower in inhibitory neurons than in excitatory neurons. Based on this, we decided to avoid making additional predictions beyond those listed above. To date, data on the degree of UNC13B protein expression in neuronal subtypes of the mouse or human brain is largely missing. Expression of mRNA, while likely indicative of protein expression, may still result in partial expression of UNC13B at the synapse level, as in the case of cultured hippocampal excitatory neurons. We believe that independent studies on the expression of UNC13B will be needed to provide more accurate predictions.

(2) Genotype-phenotype relations: The first part of the Discussion (line 509-534) contains valuable leads on how different classes of mutations give rise to distinct symptoms. However, there is no clear synthesis on what are the emerging differences in the symptom spectrum among the three classes. This is a key issue that will interest the majority of the readership and should be defined in the Discussion and probably also the Abstract and end of the Introduction.

We would like to thank the reviewer for pointing this out. In the introduction and in the respective parts of the discussion section, we now define hallmark clinical features for each of the syndrome subtypes:

- Myasthenic presentation as characteristics of the Type A condition
- Ataxia, tremor, or a dyskinetic movement disorder as a hallmark of the Type B condition
- Mild symptoms and heritability in the Type C condition
- We also note differences in the degree to which epileptic seizures can be controlled by medication in all three conditions.

In addition to the respective text passages in the discussion and introduction sections (see below), we also included a new summary figure (Extended Figure 8), which provides a comparative overview of the clinical, genetic, and experimental findings in our study for the three conditions.

Introduction (Lines 93-102): Patients with the Type A condition present with profound global developmental delay and early-onset seizures. These patients harbour biallelic loss-of-function missense, truncating or splice site variants, that lead to a >50% reduction of UNC13A expression in experimental models, and to a severe reduction in neurotransmission. Patients with the Type B condition exhibit developmental delays, particularly in speech acquisition, and ataxia, tremor, or a dyskinetic movement disorder as hallmarks of the condition. These patients harbour de novo missense variants that result in a gain of UNC13A function, leading to enhanced neurotransmission. The Type C condition is caused by a familial heterozygous missense variant that results in altered regulation of UNC13A function. The patients are mildly affected, exhibiting learning difficulties to moderate ID and seizures.

Discussion (Lines 379-387): These variants severely affect developmental milestones leading to profound GDD in all cases, and in some cases cause death in early childhood due to respiratory failure after pneumonia (Fig. 1, Supplementary table 1). In addition, all patients develop early-onset seizures that mostly responds to antiepileptic treatment (Supplementary table 1). Based on a previously reported case¹⁰ harbouring a homozygous stop codon variant, who was diagnosed with fatal myasthenia according to electromyography findings, and on our patients 3 and 7 (Supplementary table 1, Extended data figure 2 and 3), we suggest considering myasthenic presentation in Type A patients. To clarify whether this is a typical presentation requires further investigations.

(3) Data presentation can be improved. It is hard to compare different mutants without memorizing the amino acids. Why are similar mutants kept apart in Fig 2? Even if they were tested in separate experiments and have their own control groups, they can still be together. This makes it easier to conclude how similar they behave. At the very least make the scales the same.

To facilitate data comparison, we now

- included a new summary figure (Extended Figure 8), where the data obtained in this study are presented as summary bar graphs, trusting that this enables a quick comparison of conditions as well as an overall evaluation of the range of changes per condition. In the new Extended

Figure 8, we normalize data for each condition to the respective control measurements, summarizing magnitude and direction of change for comparison.

- re-scaled the majority of graphs in the manuscript to have an identical scale.
- reorganized Figure 2 to enable a better comparison between conditions.
- combined figures 5 and 6 into a single figure (see below).

We trust that these changes substantially improved the data presentation in the manuscript and thank the reviewer again for this comment.

Organization of Fig 5-6 is not great. Now Fig 5 combines a family tree of one mutation with synaptic plasticity data from three and only in Fig 6 we find the basic transmission data of the mutation of the family tree in Fig 5. Why not present these two together in Fig 5 and move synaptic plasticity data from three mutations to Fig 6?

We followed the reviewer's suggestion and re-organized the figure. We present all relevant data for the C587F mutation in one figure (Figure 5). Because this variant interferes with DAG/PDBu sensitivity, we also include in this figure additional data for the DAG/PDBu sensitivity of additional variants examined in this study, and make sure the data are presented in a way that enables visual comparison. All synaptic short-term plasticity data are now included in Figure 6. We hope this will increase the readability of the manuscript and improve the flow of the data presentation.

It would be great to add some summary data at the end of the manuscript, bringing (normalized?) key data of all mutations studied together so the readership can evaluate the defining differences in synaptic parameters in a single figure/panel.

We followed the reviewer's proposal and prepared Extended Data Figure 8. In this figure, we provide (i) an illustration of the main active zone proteins that have been linked to the group of SNAREopathies (Extended Data Figure 8a), (ii) a plot depicting the spectrum of pathogenic variants, patterns of inheritance and associated phenotypes reported for genes encoding neuronal SNAREs and associated AZ proteins, with frequency of diverse disease-causing variants and their patterns of inheritance (Extended Data Figure 8b), (iii) a short summary of the key genetic and clinical findings for each disease subtype (Extended Data Figure 8c), and (iv) a histogram plotting summarized key data for each tested variant (Extended Data Figure 8d). We trust that this figure provides an easily accessible overview about the UNC13A condition to the broad readership, and a guide for the experimental neuroscientists as well as geneticists, that can facilitate the accurate classification of newly-identified variants.

(4) The discussion is currently oriented towards molecular mechanisms and delivers relatively little scope (except the comparison between effects of UNC13A mutations early and later in life, which is great but also long). It would be great to touch upon a few more general topics, e.g. (i) genotype-phenotype relations (see above), (ii) is the classification into three groups definitive or are more classes predicted to emerge based on what we know about UNC13A function and the fact that currently known mutations are both in tolerant and intolerant regions? (iii) a comparison to developmental disorders caused by mutations in genes that operate in the same molecular machine as UNC13A and their fascinating genetic complexity.

We re-wrote large parts of the discussion to address the reviewer's criticism, acknowledging that the detailed description of the synapse physiology can be shortened. The discussion now includes (according to this reviewer's suggestions):

- references to genotype-phenotype relations, where we emphasize the hallmark presentation for each disease subtype (Lines 376-414), and
- a paragraph on additional disease subtypes we still anticipate to identify based on the known Munc13 biology (Lines 415-426): **We anticipate that additional UNC13A pathogenic variants and disease subtypes will emerge. In particular, loss of function without loss of protein is expected for variants that interfere with UNC13A function in SNARE complex assembly (e.g. the MUN or C2C domain), which may lead to a Type A-like condition. Based on AlphaMissense**

scores and the rareness of variants in the UNC13A hinge region (frequency in gnomADv4.1 database), almost all possible hinge amino acid exchanges (42/45=93%) show pathogenic predictions, and we anticipate additional pathogenic hinge variants to be identified as causing the Type B condition. Moreover, UNC13A hyperfunction has been observed in several structure-function studies¹¹⁻¹³, and variants in these regions may also result in a Type B condition. Finally, missense or in-frame variants in other critical domains involved in UNC13A regulation, including in the Ca²⁺-calmodulin binding sequence (aa 446-466)¹⁴ and in the Ca²⁺-phospholipid binding residues of the C2B domain¹⁵ may cause a Type C condition.

In addition, the new Extended Data Figure 8 includes

- (iii) a plot depicting the Spectrum of pathogenic variants, patterns of inheritance and associated phenotypes reported for genes encoding neuronal SNAREs and associated AZ proteins, with frequency of diverse disease-causing variants and their patterns of inheritance (Extended Data Figure 8b).

We thank this reviewer again for the constructive criticism and insightful comments, which we believe, we were able to fully address.

Reviewer #2:

Remarks to the Author:

The (many) authors of an international collaboration went through a concerted effort which resulted in describing a novel neurodevelopmental syndrome(s) linked to germline coding or splice site variants in the UNC13A gene, which can follow autosomal dominant (de novo or inherited heterozygous pathogenic variants) or autosomal recessive (biallelic pathogenic variants) inheritance patterns. The syndrome presents with a spectrum of symptoms, including developmental delay or intellectual disability, epilepsy, tremor, dyskinetic movements, and, in severe cases, early childhood mortality.

They use a broad array of assays to evaluate UNC13A protein stability and abundance at synapses, the strength and plasticity of neurotransmitter release using electrophysiological recordings in mouse hippocampal excitatory neurons, movement in *C. elegans* knock-in worms, synaptic responses to second messenger signaling, and structural changes through molecular dynamic modeling. Thus, they describe three distinct pathogenic mechanisms:

- Reduction in synaptic strength due to decreased UNC13A protein levels
- Increased neurotransmission resulting from a gain of function in UNC13A
- Impaired synaptic response to second messenger signaling

Based on this analysis (genotype, phenotype, physiological phenotype), they further classify UNC13A syndrome subtypes.

I am in the rare situation of having little to nothing to criticize here. The identified mutations significantly advance our understanding of UNC13A biology in humans and might potentially lay a foundation for developing therapeutic interventions. Clearly, this material apart from its clinical implications will also be of importance concerning our mechanistic understanding of Unc13 function in a generic manner. This also given that a bulk of the physiological information is retrieved from autaptic recordings, and much remains to be learned concerning the truly in vivo impact of these mutations (e.g. in the transmission across frequency space). Equally, it will (in the future) be important to address at which synapses these mutations ultimately execute their pathologically relevant dysfunctions. Still, given the major progress this paper marks, I do unreservedly suggest publication of the manuscript in its current form.

We are grateful to this reviewer for the positive evaluation of our work. This type of in-depth analysis and mechanistic investigation benefits from a great many studies in the field, which sharpened our

understanding of UNC13A function, and we are in debt to many synapse biology laboratories that paved the way for our study.

Reviewer #3:

Remarks to the Author:

This is very interesting study showing that pathogenic variants in UNC13A are (1) either biallelic or monoallelic with de novo or familial inheritance and (2) that pathogenic variants affect different UNC13A protein domains that participate in defining different properties of synaptic transmission, with evidence for at least three independent mechanisms: loss of function, gain-of-function (increased synaptic transmission) and dysregulation of transmission.

In OMIM two early onset syndromes are linked to UNC13A variants, albeit not uncontroversial: a congenital myasthenic syndrome and a dyskinetic movement disorder associated with delayed development and behavioral abnormalities. Could the authors discuss how their reported syndromes relate to these prior reported syndromes, and if not, how we should interpret these prior reports?

Indeed, few publications describing patients with UNC13A variations have been published, giving rise to the OMIM entries. A thorough and detailed examination of a patient with a premature homozygous stop codon by Engel et al.,¹⁰ led to the diagnosis of a congenital myasthenic syndrome, which implies that the disorder is at least in part of neuromuscular nature. This diagnosis is consistent with current knowledge on UNC13A expression at the neuromuscular junction¹⁶. In our Type A patients, we indeed observed symptoms that agree with the finding that complete or partial loss of UNC13A can result in expression of myasthenic symptoms. We have now added a sentence in the corresponding section of the Discussion to emphasize this connection: **Based on a previously reported case¹⁰, harbouring a homozygous stop codon variant and diagnosed with fatal myasthenia based on electromyography findings, and based on our patients 3 and 7 (Supplementary table 1, Extended data figure 2), we suggest considering myasthenic presentation in Type A patients. To what extent this is a typical presentation requires further investigations.** (lines 383-388).

The report of a single patient with a dyskinetic movement disorder, delayed development and behavioural abnormalities, refers to our previous work. In the present study, we now identified thirteen patients with variants in the same region (UNC13A hinge; aa 808-814, Type B) causing similar symptoms. This allows us to conclude that **A hallmark of the Type B condition is ataxia and tremor or dyskinetic movements (Fig. 1e and⁹)** (lines 399-400).

We accentuated this point more clearly in the Introduction and Discussion sections of the manuscript, and also illustrate this in the new overview figure (Extended Data Figure 8). Taken together, we see no contradiction between the available clinical descriptions so far and our new findings. In fact, our present study validates them in the context of a much larger cohort of patients, which enabled us to propose a sub-classification of the UNC13A syndrome.

The authors frequently refer to the recent ALS/FTD literature on UNC13A cryptic exon splicing induced by TDP-43 mislocalisation. Indeed, the question is, if this study helps to understand the mechanisms at play in ALS/FTD, since this has not yet been resolved. The cryptic exon is located between exon 20 and 21, so sort of right in the "UNC13A Hinge" as the authors call it. Still, we "think" the effect in ALS/FTD is "loss of function" as UNC13A is misspliced and the aberrant mRNA species are degraded by non-sense mediated decay. Nevertheless, we know that in ALS/FTD glutamate excitotoxicity is one of many pathogenic mechanisms, so I wondered if the authors could reflect on which of their presented mechanisms could be at play in ALS/FTD? It is very intriguing to see that coding mutations in UNC13A could lead to a toxic gain of increased synaptic transmission, but how can one this reconcile with the presumed loss of function following missplicing of UNC13A? It would be great if the authors could reflect on this, and dedicate a paragraph to this.

We believe the major findings that are relevant for a better understanding of the role of UNC13A in ALS/FTD concern the Type A condition, in which reduced UNC13A levels are observed. We report that (i) UNC13A haploinsufficiency in humans is well-tolerated. This is consistent with previous experimental findings indicating that (ii) UNC13A heterozygosity is fully-tolerated in mice, with no changes in synaptic transmission even though UNC13A expression is reduced to about 50% of WT levels³. Here, we experimentally determined (iii) a reduction of UNC13A expression to 20-30% of WT levels and aberrant patterns of synaptic transmission and short-term plasticity in cultured hippocampal neurons expressing UNC13A^{R202H} (Figs 2, 3, 5, 6), and, importantly, (iv) that UNC13A^{R202H} severely interferes with human motor function (Supplementary table 1). Taken together, these data allow us to suggest that declining UNC13A expression in neurons affected by ALS/FTD may cause aberrant patterns of synaptic transmission. Based on the above, we hypothesize that therapeutic approaches that stabilize UNC13A expression at about 50% of the respective WT levels (but not necessarily 100%) are desirable. Moreover, we also report that (v) UNC13A^{R202H}-expressing neurons maintain about 60% of the synaptic strength as compared to healthy neurons, and that (vi) even low UNC13A expression levels (20-30%) are clearly beneficial in comparison to a complete loss of expression, as patients expressing the R202H variant (and patients with (possibly leaky) splice site variants) have higher chances of survival as compared to patients carrying a homozygous premature stop codon in the *UNC13A* gene - who die in early childhood^{10,17}. We discuss these data to indicate that *any* increase in the levels of UNC13A could be advantageous to patients, with the hope that this will be constructive to any initiative aimed at developing UNC13-targeting pharmacology for ALS/FTD patients.

This reviewer correctly points out the physical proximity between the UNC13A hinge sequence (exon 20), variations in which cause a Type B UNC13A condition, and the single nucleotide polymorphisms identified to confer ALS/FTD risk (intron sequence between exons 20 and 21). We do not have a mechanistic explanation for this link, nor a hypothesis that is well-supported by current literature. It is possible that this region of the *UNC13A* gene is particularly susceptible to genetic alterations, and it would be fascinating to explore this possibility. Finally, we fully agree with this reviewer that the UNC13A gain of function may also result in excitotoxicity, a mechanism that has been extensively discussed as promoting nerve cell degeneration in ALS/FTD. However, patients carrying UNC13A hinge variations do not show signs of increased neurodegeneration up to an age of thirty years. We will continue to follow these patients as they grow older to monitor whether such a phenotype may arise. In the revised manuscript however, we refrain from making the statement that UNC13A gain of function does not result in excitotoxicity.

Lines 464-480: The characterization of UNC13A^{R202H}-expressing neurons revealed that expression levels of 20-30% of WT levels strongly impair synaptic function, particularly the pattern of plasticity and the responses to DAG/PDBu (Figs. 2, 3, 5, 6). Although we cannot exclude that R202H has additional effects on UNC13A function beyond reducing its expression levels, these data suggest that UNC13A levels below 50% profoundly change synaptic transmission and plasticity properties in hippocampal neurons in culture¹⁸, and severely interfere with human motor function, as seen in all Type A patients (Fig. 1e). This raises the possibility that such an altered neurotransmission pattern could accompany the cellular pathology in ALS/FTD as UNC13A levels decline, and potentially even exacerbate disease symptoms. However, we also note that 20-30% of UNC13A expression levels are already sufficient to support ~60% of neurotransmitter release in cultured neurons during low AP frequencies, and that patients with low expression of UNC13A have improved chances of survival as compared to patients with no functional UNC13A, who die in early childhood^{10,17}. Taken together, we propose that therapeutic strategies that stabilize even a minimal level of UNC13A expression may already be beneficial, and, because there are no indications for pathological consequences of UNC13A haploinsufficiency in humans (this study and few published cases^{17, 10}) or in heterozygous mice³, that restoration of UNC13A levels approaching 50% is a sufficient therapeutic target.

I have one methodological question: how exactly did the authors quantify synapse numbers and in which (non)human systems? This is a bit unclear from the methods section. Is it done using antibodies against SHANK2 and VGLUT1? In *C. elegans*?

Synapse numbers were determined in murine autaptic hippocampal cultures. We stained neurons with antibodies against Shank2, VGLUT1, Map2 and Munc13-1, and imaged entire neurons. Following established procedures, synapses were defined as puncta exhibiting co-localization of Shank2 and VGLUT1 immunofluorescence at a restricted distance from the MAP2 signal (Fig. 2D). We then determined the fraction of synapses showing expression of UNC13A (Fig. 2E). Next, we measured the immunofluorescence intensities of UNC13A within all puncta identified at synapses for a given neuron. For each condition, the average immunofluorescence intensity distribution is shown (Fig. 2F). We revised the main text, figure legends, and Materials and Methods section accordingly to improve clarity.

Textual:

The introduction has a strange start with "In a ground-breaking screen for genes controlling *C. elegans* behaviour¹, uncoordinated (UNC) strain number 13 (*unc-13*) was scored as severely affected." I would start with a combination of the second sentence, e.g. In a groundbreaking.... worms with a"

These sentences have now been revised and shortened to comply with manuscript length requirements.

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A Novel Neurodevelopmental Syndrome Caused by Pathogenic *UNC13A* Variants that Impair Synaptic Function (Asadollahi et al.)

We would like to thank all reviewers again for their positive assessment of our work.

Reviewer #1 (Remarks to the Author):

the authors provide adequate responses to all my prior concerns and made adequate adjustment to the manuscript. The authors deserve a big complement on this excellent study

We thank this reviewer for the support and for the complements

Reviewer #2 (Remarks to the Author):

I am impressed by the revision and suggest publication unreservedly.

We appreciate the support and time invested in assessing our manuscript.

Reviewer #3 (Remarks to the Author):

The authors have addressed all reviewers comments more than adequately, I appreciate the additions in the Discussion further placing the findings into context.

I only noticed on typo: Extended data Figure 8: the table says "Inheritance", needs to be "Inheritance". Very well done all in all.

We appreciate the attentive review and the great suggestion that drove the generation of the overview figure. The spelling mistakes in the figure have been corrected.

Pathogenic *UNC13A* variants cause a neurodevelopmental syndrome by impairing synaptic function

In the format provided by the
authors and unedited

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Supplementary Table 1: Plasmid and primer sequence information

Plasmid and primer sequence used to generate the mutation	Domain	Corresponding variation in the Human UNC13A	Figure
f(syn)wrbn- <i>Unc13a</i> ^{WT} -GFP	-	-	Figs. 2-6, ExD Figs. 4, 8
f(syn)wrbn- <i>Unc13a</i> ^{E52K} -GFP 5'-GCTGGGAGCAGGACTTCATGTTTAAGATCAACCGCC -3'	C2A	E52K	Fig. 2
f(syn)wrbn- <i>Unc13a</i> ^{R201H} -GFP 5'-CAGTGATTATCATAGTGAGACGA -3'	IDR	R202H	Figs. 2, 3, 6, ExD Fig. 8
f(syn)wrbn- <i>Unc13a</i> ^{C600F} -GFP 5'-GCATGCGCTGCACCGAGTTCGGCGTTAAG -3'	C1	C587F	Figs. 2, 5, 6
f(syn)wrbn- <i>Unc13a</i> ^{R812Q} -GFP 5'-GTCGGGCGCCATTCAGCTTCACATCAGTG -3'	C2B	R799Q	Fig. 2, ExD Fig. 4
f(syn)wrbn- <i>Unc13a</i> ^{G821D} -GFP 5'-CAGTGTGGAGATCAAAGACGAGGAGAAGGTGGCACC -3'	UNC13A hinge	G808D	Figs. 2, 4, 6
f(syn)wrbn- <i>Unc13a</i> ^{N1026S} -GFP 5'-CCTACGAGTACATCTTCAGCAACTGTCATGAGCTCTA -3'	MUN	N1013S	Fig. 2, ExD Fig. 4
f(syn)-NLS.GFP-P2A- <i>Unc13a</i> ^{WT} -Flag	-	-	Fig. 3 g-m
pEGFP-N1-NLS.GFP-P2A- <i>Unc13a</i> ^{WT} -Flag			ExD Fig. 5
pEGFP-N1-NLS.GFP-P2A- <i>Unc13a</i> ^{T22M} -Flag 5'-AAGTTCAACATGTACGTGACG-3'	C2A	T22M	ExD Fig. 5
f(syn)-NLS.GFP-P2A- <i>Unc13a</i> ^{E52K} -Flag	C2A	E52K	Fig. 3 g-m
pEGFP-N1-NLS.GFP-P2A- <i>Unc13a</i> ^{E52K} -Flag	C2A	E52K	ExD Fig. 5
pEGFP-N1-NLS.GFP-P2A- <i>Unc13a</i> ^{G183S} -Flag 5'-CTTTGGCTGGAGTGAACAGAATG -3'	IDR	G183S	ExD Fig. 5
pEGFP-N1-NLS.GFP-P2A- <i>Unc13a</i> ^{R201H} -Flag	IDR	R202H	ExD Fig. 5
pEGFP-N1-NLS.GFP-P2A- <i>Unc13a</i> ^{E246K} -Flag 5'-CATGCACAGCTATAAAGAGTTCTCTGAG-3'	IDR	E247K	ExD Fig. 5
pEGFP-N1-NLS.GFP-P2A- <i>Unc13a</i> ^{C600F} -Flag	IDR	C587F	ExD Fig. 5

Supplementary Table 2: Antibody information

The following antibodies were used in this study:

Primary and secondary antibodies for immunostaining (Fig.2)

Antibody	Source	Dilution	RRID	Identifier
Mouse monoclonal GFP	Merck Millipore	1:250	AB_94936	MAB3580
Rabbit polyclonal VGLUT1	Synaptic Systems	1:1000	AB_887877	135 302
Guinea pig polyclonal Shank2	Synaptic Systems	1:250	AB_2619861	162 204
Chicken polyclonal MAP2	Novus Biologicals	1:1000	AB_2138178	NB300-213
Goat anti-Mouse Alexa 488	Thermo Fisher	1:2000	AB_2534088	A11029
Goat anti-Rabbit Alexa 633	Thermo Fisher	1:2000	AB_141419	A21071
Goat anti-Guinea Pig Alexa 568	Abcam	1:2000	AB_2864763	Ab175714
Goat anti-Chicken Alexa 405	Abcam	1:1000	AB_2890171	Ab175674

Primary and secondary antibodies for Western Blot analysis (Extended Fig. 5):

Antibody	Source	Dilution	RRID	Identifier
Polyclonal rabbit anti-FLAG	Sigma-Aldrich	1:2000	AB_439687	F7425
Monoclonal mouse anti- Green Fluorescent Protein (1E4)	Enzo Life Sciences	1:1000		ADI-SAB-500-E
Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson immunoResearch	1:5000	AB_2307392	115-035-146
Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)	Jackson immunoResearch	1:30000	AB_2307391	111-035-144

Supplementary Note 1: Sequence information for *C. elegans* strains

Sequence information (Bold= mutation; Underline = RE)

Strain	<i>unc-13</i> allele	Description	Human UNC13A	<i>C. elegans</i> UNC-13
JSD1433	<i>nu641</i>	C-terminal mScarlet	-	-
JSD1381	<i>tau45</i>	Hinge proline	P814L	P956L
JSD1422	<i>tau48 nu641</i>	Hinge glycine	G808C	G950C
JSD1440	<i>tau64 nu641</i>	C1 domain cysteine	C587F	C729F

unc-13(tau45)

crRNA: CATGTATACTGAACATGATA

AAATTGACTCGTGAATCTGATGATTTCTTGGGACAAACAGTAATTGAAGTTCGAACTTTA
TCTGGTGAAATGGATGTCTGGTATAATCTTGAAAAGAGAACTGATAAATCTGCTGTATCC
GGAGCAATTCGATTGCATATCAATGTTGAAATCAAGGGAGAAGAGAAGCTAGCATTGTA
TCATGTTCAGTATACATGTCTTCATGAACATCTTTTTGCTGCTCATTGTGTAGA

unc-13(tau48 nu641)

crRNA: GCATATCAATGTTGAAATCA

AAATCTGCTGTATCCGGAGCAATTCGATTGCATATCAATGTTGAAATCAAGT**GC**GAAGA
GAAGCTTGCACCGTATCATGTTCAGTATACATGTCTTCATGAACATCTTTTTGCTGCTCA
T

unc-13(tau64 nu641)

Two crRNAs used:

crRNA 1: CGAAGGATTATTGTGGGGAT

crRNA 2: TCCATGTTTCGTCGACTTCT

TTCCAAACACCCACGTTTTGTTACGAATGCGAAGGATTATTGTGGGGATTaGCTAGACA
AGGATTGCGATGTACTCAGT**t**TCAAGTGAAAGTTCACGATAAATGTCGTGAATTGCTCAG
CGCTGATTGTCTgC**Ag**AGAGCAGCtGAGAAGTCGACGAAACATGGAGAAGCTGATAGAA
CACAGTCACTTGTC

Co-CRISPR, *unc-58 (L428F)*

crRNA: ATCCACGCACATGGTCACTA

ATAGCCGAGTTAGGAAACAAATTTTTCTTTCAGGTT**TTCT**CAGTAGTGACCATGTGCGTG
GATCTTGCGTCCACACA

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