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Peer Review File

Sex-specific perturbations of neuronal development caused by mutations in the autism risk gene DDX3X

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the manuscript “Sex-specific perturbations of neuronal development caused by mutations in the autism risk gene DDX3X”, Mossa and colleagues assess the sex-dependent effects of mutations in DDX3X, an X-linked gene that encodes an RNA helicase using mouse models of DDX3X haploinsufficiency and null mutants. Ddx3x deficiency was shown to reduce dendritic complexity with a gene dosage effect, and Ddx3y is unable to fully compensate for Ddx3x in neuronal morphogenesis. Quantitative proteomics indicates that generally, the sex-specific Ddx3x signatures in females are specifically relevant to changes in ribosomal proteins and translational regulators. Dendritic spine density is increased in null female Ddx3x mutants, and neck length and head width of spines are increased. An Emx1-Cre mouse model demonstrates the expected growth delay, with significant motor function deficits. The results are interesting, and the findings are significant for the field. Overall, the scientific design is sound, though the presentation of the data needs to be edited to more clearly convey the significance of the results.

1. In the text of the Results, the quantitative findings are generally not presented; wherever applicable, the mean ± SEM should be stated. The captions indicate that the n is in the legend, but the means/SEMs are not clearly listed in the legends either.

2. In addition to the number of dendrites, was the length of these dendrites significantly different between genotypes?

3. Is there any quantification of the normalized DDX3X immunofluorescence (Fig. 1) between conditions?

4. Were there any changes in nucleoli of neurons that, unlike CTIP2+ neurons, were not altered in the Sox2 mice?

5. The “statistical analysis” section of the Methods does not mention the Kruskal-Wallis rank sum test, which is used repeatedly throughout the manuscript.

6. The jump from dendrites to ribosomal proteins to dendritic spines is a bit jarring; it seems as if it would flow better to discuss the dendritic spines when discussing the dendritic complexity before presenting the ribosomal protein data.

7. Did the spine density differ between primary, secondary, or tertiary dendrites?

8. Further description of the “distinguishable patterns” (line 245) would be helpful, as the figure alone does not clearly convey that.

9. More explanation of the significance of the alterations in dendritic spine morphology would strengthen this section of the manuscript.

10. The value of the empirical distribution frequency figures for spine neck length and head width (Figs. 3H and 3I) is unclear, especially since Figures 3F and 3G convey the data clearly. A histogram may be more effective.

11. In the Emx1 line, were there any differences in the latency to cross the same length of the balance beam?

Reviewer #2

(Remarks to the Author)
Mossa et al use elegant mouse genetics to define the role of Ddx3x in neuronal development and identify sex-specific differences and dosage dependent differences with respect to neuronal arborization, they identify a molecular signature that is distinct between males and females in relation to the control of translation. The data is presented in a clear and logical manner and the statistical analysis are conducted with appropriate tests and the experiments were well powered. Their findings represent an important advancement and a novel advancement in the field of neuroscience and provide mechanistic understanding of pathways that could underlie the sex-bias of several neuropsychiatric and neurodevelopmental disorders.
However, there are some points that warrant additional explanations.

1- Because of the strong translation signature, this warrants a bit further interrogation in terms of what is happening in the neurons. For example, using a puromycilation assay to measure translation rates would add to the work that is already presented in this paper.

2- The nucleoli and additional protein analysis was done on Sox2 mutant mice did they also look at whether the SOX2 mice have arborization defects as in their primary cultures in figure 1? This would provide a more direct correlation between defects in neuronal arborization and the nucleoli defects they observed.

3- Did they quantify the mean length? From the representative neurons they seem a lot smaller but only branching measurements are shown on figure 1

4- For figure 2 they use the same labelling method as figure 1 where they sparsely label the neurons so did they FACS sorted the cells? or how did they enrich for the specific genotypes? This just need clarification in the text or figure legends

5- Could the authors comment on whether there are shared signatures between the male and female mutants that are differentially expressed could they add this to the supplementary figures

6- In Figure 2I to 2L - DDX3 is driving the sex-specific differences in nucleoli in terms of volume as well as nuclear volume, and then as proportion of nuclei but not the numbers? with larger nuclear size and nucleoli volume in females compared to males but once mutated in females it goes back to levels similar to the control males, this is not clear should the proportion not be a representation of the number of nucleoli or how is this number being calculated, this should be explained because it contradicts the no changes in nucleoli number

7- In supplementary figure 1D there are a lot more significant proteins that are from the DDG2P gene list for the SOX2 samples as opposed to the samples from the Ddx3x het vs wild type can they explain or discuss the potential reason for this? Are the cell types they are analyzing different?

8- Differences between nucleoli number and nucleolar volume in supplementary and in main figure there is a discrepancy depending on the model used in particular in nucleoli number could the authors clarified why this is the case?

9- Not clear the connection between nuclear size and nucleoli size with sex specific differences, there are clear differences what is driving the molecular differences? Could they add more of a discussion on potential mechanisms they are proposing that are driving the sex specific differences.

Reviewer #3

(Remarks to the Author)
This paper by Mossa et al. presents an interesting story that intends to show that DDX3X mediates sexual dimorphisms in brain development at a molecular, cellular, and behavioral level. Overall, this work provides important mouse models, which is very exciting. One key finding is that DDX3X regulates dendritic outgrowth in a sex- and dose-dependent manner in both female and male neurons, while dendritic spine development is influenced only in female neurons. However, it should be noted that the study is mainly descriptive and does not provide new insights into the underlying molecular mechanisms. Additionally, some parts of the proposed mechanism need to be improved to avoid misleading. The manuscript also requires extensive proofreading to address issues such as using italics or bold formatting properly and correcting spelling errors.

Figure 1 shows significant differences in arborization, particularly in the secondary and tertiary dendrites of male and female neurons. However, several concerns need to be addressed. First, the transfection rate of the mCherry reporter is extremely low, which could significantly impact the results. Additionally, there are substantial variations within the same group (Fig. 1D-1G). Can the authors provide an explanation for this? Lastly, the entire figure requires proofreading to correct issues such as the notation (e.g., Ddx3x+/+).

The authors assert that DDX3X mediates sex dimorphisms in neuronal ribosomal biogenesis. Using proteomics, they aim to demonstrate that DDX3X influences female-biased expression of ribosomal proteins and mRNA translation regulators. This effect is reportedly absent in male neurons when DDX3X is removed. While the effort to perform proteomics is commendable and provides a valuable resource to the field, the data do not convincingly support the conclusion that DDX3X supports

ribosome biogenesis. This claim may be an overstatement.

1. The authors only showed proteome changes, but no data on ribosome biogenesis or translation exists. Additionally, no direct link is provided between Ddx3x and the nucleolar phenotype, possibly due to any number of proteins affected by Ddx3x insufficiency. The phase separation connection is speculative, and no evidence is provided.
2. Fig. 2H-L is not convincing. FBL staining is problematic (many in the cytoplasm); it did not support the conclusion of nucleoli dynamics. There is no real connection between nucleolar size and DDX3X, just correlation.
3. It is unclear how many replicates were performed? It is surprising to see that almost no proteome changes between Ddx3x-/y and Ddx3x+/y neurons since DDX3X expression is critical for male neuron development.
4. The authors might consider comparing the proteomics data with ribo-seq data to determine whether the observed proteomic effects are secondary or if the proteins/mRNAs are directly regulated by DDX3X. Although the authors compared their data to iCLIP results, it is well-known that DDX3X interacts with the small subunit of the ribosome and binds extensively across mRNAs.

In Fig. 3B, how do the authors explain that Ddx3x+/+ exhibits stronger dendritic spines than Ddx3x+/- but weaker dendritic spines than Ddx3x-/-? This seems contradictory and suggests that DDX3X expression may not be directly relevant to female dendritic spine strength.

Minor:
Line 58 – The sentence beginning with “Over 95% of affected…” is unclear and needs to be rewritten

Line 63 – Needs to cite Shih et al. 2012 for DDX3X LLPS into SGs (PMID 21883093)

Line 67 – in utero should be italicized.

Line 77 – Also need to cite Cortez et al. 2014 (21883093)

General comment – The introduction needs copy editing

Line 100 – in vitro needs to be italicized

Line 300 – Ddx3x, not DDX3X

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have comprehensively addressed my concerns and significantly strengthened the manuscript. I have no further comments.

Reviewer #2

(Remarks to the Author)
The authors have added several key experiments to the original manuscript that further contribute to the current understanding on how sex specific differences are driven by DDX3 and make this work of high importance and novelty. Their work is highly significant to the field of neurodevelopmental disorders as mechanisms underlying sex-specific differences across different disorders are poorly understood. The data analysis and interpretation of the results is scholastically sound and rigorous. The authors have satisfactorily addressed all my previous concerns.

Reviewer #3

(Remarks to the Author)
I appreciate the significant effort and additional experiments undertaken during the revision process; however, the quality of the analyses and the interpretations remains inadequate. Many of the analyses, particularly in proteomics, nucleoli quantification, and LLPS, are overly descriptive, and certain conclusions appear to be inaccurate. While the biological questions posed are undoubtedly fascinating, the biochemical and ribosome biogenesis experiments are not executed to a sufficient standard.

A central conclusion regarding ribosome biogenesis is based on proteomics data. There are key concerns about the proteomics analysis, including a lack of clarity around data coverage and consistency. The methodology is insufficiently detailed, and the results have not been validated, which significantly undermines the reliability of the findings.

The authors state that DDX3X- and DDX3Y-specific antibodies were used. However, to my knowledge, such specificity does not exist in commercially available antibodies. No evidence supporting this claim has been provided, which raises serious concerns about the robustness of the data, particularly given the emphasis on sex-specific expression of DDX3X and DDX3Y.

Regarding ribosome biogenesis, it remains unclear which antibody was used to detect 5.8S rRNA, and the rationale for focusing on 5.8S rRNA is not adequately explained. If ribosome biogenesis is indeed a central aspect of the study, working with an RNA biologist with expertise in this area would substantially strengthen the findings.

There is also a disconnect between the proteomics data and the reported effects on ribosome biogenesis and protein synthesis. While the proteomics data suggest alterations in both small and large subunits, the decision to focus solely on 5.8S rRNA is difficult to justify. The statement, “These observations suggest that Ddx3x defines aspects of ribosomal biogenesis that depend on sex and neuronal subtype. Further, Ddx3x mutations in neurons affect ribosome production,” feels overly descriptive and premature given the limited supporting evidence.

Figure 5H raises concerns, as it lacks adequate explanation and validation.

The protein purification and LLPS assays are also problematic. The experimental approach appears inadequate, and the conclusions drawn contradict existing literature. For example, MBP cleavage is known to destabilise DDX3, and the results in Figure 6I may simply reflect protein aggregation or precipitation rather than meaningful biological properties. Moreover, critical parameters for LLPS assays, such as protein purity and RNA content, are not presented, which are essential for reliable interpretation.

I do not wish to delay the authors, but I cannot support the manuscript in its current form because the biochemistry and ribosome biogenesis work could mislead the field.

In summary, while the biological premise is compelling, the experimental design and execution, particularly in biochemistry and ribosome biogenesis, require considerable improvement to ensure the conclusions are accurate.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
I appreciate the thoroughness of the newly-inserted methods, which significantly strengthen the manuscript. However, I have concerns regarding the biochemistry presented in the study, particularly in Figure 6I and related conclusions.

The images for DDX3X at 200 ng/μL RNA and DDX3Y with no RNA appear more like aggregation than liquid-liquid phase separation. This is a major sticking point, as it calls into question the authors’ conclusion. There is a clear contrast between the rounded droplets seen in DDX3X at 0 ng/μL RNA and the jagged structures observed in DDX3Y under the same condition, as well as in the 200 ng/μL condition of DDX3X. If the authors stand by their conclusion that Figure 6I represents LLPS, they should provide additional evidence, such as fluorescence recovery after photobleaching (FRAP) to demonstrate molecular exchange within the droplets, as well as sensitivity tests using 1,6-hexanediol. The LLPS experiments appear to be based on a single replicate, as no statement regarding sample size or quantification is provided. Even if the experiment has been repeated, quantification is essential, especially when making a claim that challenges existing literature.

The authors’ conclusion that DDX3X undergoes stronger phase separation than DDX3Y contradicts existing in-cell data under physiological conditions. Moreover, sequence composition analysis of the N-terminal intrinsically disordered regions (IDRs) suggests the opposite—DDX3Y has more LLPS-promoting residues than DDX3X.

The current data in Figure 6I detracts from the manuscript. Readers may focus on the apparent aggregation of DDX3Y (and, to a lesser extent, DDX3X) rather than appreciating the important biological insights presented elsewhere. Given the strength of the biological findings, the paper would not suffer without the LLPS experiments, and their removal might allow the manuscript’s key advances to stand out more clearly.

The claim regarding rRNA biogenesis is made without performing classical biochemical assays such as northern blots, which is surprising. The current presentation of rRNA biogenesis is very descriptive.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The reviewers have addressed all the questions. I appreciate their efforts and dedication in completing the work.

Congratulations!

We thank the Reviewers for the constructive comments, which are addressed in the point-by-point response below and in the revised version of the manuscript.

Given that the new body of work is substantial, we wish to provide all Reviewers with a concise overview of new findings:

- *Ddx3x* regulates dendritogenesis *in vivo* (**new Fig. 2**).
- *Ddx3x* regulates dendritogenesis only if its ATPase activity is intact (**new Fig. 6A-C**).
- *Ddx3x* affects ribosome biogenesis (**new Fig. 5A-E**) rather than global protein synthesis (**new Fig. S5**).
- *Ddx3x* defines sexually dimorphic nucleolar properties specifically in $CTIP2^{+}$ neurons (**new Fig. 5F-J**).
- *Ddx3x* regulates nucleolar dynamics only if its catalytic activity is intact (**new Fig. 6D-H**).
- Re-expressing human DDX3X or DDX3Y alleviates dendritic deficits caused by *Ddx3x* haploinsufficiency (**new Fig. 6A-C**).
- DDX3X and DDX3Y differ in their impact on nucleolar properties (**new Fig. 6D-H**).
- DDX3X and DDX3Y have distinct features related to liquid-liquid phase separation (**new Fig. 6I**).

In addition, we also provide new ancillary analyses that are included in new Fig. 1C, Fig. 1I, Fig. S1, Fig. S2, Fig. S3, Fig. S4E, Fig. S6A, Fig. S6C, Fig. S6E, and Fig. S12B.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the manuscript “Sex-specific perturbations of neuronal development caused by mutations in the autism risk gene *DDX3X*”, Mossa and colleagues assess the sex-dependent effects of mutations in *DDX3X*, an X-linked gene that encodes an RNA helicase using mouse models of *DDX3X* haploinsufficiency and null mutants. *Ddx3x* deficiency was shown to reduce dendritic complexity with a gene dosage effect, and *Ddx3y* is unable to fully compensate for Ddx3x in neuronal morphogenesis. Quantitative proteomics indicates that generally, the sex-specific *Ddx3x* signatures in females are specifically relevant to changes in ribosomal proteins and translational regulators. Dendritic spine density is increased in null female *Ddx3x* mutants, and neck length and head width of spines are increased. An *Emx1-Cre* mouse model demonstrates the expected growth delay, with significant motor function deficits. The results are interesting, and the findings are significant for the field. Overall, the scientific design is sound, though the presentation of the data needs to be edited to more clearly convey the significance of the results.

1. In the text of the Results, the quantitative findings are generally not presented; wherever applicable, the mean ± SEM should be stated. The captions indicate that the n is in the legend, but the means/SEMs are not clearly listed in the legends either.

We have now included the mean ± SEM values in the figure legend for statistically significant differences, whenever possible. We have also demarcated a *Statistics* section in each figure legend to clearly indicate the statistical considerations for each panel.

2. In addition to the number of dendrites, was the length of these dendrites significantly different between genotypes?

We thank the Reviewer for the question, which was also raised by Reviewer #2.

We have now quantified the total length of all dendrites. In line with the other measures presented in Fig. 1E-H, we found that *Ddx3x* haploinsufficiency or loss reduces the overall length of the dendritic arbor (**new Fig. 1I**).

We also note that our new results (**new Fig. 2**) corroborate *in vivo* our findings of altered morphogenesis and changes in dendritic length.

3. Is there any quantification of the normalized DDX3X immunofluorescence (Fig. 1) between conditions?

We have now quantified the DDX3X expression, as requested (**new Fig. 1C**).

To enhance the rigor of these quantifications, we compared the DDX3X immunostaining signal of the sparsely transfected neurons ($CRE^{+}mCherry^{+}$) from *Ddx3x*$^{+/+}$, *Ddx3x*$^{flox/+}$, *Ddx3x*$^{flox/flox}$, *Ddx3x*$^{+/y}$, or *Ddx3x*$^{flox/y}$ embryos with the signal of neighboring untransfected neurons ($CRE^{-}mCherry^{-}$) within the same coverslip.

As expected, DDX3X expression in CRE^{+}mCherry^{+} *Ddx3x*$^{+/+}$ or *Ddx3x*$^{+/y}$ neurons is indistinguishable from that of their neighboring cells.

As intended, DDX3X shows protein levels indicative of monoallelic *Ddx3x* expression in CRE^{+}mCherry^{+} *Ddx3x*$^{flox/+}$ (hence *Ddx3x*$^{+/-}$) neurons. As intended, DDX3X is not detected in CRE^{+}mCherry^{+} *Ddx3x*$^{flox/flox}$ or *Ddx3x*$^{flox/y}$ neurons (hence *Ddx3x*$^{-/-}$ or *Ddx3x*$^{-/y}$).

We have also revised Fig. 1B to clearly point to the DDX3X expression in the sparse mCherry^{+} neurons.

4. Were there any changes in nucleoli of neurons that, unlike CTIP2+ neurons, were not altered in the Sox2 mice?

We thank the Reviewer for raising this interesting thought.

We have now examined nucleoli in CTIP2^{-} cells. We found that CTIP2^{-} cells display no significant sex- or genotype-driven changes (**new Fig. 5F-J**). To enhance rigor, these analyses were conducted on the same confocal images used to quantify changes in CTIP2^{+} neurons.

We have also conducted orthogonal analyses on ribosome biogenesis on both CTIP2^{+} and CTIP2^{-} cells, as suggested by the Reviewer. We replicated a specific signature in CTIP2^{+} neurons coherent with the rest of the data (**new Fig. 5A-E**).

These new observations add on new evidence that the translation rate is an inherent feature of cortical neuronal subtypes, with layer V CTIP2^{+} neurons displaying higher translation rates than other deep-layer neurons (reference #18 cited in the manuscript).

5. The “statistical analysis” section of the Methods does not mention the Kruskal-Wallis rank sum test, which is used repeatedly throughout the manuscript.

We apologize for the oversight.

We have now carefully revised and expanded the *Statistical Analyses* section.

Further, in an effort to streamline the figures, we have moved the information about statistical testing employed under each panel from the figure to the *Statistics* section at the end of each figure legend.

6. The jump from dendrites to ribosomal proteins to dendritic spines is a bit jarring; it seems as if it would flow better to discuss the dendritic spines when discussing the dendritic complexity before presenting the ribosomal protein data.

We thank the Reviewer for this helpful suggestion. The manuscript has been rearranged accordingly.

7. Did the spine density differ between primary, secondary, or tertiary dendrites?

We thank the Reviewer for this question.

As requested, we have stratified spine density based on dendrite hierarchy (**new Fig. S2A**).

We see that *Ddx3x*$^{-/-}$ neurons have significantly more spines on secondary dendrites. This is coherent with our previous data showing that spine density for *Ddx3x*$^{-/-}$ neurons begins to diverge from other genotypes at >60 μm from the soma (revised Fig. 3D) (see also next response).

We also examined spine density based on dendrite diameter (**new Fig. S2A**), and replicated the differences we see in the cumulative counts for dendrites with 0.2-0.5 μm diameter (the analyses are underpowered for dendrites with 0.5-0.8 μm diameter).

For transparency, we also provide an overview of the metrics that went into the spine analysis presented in Fig. 3, including the cumulative distribution of spines number per 10 μm segments as a function of the distance from the soma or thickness of the dendrite across all genotypes (**new Fig. S1A-B**), as well as the distribution of the 10 μm segments over the dendrite hierarchy and diameter in each genotype (**new Fig. S1C-D**).

8. Further description of the “distinguishable patterns” (line 245) would be helpful, as the figure alone does not clearly convey that.

We apologize for the lack of clarity.

In the revised Fig. 3D, we have now included analysis at 60-70 μm away from the soma, where we observe that that the increase in the number of dendritic spines in *Ddx3x*$^{-/-}$ neurons is most prominent. We have rephrased the text accordingly.

9. More explanation of the significance of the alterations in dendritic spine morphology would strengthen this section of the manuscript.

We have now included the following sentence in the section related to the dendritic spines:

“These observations suggest that the *Ddx3x* is required for both the formation and the maturation of dendritic spines: *Ddx3x* monoallelic expression is sufficient to repress excessive spines formation at an early phase and/or facilitate pruning at a later phase, but biallelic expression is required throughout spine maturation.”

10. The value of the empirical distribution frequency figures for spine neck length and head width (Figs. 3H and 3I) is unclear, especially since Figures 3F and 3G convey the data clearly. A histogram may be more effective.

We apologize for the lack of clarity.

These plots (revised Fig. 3G and 3I) are the empirical cumulative distribution function (ECDF) of the same data shown as violin plots in Fig. 3F and 3H (for neck length and head width, respectively).

The curves in the plot are interpreted as the distribution of the cumulative percentage of spines (Y axis) whose measure is lower than the empirically measured value (X axis). For example, in Fig. 3G the right shift of the curve

representing *Ddx3x*$^{+/-}$ (purple) compared to *Ddx3x*$^{+/+}$ (black) indicates that there is a larger pool of spines with longer necks, as corroborated by the violin plot in Fig. 3F.

We feel that the visualization modality and statistical approach via ECDF are necessary complements to those in the violin plots.

To ensure that these plots are readily interpreted, we have now modified the Y axis legend on the revised Fig. 3G and 3I to read “cumulative probability” and added the following sentence in the *Statistical Analyses* section of the Methods:

“Plots showing empirical cumulative distribution function (ECDF) display data points from lowest to highest (X axis) against their percentiles (Y axis).”

11. In the Emx1 line, were there any differences in the latency to cross the same length of the balance beam?

To address the Reviewer’s question, we have now examined the latency to cover 35 frames of the balance beam, as in our previous analyses (reference #19 cited in the manuscript).

We found no statistically significant differences across genotypes (**new Fig. S12B**).

Reviewer #2 (Remarks to the Author):

Mossa et al use elegant mouse genetics to define the role of Ddx3x in neuronal development and identify sex-specific differences and dosage dependent differences with respect to neuronal arborization, they identify a molecular signature that is distinct between males and females in relation to the control of translation. The data is presented in a clear and logical manner and the statistical analysis are conducted with appropriate tests and the experiments were well powered. Their findings represent an important advancement and a novel advancement in the field of neuroscience and provide mechanistic understanding of pathways that could underlie the sex-bias of several neuropsychiatric and neurodevelopmental disorders.

However, there are some points that warrant additional explanations.

1- Because of the strong translation signature, this warrants a bit further interrogation in terms of what is happening in the neurons. For example, using a puromycilation assay to measure translation rates would add to the work that is already presented in this paper.

We appreciate the Reviewer's perspective and constructive feedback. We have now expanded our initial investigation of the translational signature with two approaches.

1) First, following the Reviewer's recommendation, we performed a puromycin-based pulse-chase experiment in cultured neurons (**new Fig. S5**).

   We infected DIV0 cortical neurons from *Ddx3x*$^{+/+}$, *Ddx3x*$^{+/flox}$, or *Ddx3x*$^{+/y}$ E15 embryos with AAV8-Ef1a-mCherry-IRES-Cre viral particles. At DIV9, we performed a 10-min puromycin pulse followed by a 30-min chase. This approach was effective in detecting newly synthesized proteins, as shown by abrogated puromycin incorporation in neurons treated with the protein synthesis inhibitor cycloheximide 15 min prior to the pulse. When comparing the three genotypes, we did not detect significant differences in the puromycin labeling, suggesting that there are no overt changes in global protein synthesis detectable with this approach.

2) Second, we examined ribosome biogenesis using as a proxy the ribosomal RNA (rRNA) 5.8S in the developing motor cortex of *Sox2-Ddx3x*$^{+/+}$, *Sox2-Ddx3x*$^{+/-}$, or *Sox2-Ddx3x*$^{+/y}$ neonates (**new Fig. 5A-C**), in keeping with the design of our prior analyses of nucleolar features.

   We found that *Sox2-Ddx3x*$^{+/-}$ cortices had a reduction in both the cytoplasm-to-nuclear distribution and expression of 5.8S rRNA, supporting the ribosome biogenesis signature emerging from the proteomic analyses in Fig. 4 and the nucleolar phenotype in Fig. 5F-J.

2- The nucleoli and additional protein analysis was done on Sox2 mutant mice did they also look at whether the SOX2 mice have arborization defects as in their primary cultures in figure 1? This would provide a more direct correlation between defects in neuronal arborization and the nucleoli defects they observed.

We thank the Reviewer for this suggestion.

We now provide *ex vivo* analyses of dendritic arborization in cortical neurons from *Sox2-Ddx3x* mutant mice (**new Fig. 2**). These new findings corroborate the defects we have previously seen in cultured neurons.

For this new set of analyses, layer V pyramidal neurons were *in vivo* labeled using retrograde tracing combined with brain tissue clearing via iDISCO and light-sheet microscopy (**new Fig. 2A**). We found no overt genotype-driven changes in the overall dendritic arborization, but significantly fewer intersections at 600-700 μm from the somata in *Ddx3x*$^{+/-}$ neurons compared with control *Ddx3x*$^{+/+}$ neurons (**new Fig. 2A-B**). We also found that *Ddx3x*$^{+/-}$ neurons had longer proximal apical dendrite at the expense of the distal apical dendrite and tuft (**new Fig. 2C-D**) and simpler terminal tufts (**Fig. 2E-F**).

We note that these analyses focus on cortical neurons that project to the pontine nuclei in the brainstem to form circuits important for motor function. Therefore, we believe that these new findings offer a new insight into our earlier observations that motor deficits caused by *Ddx3x* mutations depend on the forebrain (Fig. 7).

3- Did they quantify the mean length? From the representative neurons they seem a lot smaller but only branching measurements are shown on figure 1

We thank the Reviewer for the question, which was also raised by Reviewer #1.

We have now quantified the total length of all dendrites. In line with the other measures presented in Fig. 1E-H, we found that *Ddx3x* haploinsufficiency or loss reduces the overall dendrite length (**new Fig. 1I**).

As noted in the comment above, we replicated observations of changes in dendritic length also *in vivo* (**new Fig. 2**).

4- For figure 2 they use the same labelling method as figure 1 where they sparsely label the neurons so did they FACS sorted the cells? or how did they enrich for the specific genotypes? This just need clarification in the text or figure legends.

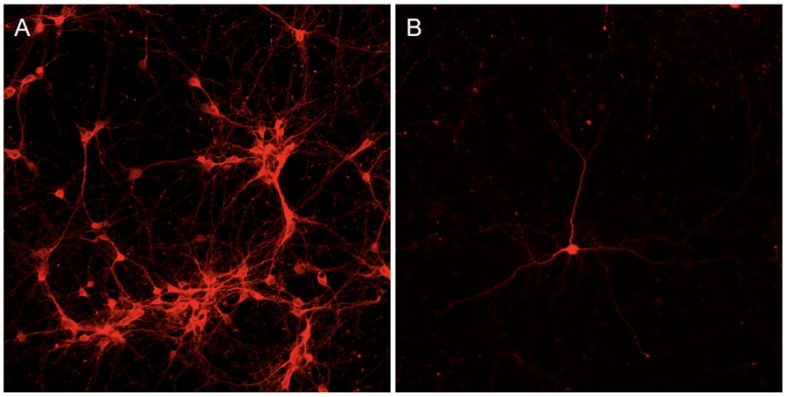
We apologize for the lack of clarity and thank the Reviewer for giving us the chance to iron this out.

As now clarified in the manuscript and outlined in the scheme in **new Fig. 4A,** we delivered the same IRES-mCherry-CRE construct but through viral transduction (as opposed to sparse DNA transfection for Fig. 1). Specifically, we infected DIV0 cortical neurons with AAV8-Ef1a-mCherry-IRES-Cre viral particles and then collected lysates at DIV9.

This strategy allows us to obtain homogenous populations. In fact, as shown in the figure provided here below for the Reviewer's consideration, DIV9 cortical neurons virally infected at DIV0 with AAV8-Ef1a-mCherry-IRES-Cre viral particles show widely distributed mCherry expression (panel A) unlike those transfected with pAAV-Ef1a-mCherry-IRES-Cre (panel B).

To further show the efficacy of the viral-mediated strategy, we have now included Western blot data showing complete loss of DDX3X in DIV9 *Ddx3x*$^{flox/flox}$ and *Ddx3x*$^{flox/y}$ cortical neurons virally transduced with AAV8-Ef1a-mCherry-IRES-Cre at DIV0 (**new Fig. S3**).

We also note that the downregulation of DDX3X also emerges from the proteomic data (Fig. 4B-D).



DIV9 cortical culture virally transduced with AAV8-Ef1a-mCherry-IRES-Cre at DIV0, as in **Fig. 4**.

DIV9 cortical culture transfected with the plasmid pAAV-Ef1a-mCherry-IRES-Cre at DIV0, as **Fig 1**.

5- Could the authors comment on whether there are shared signatures between the male and female mutants that are differentially expressed could they add this to the supplementary figures

There is no shared signature between male and female mutants, as now shown in the Venn diagram in **new Fig. S4E**. Beyond *Ddx3x* (downregulated in both datasets), there are only two shared proteins, Hip1r and Hsdl1 (Table S1).

We elaborate on this point as follows in the Discussion:

"Our data show distinct molecular signatures based on sex. *Ddx3x* loss in male neurons result in a distinct and narrowed impact on proteins directly necessary for neurite outgrowth (Fig. 4), with no noticeable nucleolar alterations (Fig. S6). *Ddx3x* haploinsufficiency in females causes a downregulation of constitutive components of the protein synthesis machinery (Fig. 4), orthogonally corroborated by a reduction in nucleoli volume and 5.8S-containing ribosomes (Fig. 5). The defects in ribosome biogenesis might in turn alter the synthesis of specific proteins required to sustain dendritic growth, yielding the reduced dendritic branching resulting from *Ddx3x* perturbations (Fig. 1-2)."

6- In Figure 2I to 2L - DDX3 is driving the sex-specific differences in nucleoli in terms of volume as well as nuclear volume, and then as proportion of nuclei but not the numbers? with larger nuclear size and nucleoli volume in females compared to males but once mutated in females it goes back to levels similar to the control males, this is not clear should the proportion not be a representation of the number of nucleoli or how is this number being calculated, this should be explained because it contradicts the no changes in nucleoli number

We regret a lack of clarity.

The plot in Fig. 5G (former Fig. 2I) represents the empirical cumulative distribution function (ECDF) of the volumes of all nucleoli quantified in each genotype. As such, the curves in the plot are interpreted as the distribution of the cumulative percentage of nucleoli (Y axis) whose volume is lower than an empirically measured value (X axis).

A right shift in the curve (e.g., black curve for the *Ddx3x*$^{+/+}$ genotype) indicates that there is a larger pool of nucleoli with large volumes. For example, for CTIP2^{+} neurons, 75% of nucleoli in *Ddx3x*$^{+/y}$ neurons (grey curve)

have a volume below ~1.49, but only 50% of the of nucleoli in *Ddx3x*$^{+/+}$ neurons (black curve) have a volume below ~1.49. Hence, the distribution of nucleoli in CTIP2^{+} *Ddx3x*$^{+/+}$ neurons is skewed towards larger nucleoli compared to all other genotypes.

These data are therefore consistent with those in Fig. 5H (former Fig. 2J), where the summed volume of all nucleoli per neuron is represented, showing on average larger nucleoli in CTIP2^{+} *Ddx3x*$^{+/+}$ neurons compared to all other genotypes.

To ensure that the ECDF plots are readily interpreted, we have now modified the Y axis legend on revised Fig. 5G to read “cumulative probability” and added the following sentence in the *Statistical Analyses* section of the Methods:

“Plots showing empirical cumulative distribution function (ECDF) display data points from lowest to highest (X axis) against their percentiles (Y axis).”

7- In supplementary figure 1D there are a lot more significant proteins that are from the DDG2P gene list for the SOX2 samples as opposed to the samples from the Ddx3x het vs wild type can they explain or discuss the potential reason for this? Are the cell types they are analyzing different?

We apologize for the lack of clarity.

In the label of revised Fig. S4D (former Fig. S1D), we have now clearly indicated that the *Sox2-Ddx3x*$^{+/-}$ dataset is from synaptosomes. The corresponding volcano plot, synaptic enrichment, and list of proteins are in Fig. 4H, 4I, and Table S2, respectively.

8- Differences between nucleoli number and nucleolar volume in supplementary and in main figure there is a discrepancy depending on the model used in particular in nucleoli number could the authors clarified why this is the case?

We agree with the Reviewer and appreciate the opportunity to explain this difference. We interpret this apparent discrepancy as follows:

“We examined nucleolar measures also in cultured cortical neurons, which are mostly expressing CTIP2 (Fig. S6A). We found that the *Ddx3x*-dependent female bias manifested in the number of nucleoli, and that *Ddx3x*$^{-/y}$ neurons did not show changes in nucleoli compared with male controls (Fig. S6). We note that although there is convergence in a *Ddx3x*-dependent female-biased nucleolar signature ex vivo (Fig. 5) and in vitro (Fig. S6), the nature of this signature differs. These differences might result from the fact that the *Ddx3x* deletion in the cultured system ensues at E15, plausibly post-mitotically, while it starts at gastrulation (thus also in the neural progenitors) in *Sox2-Ddx3x*$^{+/-}$ mice.”

In the revised Fig. S6, we have also included all four measures used for the *ex vivo* model to allow the reader to compare the two experimental systems side by side.

9- Not clear the connection between nuclear size and nucleoli size with sex specific differences, there are clear differences what is driving the molecular differences? Could they add more of a discussion on potential mechanisms they are proposing that are driving the sex specific differences.

We thank the Reviewer for raising this interesting point, also considering new data we have collected (**new Fig. 6H, new Fig. S6E**).

We see changes in nuclear volume only in *ex vivo* cortices from *Sox2-Ddx3x*$^{+/-}$ mice, and therein only in CTIP2^{+} neurons (new subpanel in revised Fig. 5I). We do not see changes in nuclear volume in *Ddx3x*$^{+/-}$ cultured neurons (**new Fig. S6E**) or *Ddx3x*$^{+/-}$ cultured neurons re-expressing human DDX3X (**new Fig. 6H**), in spites of changes in nucleoli. We interpret these data as a lack of causal relationship between nuclear and nucleolar phenotypes.

Given that the nuclear phenotype is seen only in *Sox2-Ddx3x*$^{+/-}$ mice (where *Ddx3x* is mutated since gastrulation and thus also in neural progenitors) and not when manipulations incur post-mitotically, the nuclear phenotype might be secondary to the neurogenesis defects that have been documented as a consequence of *Ddx3x* loss (references #3 and #12 cited in the manuscript).

Reviewer #3 (Remarks to the Author):

This paper by Mossa et al. presents an interesting story that intends to show that DDX3X mediates sexual dimorphisms in brain development at a molecular, cellular, and behavioral level. Overall, this work provides important mouse models, which is very exciting. One key finding is that DDX3X regulates dendritic outgrowth in a sex- and dose-dependent manner in both female and male neurons, while dendritic spine development is influenced only in female neurons. However, it should be noted that the study is mainly descriptive and does not provide new insights into the underlying molecular mechanisms. Additionally, some parts of the proposed mechanism need to be improved to avoid misleading. The manuscript also requires extensive proofreading to address issues such as using italics or bold formatting properly and correcting spelling errors.

We appreciate the Reviewer's perspective about the need of mechanistic insights. We now provide additional data that we believe add on our mechanistic understanding of DDX3X. Specifically, we found that:

- the ATPase activity of DDX3X is required for regulating both neuronal morphogenesis and nucleolar properties (**new Fig. 6**).
- The sex differences and *Ddx3x* translational signature are not impacting global protein synthesis rates (**new Fig. S5**) but rather ribosome genesis and maturation (**new Fig. 5A-E**).
- Human DDX3X and DDX3Y partially differ in their functions (**new Fig. 6**).
- *Ddx3x* haploinsufficiency causes defective dendritic arborization in cortical neurons that project to the brainstem pontine nuclei to form circuits important for motor function (**new Fig. 2**), offering new insights into our observation that motor deficits depend on the forebrain (Fig. 7).

Figure 1 shows significant differences in arborization, particularly in the secondary and tertiary dendrites of male and female neurons. However, several concerns need to be addressed. First, the transfection rate of the mCherry reporter is extremely low, which could significantly impact the results.

We regret a lack of clarity.

The extremely low transfection rate is intentional (see scheme in Fig. 1A): by manipulating *Ddx3x* only in a few neurons in the dish, we can assess the cell-autonomous functions of *Ddx3x*.

To further clarify this, we now provide a quantification of the DDX3X expression in the sparsely transfected neurons compared to neighboring neurons (**new Fig. S1C**). Specifically, we compared the DDX3X immunostaining signal of the sparsely transfected neurons ($CRE^+mCherry^+$) from $Ddx3x^{+/+}$, $Ddx3x^{flox/+}$, $Ddx3x^{flox/flox}$, $Ddx3x^{+/y}$, or $Ddx3x^{flox/y}$ embryos with the signal in neighboring untransfected neurons ($CRE^-mCherry^-$) within the same coverslip.

As expected, DDX3X expression in $CRE^+mCherry^+$ $Ddx3x^{+/+}$ or $Ddx3x^{+/y}$ neurons is indistinguishable from that of neighboring cells. As intended, DDX3X shows protein levels indicative of monoallelic *Ddx3x* expression in $CRE^+mCherry^+$ $Ddx3x^{flox/+}$ (hence $Ddx3x^{+/-}$) neurons. As intended, DDX3X is not detected in $CRE^+mCherry^+$ $Ddx3x^{flox/flox}$ or $Ddx3x^{flox/y}$ neurons (hence $Ddx3x^{-/-}$ or $Ddx3x^{-/y}$).

We have also revised Fig. 1B to clearly show DDX3X expression in the sparse mCherry^{+} neurons.

Further, we now provide orthogonal *in vivo* evidence of defective dendritic development in *Ddx3x* haploinsufficient mice (**new Fig. 2**).

We also note that the sparse transfection approach was only employed for cellular analyses (Fig. 1, Fig. 3). For the molecular analyses in Fig. 4, we delivered the same IRES-mCherry-CRE construct but through viral transduction, to obtain homogenous populations, as now clarified in the scheme in **new Fig. 4A**.

Additionally, there are substantial variations within the same group (Fig. 1D-1G). Can the authors provide an explanation for this?

In the previous version of the manuscript, the lower limit of the Y axis had not been set to 0, hence the apparently larger spread of the data. We apologize for this oversight. We have now made sure that all plots have the lower limit of the Y axis set to 0.

We hope that the Reviewer will appreciate that the distribution of the data in the revised panels 1E-H (former 1D-1G) is more compact.

We also note that most studies display aggregated data, without showing individual data points. When considering the mean ± SEM, the distribution of our data is comparable to that reported in other studies showing aggregated data (e.g., PMID: 22445341; 33727673; 21745642).

Lastly, the entire figure requires proofreading to correct issues such as the notation (e.g., Ddx3x+/+).

We apologize for the oversight. All figures have been proofread.

The authors assert that DDX3X mediates sex dimorphisms in neuronal ribosomal biogenesis. Using proteomics, they aim to demonstrate that DDX3X influences female-biased expression of ribosomal proteins and mRNA translation regulators. This effect is reportedly absent in male neurons when DDX3X is removed. While the effort to perform proteomics is commendable and provides a valuable resource to the field, the data do not convincingly support the conclusion that DDX3X supports ribosome biogenesis. This claim may be an overstatement.

1. The authors only showed proteome changes, but no data on ribosome biogenesis or translation exists.

We thank the Reviewer for raising this important point. We now provide two additional pieces of evidence that add on translation (**Fig. S5**) and ribosome biogenesis (**new Fig. 5A-C**), as requested.

1) We performed a puromycin-based pulse-chase experiment in culture (**new Fig. S5**).

   We infected DIV0 cortical neurons from *Ddx3x*$^{+/+}$, *Ddx3x*$^{+/flox}$, or *Ddx3x*$^{+/y}$ E15 embryos with AAV8-Ef1a-mCherry-IRES-Cre viral particles. At DIV9, we performed a 10-min puromycin pulse followed by a 30-min chase. This approach was effective in detecting newly synthesized proteins, as shown by abrogated puromycin incorporation in neurons treated with the protein synthesis inhibitor cycloheximide 15 min prior to

the pulse. When comparing the three genotypes, we did not detect significant differences in the puromycin labeling, suggesting that there are no overt changes in global protein synthesis detectable with this approach.

2) We examined ribosome biogenesis using as a proxy the ribosomal RNA (rRNA) 5.8S in the developing motor cortex of *Sox2-Ddx3x*$^{+/+}$, *Sox2-Ddx3x*$^{+/-}$, or *Sox2-Ddx3x*$^{+/y}$ neonates, in keeping with the design of our prior analyses of nucleoli. We found that *Sox2-Ddx3x*$^{+/-}$ cortices had a reduction in both the cytoplasm-to-nuclear distribution and expression of 5.8S rRNA (**new Fig. 5A-C**). These data support the ribosome biogenesis signature emerging from the proteomic analyses in Fig. 4 and the nucleolar phenotype in Fig. 5F-J.

We also note that motivated by a question raised by Reviewer #1, we have examined both CTIP2^{+} and CTIP2^{-} cells and the phenotypes we see are specific to CTIP2^{+} neurons.

Additionally, no direct link is provided between Ddx3x and the nucleolar phenotype, possibly due to any number of proteins affected by Ddx3x insufficiency. The phase separation connection is speculative, and no evidence is provided.

We thank the Reviewer for raising these two important points. We now provide new data that we believe begin to unravel the relationship between DDX3X, nucleoli, and LLPS. We have also rephrased some of our claims to ensure that the data are not overinterpreted.

1) Re-introducing human DDX3X or human DDX3Y in *Ddx3x* haploinsufficient neurons has a profound but divergent impact on neuronal nucleoli (**new Fig. 6D-G**).

2) Re-introducing an ATPase-deficient human DDX3X (E348Q mutant), which has been shown to affect the formation of RNA-containing phase-separated granules plausibly via LLPS, has no effect on nucleolar features (**new Fig. 6D-G**).

3) DDX3X and DDX3Y have distinct LLPS propensity (**new Fig. 6I**).

2. Fig. 2H-L is not convincing. FBL staining is problematic (many in the cytoplasm); it did not support the conclusion of nucleoli dynamics. There is no real connection between nucleolar size and DDX3X, just correlation.

As it pertains to the FBL staining, we regret a lack of clarity on how the immunostaining was presented (revised Fig. 5F, former Fig. 2H),

We believe that the FBL staining in CTIP2^{-} nuclei might have been misinterpreted as cytoplasmatic staining. We have now added graphical elements to distinguish the nuclei of CTIP2^{+} neurons (CTIP2^{+}DAPI^{+}, white arrowheads) and those of other cells (CTIP2^{-}DAPI^{+}, yellow arrowheads). We hope the Reviewer will now appreciate the nuclear specificity of the FBL staining in the revised Fig. 5F.

We also note that we have extended these analyses to CTIP2^{-} nuclei, in response to a comment by Reviewer #1. Interestingly, there are no sex- or genotype-driven differences in the nucleoli of CTIP2^{-} cells.

As for the lack of evidence about connection between nucleolar size and DDX3X, we have now incorporated a new set of experiments (**new Fig. 6**) that we believe strengthen the relationship between DDX3X and the nucleolar phenotypes. Specifically, we found that re-introducing human wild-type DDX3X in *Ddx3x*$^{+/-}$ neurons changes nucleolar volume and number. However, an ATPase-deficient DDX3X with a single point mutation in the DEAD box domain (E348Q) has no impact on nucleoli.

3. It is unclear how many replicates were performed? It is surprising to see that almost no proteome changes between Ddx3x-/y and Ddx3x+/y neurons since DDX3X expression is critical for male neuron development.

As indicated in the legend and above each plot (Fig. 4, former Fig. 2), the proteomic analyses were conducted on 5 independent cultures (thus 5 independent embryos) per genotype.

We were also surprised to see a smaller pool of proteins affected by *Ddx3x* loss in male neurons, given the morphological alterations. Intriguingly though, the proteins specifically dysregulated in *Ddx3x*$^{-/y}$ neurons are direct regulators of dendrite outgrowth and/or neuronal migration (e.g., *Dcx, Plxnd1 Rab23*). Hence, there are fewer dysregulated proteins, but their alteration plausibly has larger effect size on dendritic development.

We elaborate on this point in the Discussion:

“Our data show distinct molecular signatures based on sex. *Ddx3x* loss in male neurons result in a distinct and narrowed impact on proteins directly necessary for neurite outgrowth (Fig. 4), with no noticeable nucleolar alterations (Fig. S6). *Ddx3x* haploinsufficiency in females causes a downregulation of constitutive components of the protein synthesis machinery (Fig. 4), orthogonally corroborated by a reduction in nucleoli volume and 5.8S-containing ribosomes (Fig. 5). The defects in ribosome biogenesis might in turn alter the synthesis of specific proteins required to sustain dendritic growth, yielding the reduced dendritic branching resulting from *Ddx3x* perturbations (Fig. 1-2).”

4. The authors might consider comparing the proteomics data with ribo-seq data to determine whether the observed proteomic effects are secondary or if the proteins/mRNAs are directly regulated by DDX3X. Although the authors compared their data to iCLIP results, it is well-known that DDX3X interacts with the small subunit of the ribosome and binds extensively across mRNAs.

Motivated by the Reviewer’s question, we have compared the set of 182 proteins dysregulated in female *Ddx3x*$^{+/-}$ neurons and 98 proteins dysregulated in male *Ddx3x*$^{-/y}$ neurons with the 147 mRNAs with differential translational efficiency in a Ribo-seq dataset on E11.5 cortices from pooled *Emx1-Ddx3x*$^{+/-}$ and *Emx1-Ddx3x*$^{-/y}$ (“cKO” in the study) contrasted with pooled female and male controls (reference #12 cited in the manuscript).

We found that of the mRNAs that show changes in their translation efficiency in cKO E11.5 cortices, 3 encode for proteins that are dysregulated in female *Ddx3x*$^{+/-}$ neurons (Txnl1, Numbl, Psmd6) and another 3 for proteins that are dysregulated in male *Ddx3x*$^{-/y}$ neurons (Srsf6, Pcsk1n, Map1s).

We note that there are considerable differences between our dataset and the one in study #12, including the developmental stage and the lack of separation by sex in the published study. For this reason, we feel that more formal analyses with the published dataset might not be informative. However, if the Reviewer deems necessary, we can include a sentence about the overlap mentioned above.

In Fig. 3B, how do the authors explain that Ddx3x+/+ exhibits stronger dendritic spines than Ddx3x+/- but weaker dendritic spines than Ddx3x-/-? This seems contradictory and suggests that DDX3X expression may not be directly relevant to female dendritic spine strength.

We interpret the Reviewer's comment as a question about the pathological relevance of higher density of dendritic spines in *Ddx3x*$^{-/-}$ neurons.

Overexuberant dendritic spines have been reported in a range of mouse models of neurodevelopmental disorders (e.g., Fragile X syndrome; PMIDs: 20534828, 11223852, 20434996, 20434996). Increased spine density does not equate with enhanced synaptic transmission or plasticity, as can be the manifestation of defective synaptic pruning (e.g., PMID: 20434996) and/or delayed stabilization during synaptic maturation (PMID: 20534828).

To contextualize our observations, we have included the following interpretation in the section about the dendritic spines:

"These observations suggest that the *Ddx3x* is required for both the formation and the maturation of dendritic spines: *Ddx3x* monoallelic expression is sufficient to repress excessive spines formation at an early phase and/or facilitate pruning at a later phase, but biallelic expression is required throughout spine maturation."

Minor:

Line 58 – The sentence beginning with "Over 95% of affected…" is unclear and needs to be rewritten.

We have now rephrased the sentence as follows:

"Over 95% of individuals with DDX3X mutations are females, and they have *de novo* loss-of-function mutations leading to haploinsufficiency or missense mutations."

Line 63 – Needs to cite Shih et al. 2012 for DDX3X LLPS into SGs (PMID 21883093)

Included now as reference #13.

Line 67 – in utero should be italicized.

Thank you for your careful review. All Latin words have now been italicized.

Line 77 – Also need to cite Cortez et al. 2014 (21883093)

We believe the Reviewer referred to Cortez et al. 2014 (PMID: 24759410). This study is now included as reference #29.

General comment – The introduction needs copy editing.

The Introduction has been copy-edited.

Line 100 – in vitro needs to be italicized

Thank you for your careful review. All Latin words have now been italicized.

Line 300 – Ddx3x, not DDX3X

Throughout the study, we use *Ddx3x* to indicate the murine gene, *DDX3X* to indicate the human gene, and DDX3X to use both the murine or the human protein, according to the International Protein Nomenclature Guidelines ("*for vertebrates, use an all-uppercase gene symbol in a protein name*").

We thank the Reviewers for appreciating the work included in the revised manuscript. Here below we provide a point-by-point response to their comments, alongside a revised version of the manuscript.

Reviewer #1 (Remarks to the Author):

The authors have comprehensively addressed my concerns and significantly strengthened the manuscript. I have no further comments.

We thank the Reviewer for taking the time to assess the revised manuscript and for the insightful comments to the original version, which we feel have helped strengthen the study.

Reviewer #2 (Remarks to the Author):

The authors have added several key experiments to the original manuscript that further contribute to the current understanding on how sex specific differences are driven by DDX3 and make this work of high importance and novelty. Their work is highly significant to the field of neurodevelopmental disorders as mechanisms underlying sex-specific differences across different disorders are poorly understood. The data analysis and interpretation of the results is scholastically sound and rigorous. The authors have satisfactorily addressed all my previous concerns.

We thank the Reviewer for taking the time to evaluate the revised manuscript and for appreciating the value of the new experiments and the broader significance of our study.

Reviewer #3 (Remarks to the Author):

I appreciate the significant effort and additional experiments undertaken during the revision process; however, the quality of the analyses and the interpretations remains inadequate. Many of the analyses, particularly in proteomics, nucleoli quantification, and LLPS, are overly descriptive, and certain conclusions appear to be inaccurate. While the biological questions posed are undoubtedly fascinating, the biochemical and ribosome biogenesis experiments are not executed to a sufficient standard.

We thank the Reviewer for assessing and appreciating the additional experiments included during the revision process.

As discussed below, the revised manuscript includes new data (**new Fig. S4B-C**, **new Fig. S6G**, see below) to address the Reviewer's questions about the proteomics and biochemistry experiments. We also provide detailed responses to the other points raised by the Reviewer.

Additionally, we wish to provide a concise discussion of the broader context to explain why our findings enhance the mechanistic understanding of *Ddx3x* and DDX3X syndrome.

The biochemistry of DDX3X and its role in mRNA translation have been intensively studied in non-neural experimental models. However, it is now abundantly clear that neuronal mRNA translation has its own logic. Neuronal ribosomes have distinct conformations (Anadolu et al., *PNAS*, 2024, PMID: 38315848), specific co-

regulators (Kraushar et al., *Mol Cell*, 2021, PMID: 33357414), and non-canonical mechanisms of biogenesis (Fusco et al., *Nat Commun*, 2021, ref#55 cited in the manuscript). Further, sophisticated forms of spatiotemporal regulation of mRNA translation take place during neurodevelopment (e.g., Yoon et al., *Cell*, 2017, PMID: 28965759; Salamon et al., *Nat Commun*, 2023, ref #17; Harnett et al., *Nat Struct Mol Biol*, 2022, ref #18; Duffy et al., *Nat Neurosci*, 2022, ref #19; Borisova et al., *Nat Commun*, 2024, ref #20).

To date, only three studies have examined the molecular targets of *Ddx3x* in neural tissue (ref #3, #12, and Jowhar et al., *Mol Syst Biol*, 2024, PMID: 38273160). These studies cast a model whereby *Ddx3x* regulates the translation and stability of a subset of mRNAs, based on evidence in neural progenitors. By calibrating gene dosage, accounting for sex, and integrating *in cellulo* and *ex vivo* approaches, our study shows another layer of regulation taking place in differentiated neurons.

This is important because it indicates that *Ddx3x* functions along a neurodevelopmental continuum—from neurogenesis to neuronal maturation—and operates through a multimodal regulation depending on the cellular context. As we elaborate on within the *Discussion* section, these findings expand the window of risk for *DDX3X* mutations beyond prenatal life, with repercussions for the development of strategies for therapeutic intervention.

A central conclusion regarding ribosome biogenesis is based on proteomics data. There are key concerns about the proteomics analysis, including a lack of clarity around data coverage and consistency. The methodology is insufficiently detailed, and the results have not been validated, which significantly undermines the reliability of the findings.

We respectfully note that the concerns related to the proteomics data were not raised in the previous round of revision and that we have not made changes to this body of work in the revised manuscript. We are not clear about the specific concerns on data coverage and consistency in the proteomics data, but we are happy to address specific questions.

These data were generated and analyzed based on an established pipeline at the Keck MS & Proteomics Resource at Yale School of Medicine, which adheres to the best practices for mass spectrometry studies (e.g Norton et al., *Sci Adv*, 2024, PMID: 39110807; Onuchic et al., *Nat Commun*, 2023, PMID: 36997516; Shi et al., *Sci Adv*, 2023, PMID: 36626580).

We have deposited the raw data to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier #PXD060033, as now included in both the *Methods* section and the *Resource Availability* statement. During the peer review phase, the Reviewer can access the data with the following account details:

Username: reviewer_pxd060033@ebi.ac.uk
Password: MyQPaUBrjGmR

To address the Reviewer's request for validation, we have now performed Western blot analyses for some of the translational factors and ribosomal proteins found downregulated in *Ddx3x* haploinsufficient neurons by unbiased proteomics. As shown in **new Fig. S4B-C**, *Rps5*, *Rpl11*, *Rpl36a*, and *Eif3k* all validate as downregulated proteins

in *Ddx3x* haploinsufficient neurons. We also attempted to test expression for *Rps3*, *Rpl13*, *Rpl30*, *Rps10*, *Rps19*, *Rpl22*, and *Eif3i*, but the commercial antibodies we tried did not work well in our hands.

We also note that the ribosome biogenesis signature that first emerged through unbiased proteomics (now further validated, **new Fig. S4B-C**) is corroborated by three orthogonal and completely independent lines of evidence: *1) Ddx3x* haploinsufficient neurons have reduced expression of ribosomal RNAs (Fig. 5A-E); *2) Ddx3x* haploinsufficient neurons have smaller nucleoli (Fig. 5F-J); and, *3)* Re-expressing wild-type human DDX3X in *Ddx3x* haploinsufficient neurons repristinates nucleolar volume, but an ATPase-deficient DDX3X does not (Fig. 6).

The authors state that DDX3X- and DDX3Y-specific antibodies were used. However, to my knowledge, such specificity does not exist in commercially available antibodies. No evidence supporting this claim has been provided, which raises serious concerns about the robustness of the data, particularly given the emphasis on sex-specific expression of DDX3X and DDX3Y.

We respectfully note that we did not state that the antibody used to detect DDX3Y is specific (see point *a* below), as the Reviewer is correct that there are no commercial antibodies that recognize specifically DDX3Y. Further, none of the conclusions related to the sexual dimorphisms relies on the use of these antibodies (see point *b* below). More information on both points below.

a) We did not claim that the antibody used to detect DDX3Y is specific, rather the opposite.

This antibody was only used in Fig. S4A (old Fig. S3). In the corresponding legend, we wrote "*Ddx3x*$^{-/y}$ (lanes 7-8) neurons show expression of DDX3Y, as detected by an antibody that recognizes both DDX3X and DDX3Y (see *Methods*)". Coherently, in Fig. S4A, the band detected is denoted as "DDX3X/DDX3Y".

To provide further context, we used a rabbit anti-DDX3X polyclonal antibody (ThermoFisher Scientific, #A300-474A, 1:1000) and a mouse anti-DDX3Y monoclonal antibody (Sigma-Aldrich, #WH0008653M1, 1:1000), as detailed in the *Methods* section. The Western blot in Fig. S4A shows that: *a*) the #A300-474A "anti-DDX3X antibody" does not detect a band in *Ddx3x*$^{-/-}$ (lanes 5-6) or *Ddx3x*$^{-/y}$ (lanes 7-8) neurons; and *b*) the #WH0008653M1 "anti-DDX3Y antibody" is promiscuous, as it detects a band in control female neurons that do not carry the *Ddx3y* gene (*Ddx3x*$^{+/+}$, lanes 1-2). The fact that *Ddx3x*$^{-/y}$ neurons (lanes 7-8) show a band detected by the promiscuous #WH0008653M1 "anti-DDX3Y antibody" but not the #A300-474A "anti-DDX3X antibody" indicates that that band corresponds to DDX3Y.

To ensure clarity on this point, we have edited the *Methods* section, as follows:

"After blocking, membranes were incubated with a rabbit anti-DDX3X polyclonal antibody (Thermo Fisher Scientific, #A300-474A, 1:1000), a mouse monoclonal antibody raised against the first 80 residues at the N-terminus of DDX3Y which recognizes both DDX3X and DDX3Y (Sigma-Aldrich, #WH0008653M1, 1:1000), […]".

We also note that DDX3Y protein expression in *Ddx3x*$^{-/y}$ detected by Western blot in Fig. S4A is orthogonally corroborated by proteomic evidence of DDX3Y-specific peptides in *Ddx3x*$^{-/y}$ neurons ["We identified a

DDX3Y-specific peptide (KPILVATAVAAR in DDX3Y, Uniprot #Q62095; SPILVATAVAAR in DDX3X, Uniprot #Q62167) exclusively in the *Ddx3x*$^{-/y}$ dataset"].

b) None of the conclusions related to the sexual dimorphisms relies on the use of these antibodies.
The use of the anti-DDX3X/DDX3Y antibodies is limited to Fig. S4A, for the reasons and with the interpretations stated above.
For all experiments in Fig. 6, we deliberately expressed hDDX3X or hDDX3Y as tagged proteins (HA- or FLAG-tagged, respectively, see Fig. 6B) and detected the exogenous proteins with anti-HA or anti-FLAG antibodies.

Regarding ribosome biogenesis, it remains unclear which antibody was used to detect 5.8S rRNA, and the rationale for focusing on 5.8S rRNA is not adequately explained.

We used a mouse monoclonal antibody raised against the 5.8S rRNA (clone Y10B, Abcam, #ab171119, RRID:AB_777714). We had provided references for the specificity of this antibody for rRNA (refs #55-58 of the current bibliography, listed as refs #53-56 in the previous version) and have further included refs #59-62 in the revised manuscript, as follows:

"Using a monoclonal antibody raised against the 5.8S rRNA (clone Y10b[55-62]), we quantified rRNA in the developing motor cortex of *Sox2-Ddx3x*$^{+/+}$, *Sox2-Ddx3x*$^{+/-}$, or *Sox2-Ddx3x*$^{+/y}$ neonates (Fig. 5A)."

We also implemented two additional changes to enhance clarity:

1) Revised Fig. 5 and the corresponding legend to clearly outline the antibody used.
2) Revised the *Methods* section to read: "[…, a mouse monoclonal antibody raised against the 5.8S rRNA (Y10b clone, Abcam, #ab171119, 1:50),…]".

The rationale for our approach is twofold:

1) We sought to examine rRNAs to independently corroborate the findings of reduced ribosomal proteins (Fig. 4, **new Fig. S4B-C**, see below) and reduced nucleolar volume (Fig. 5) and thus support the hypothesis of ribosome biogenesis.
There are four mature rRNAs: 28S, 18S, 5.8S and 5S. Three of them (28S, 18S, and 5.8S) are synthesized in the nucleolus and result from the processing of a single polycistronic pre-rRNA. Given the findings of altered nucleoli in *Ddx3x* haploinsufficient neurons, we assessed rRNA species that are synthesized in the nucleolus.
We prioritized the analysis of the 5.8S rRNA via Y10b immunolabeling because this approach has been previously used, in conjunction with nucleolar analyses via Fibrillarin staining (as in Fig. 5-6), to examine ribosome biogenesis in neurons (Seo, et al. *Nat Commun*, 2022, ref #56; Chau et al., *eLife*, 2018, ref #59).

2) Targeting rRNA with the Y10b antibody has been widely employed to detect and purify ribosomes in brain tissues and neurons, as in the following publications: Fusco et al., *Nat Commun*, 2021, ref #55; Seo, et al.

Nat Commun, 2022, ref #56; Akbalik et al., *RNA Biol*, 2017, ref #58; Chau et al., *eLife*, 2018, ref #59; Taylor et al., *J Neurosci*, 2013, PMID: 23536073; Costa et al., *Cell Reports*, 2019, PMID: 31340150; Metz et al., *Sci Rep*, 2022, ref #62; Salehi et al., *Nat Commun*, 2023, PMID: 37438340; Tran et al., *Biorxiv*, 2024, PMID: 39763931.

If ribosome biogenesis is indeed a central aspect of the study, working with an RNA biologist with expertise in this area would substantially strengthen the findings.

We respectfully note that the corresponding author's training was precisely on neuronal mRNA translation and RNA-binding proteins (e.g., Tassone, De Rubeis et al., *Nucleic Acids Res*, 2011, PMID: 21478165; De Rubeis et al., *Neuron*, 2014, PMID: 24050404).

Collaborator Dr. Tatzelt, whose team was engaged during revision to address the biochemical questions raised by the Reviewers, has a long-standing track record in LLPS studies (e.g., Kamps et al., *J Biol Chem*, 2021, ref #97; Gogte et al., *PNAS*, 2025, PMID: 39739794), including with RNA-binding proteins (e.g., Ahlers et al., *Biophys J*, 2021, PMID: 33515602; Polido et al., Brain, 2024, PMID: 37669322).

There is also a disconnect between the proteomics data and the reported effects on ribosome biogenesis and protein synthesis. While the proteomics data suggest alterations in both small and large subunits, the decision to focus solely on 5.8S rRNA is difficult to justify.

The Reviewer is correct that the proteomics data indicated reduced expression of both small and large ribosomal proteins.

We have now cross-validated the reduced expression for two ribosomal proteins of the large subunit (*Rpl36a* aka eL42; *Rpl11*, aka uL5), one protein of the small subunit (*Rps5*, aka uS7), and a translation initiation factor (*Eif3k*) (**new Fig. S4B-C**). We note that *Rpl11* is critical for pre-ribosome assembly, and we had included the following sentence in the *Results* section: "We note that among the ribosomal proteins that have higher expression in female neurons but is reduced in *Ddx3x* haploinsufficient neurons is *Rpl11* (**Fig. 4J**) and this protein has a critical role in pre-ribosome assembly [63,64]". We have now edited this sentence to include a reference to Fig. S4B-C.

We note that the analyses on the 5.8S rRNA do not limit the investigation to the 60S subunit. In fact, the Y10b antibody recognizes not only free rRNA species, but also allows to label and purify mature ribosomes (Garden et al., *Mol Cell Neuro*, 1995, PMID: 7496633; Metz et al., *Sci Rep*, 2022, ref #62; Deng et al., *J Cell Sci*, 2021, PMID: 34668554; Zare et al., *Nat Commun*, 2024, PMID: 39198412; Tran et al., *Biorxiv*, 2024, PMID: 39763931). In fact, in line with previous reports, the immunostaining has both nuclear and cytoplasmic compartments and we observe differences in total expression and distribution between compartments (Fig. 5A-E).

Based on these considerations, we respectfully disagree that there is a disconnect between the proteomics data and the subsequent analyses. Rather, the proteomics data have offered an unbiased approach for the discovery

phase, yielding the first evidence of ribosomal deficits. We then corroborated this evidence with hypothesis-driven analyses at the RNA (Fig. 5A-E), protein (Fig. S4B-C), and nucleolar (Fig. 5F-I, Fig. 6D-H) levels.

The statement, “These observations suggest that *Ddx3x* defines aspects of ribosomal biogenesis that depend on sex and neuronal subtype. Further, *Ddx3x* mutations in neurons affect ribosome production,” feels overly descriptive and premature given the limited supporting evidence.

We have now rephrased the sentence as follows:

“These observations suggest that *Ddx3x* mutations in neurons are associated with altered rRNA expression and ribosomal deficits.”

Figure 5H raises concerns, as it lacks adequate explanation and validation.

We are not clear about the specific concerns related to the plot in Fig. 5H. As stated in the figure legend, Fig. 5H, shows the sum volume (μm^3) of all the nucleoli in the nucleus across three genotypes in both $CTIP2^+$ and $CTIP2^-$ neurons from cortices from *Sox2-Ddx3x*$^{+/+}$, *Sox2-Ddx3x*$^{+/-}$, and *Sox2-Ddx3x*$^{+/y}$ neonates. This quantification is related to the Fibrillarin (FBL) staining shown in Fig. 5F. Additional measures related to the same immunostaining data in *ex vivo* tissues are the empirical cumulative distribution function (cumulative probability) of the nucleoli volume across the three genotypes (Fig. 5G) and the average number of nucleoli (Fig. 5J).

The protein purification and LLPS assays are also problematic. The experimental approach appears inadequate, and the conclusions drawn contradict existing literature.

As it pertains to protein purification, we now provide an SDS-PAGE gel to show the purity of the MBP-DDX3X-eGFP and MBP-DDX3Y-eGFP fusion proteins used for the LLPS assay (**new Fig. S6G**). Additional comments about protein expression and purification are addressed in the next point.

As it pertains to the LLPS assay, the experimental approach adopted here is a well-established strategy for studying phase separation by assessing liquid droplets assembly upon the removal of a solubilizing tag. In addition to ref #97 cited in the manuscript, this approach has been widely employed in the literature, as in the following selected publications: Li et al., *Mol Cell*, 2015, PMID: 26412307; Schuster et al., *Nat Commun*, 2018, PMID: 30061688; Babinchak et al, *Nat Commun*, 2020, PMID: 33149109; Birsa et al., *Sci Adv*, 2021, PMID: 34290090; Burke et al., *Mol Cell*, 2015, PMID: 26455390; Hallegger et al., *Cell*, 2021, PMID: 34380047; Hofweber, *Cell*, 2018, PMID: 29677514; Murthy et al., *Nat Struct Mol Biol*, 2021, PMID: 34759379; Yoshizawa et al., *Cell*, 2018, PMID: 29677513; Zambusi et al., *Nat Neurosci*, 2022, PMID: 36424432; Gogte et al., *PNAS*, 2025, PMID: 39739794.

As outlined in the manuscript, “we note that our observations diverge from previously published reports, in both the qualitative appearance of condensates and the effect of RNA, likely because of different experimental conditions (see *Methods*). Nevertheless, in line with previous evidence [14], we found that hDDX3X or hDDX3Y condensates differ (Fig. 6I).”

Specifically, when compared with the experimental conditions in Shen et al., *Mol Cell*, 2022, #ref14, we performed the reaction in physiological salt conditions (150 mM NaCl in our assay vs. 300 mM NaCl in #14), physiological temperature (37°C in our assay vs. RT in #14), and with naturally occurring RNA substrates (total mRNA purified from cells vs. poly(U) in #14). As stated, the study by Shen and colleagues pioneered the understanding of the differences between hDDX3X and hDDX3Y and we wished to add onto the literature by mimicking experimental conditions that could be as close as possible to physiological ones.

One of the main divergences with the results from Shen et al., *Mol Cell*, 2022, ref #14 is that we observe droplet formation without adding RNA. We note that purified proteins can have a carry-over, albeit minimal, of RNA (Shen et al., *Mol Cell*, 2022, ref #14) and that the ability of DDX3X to spontaneously form assemblies in physiological buffer without supplementing RNA has been also reported by Saito et al., *Nat Chem Biol*, 2019, ref #16. More broadly, our observations are in line with previous reports of spontaneous formation of condensates *in vitro* in physiological conditions and in cells for other RNA-binding proteins (Maharana et al., *Science*, 2018, PMID: 29650702).

For example, MBP cleavage is known to destabilise DDX3, and the results in Figure 6I may simply reflect protein aggregation or precipitation rather than meaningful biological properties.

We respectfully disagree with the Reviewer on both claims, as articulated below.

1) With respect to the claim that “MBP cleavage is known to destabilise DDX3”, we apologize if we have missed the specific study the Reviewer is alluding to, but we were not able to identify evidence in the literature supporting this statement.
   On the contrary, there are at least 6 studies (from 5 independent groups) that have employed DDX3X upon TEV-mediate MBP cleavage (Lennox et al., *Neuron*, 2020, ref #3; Shen et al., *Mol Cell*, 2022, ref #14; Floor et al., *J Biol Chem*, 2016, PMID: 26598523; Secchi et al., *Nucleic Acids Research*, 2024, PMID: 39189461; Song & Ji, *Nat Commun*, 2019, PMID: 31300642; de Bisschop et al., *Biochimie*, 2019, PMID: 30910425).
   Importantly, in one such study (Song & Ji, *Nat Commun*, 2019), the D1D2 helicase core of DDX3X obtained upon TEV cleavage of the corresponding MBP-tagged protein was employed to successfully solve the crystal structure, in direct support of preservation of folding upon TEV-mediated cleavage of the MBP tag.

   Related to this point and as explained in the manuscript, the MBP tag is widely used in the purification of misfolding-prone proteins in a soluble conformation and/or to prevent spontaneous phase separation (see considerations in Alberti et al., *Cell*, 2019, PMID: 30682370 and Holehouse & Alberti, *Mol Cell*, 2025, PMID: 39824169). In fact, MBP-tagged DDX3X has been used in several studies, including Cheng et al., *Neuron*, 2019, PMID: 31587919; Shen et al., *Mol Cell*, 2022, ref #14; Owens et al., *Nat Commun*, 2024, PMID: 39500865; Drino et al., *Nucleic Acids Research*, 2023, PMID: 36718960; Yanas et al., *Curr Biol*, 2024, PMID: 39591970.

2) In response to the concern that “results in Figure 6I may simply reflect protein aggregation or precipitation rather than meaningful biological properties”, we note that our observations add upon a compelling body of

literature showing DDX3X and DDX3Y forming liquid droplets *in vitro* (e.g., Hondele et al., *Nature*, 2019, PMID: 31435012; Saito et al., *Nat Chem Biol*, 2019, ref #16; Shen et al., *Mol Cell*, 2022, ref #14).

Moreover, critical parameters for LLPS assays, such as protein purity and RNA content, are not presented, which are essential for reliable interpretation.

In the revised version of the manuscript, we have included an SDS-PAGE gel to show the purity of the MBP-DDX3X-eGFP and MBP-DDX3Y-eGFP fusion proteins used for the LLPS assay (**new Fig. S6G**).

As for the RNA content, as detailed in the *Methods* section, we used total RNA isolated from HeLa cells. This approach was used to account for naturally occurring RNA substrates (as opposed, for example, to poly(U) RNAs).

I do not wish to delay the authors, but I cannot support the manuscript in its current form because the biochemistry and ribosome biogenesis work could mislead the field. In summary, while the biological premise is compelling, the experimental design and execution, particularly in biochemistry and ribosome biogenesis, require considerable improvement to ensure the conclusions are accurate.

As outlined throughout our response above, we believe that our interpretation of ribosomal deficits is supported by independent and concordant lines of evidence: downregulated ribosomal proteins; downregulated ribosomal RNA; and nucleolar deficits. We hope that the responses above and the new data (**new Fig. S4B-C**, **new Fig. S6G**) will reassure the Reviewer.

We also note that a role for DDX3X in ribosome biogenesis has emerged in non-neural experimental systems, including lymphoma cells (Gong et al., *Mol Cell*, 2021, ref #40) and *Leishmania* (Padmanabhan et al., *Nucleic Acids Res*, 2021, PMID: 34358325).

Specifically related to ref #40, we had included the following sentence in our original manuscript: “Strikingly, 7 of the 13 ribosomal proteins reduced in *Ddx3x*$^{+/-}$ neurons are encoded by mRNAs interacting with DDX3X based on iCLIP data in lymphoma [40] (i.e., *Rps3*, *Rps5*, *Rps8*, *Rps19*, *Rpl11*, *Rpl13*, *Rpl30*), and 5 were found to have reduced translational efficiency in lymphoma [40] (i.e., *Rps3*, *Rps5*, *Rps19*, *Rpl11*, *Rpl13*).” Of these 5 mRNAs with reduced translational efficiency, we provide validation by Western blot in addition to our original results from unbiased proteomics (*Rps5* and *Rpl11*, **new Fig. 4B-C**).

We thank the Reviewer for taking the time to assess the revised manuscript and for appreciating the changes introduced.

Here below we provide a point-by-point response to their comments, alongside a revised version of the manuscript.

Reviewer #3 (Remarks to the Author):

I appreciate the thoroughness of the newly-inserted methods, which significantly strengthen the manuscript. However, I have concerns regarding the biochemistry presented in the study, particularly in Figure 6I and related conclusions.

We are delighted to hear that the modifications made during the revision strengthened our manuscript.

In response to the Reviewer's concerns about the LLPS experiment, we have removed Fig. 6I and related conclusions.

The images for DDX3X at 200 ng/μL RNA and DDX3Y with no RNA appear more like aggregation than liquid-liquid phase separation. This is a major sticking point, as it calls into question the authors' conclusion. There is a clear contrast between the rounded droplets seen in DDX3X at 0 ng/μL RNA and the jagged structures observed in DDX3Y under the same condition, as well as in the 200 ng/μL condition of DDX3X. If the authors stand by their conclusion that Figure 6I represents LLPS, they should provide additional evidence, such as fluorescence recovery after photobleaching (FRAP) to demonstrate molecular exchange within the droplets, as well as sensitivity tests using 1,6-hexanediol. The LLPS experiments appear to be based on a single replicate, as no statement regarding sample size or quantification is provided. Even if the experiment has been repeated, quantification is essential, especially when making a claim that challenges existing literature. The authors' conclusion that DDX3X undergoes stronger phase separation than DDX3Y contradicts existing in-cell data under physiological conditions. Moreover, sequence composition analysis of the N-terminal intrinsically disordered regions (IDRs) suggests the opposite—DDX3Y has more LLPS-promoting residues than DDX3X.

The current data in Figure 6I detracts from the manuscript. Readers may focus on the apparent aggregation of DDX3Y (and, to a lesser extent, DDX3X) rather than appreciating the important biological insights presented elsewhere. Given the strength of the biological findings, the paper would not suffer without the LLPS experiments, and their removal might allow the manuscript's key advances to stand out more clearly.

We are thankful that the Reviewer appreciates the "important biological insights" and "key advances" of our study.

We agree that the LLPS experiment is not central to the message of the paper. Following the Reviewer's suggestion, we have now removed Fig. 6I (and the related Fig. S6G) and the text associated with these experiments in Methods, Results, and Supplemental Note.

Further, to provide the Reader with the references to the most updated literature on DDX3X and LLPS, we have introduced two new citations (Yanas et al., *Curr Biol*, 2024, ref #18; Owens et al., *Nat Commun*, 2024, ref #19), as follows:

1) Introduction: "*DDX3X* encodes an ATP-dependent DEAD-box RNA helicase with broad functions in RNA metabolism [3,10-14], including regulating mRNA translation [10,11] and RNA-containing condensates and granules via liquid-liquid phase separation (LLPS) [15-19]."
2) Results (related to Fig. 6): "Considering that the nucleolus is a phase-separated organelle, our data align with prior evidence showing that human DDX3X and DDX3Y have distinct propensities to LLPS [15,18,19]".
3) Discussion: "Human DDX3X and DDX3Y differ in their catalytic properties and propensity to LLPS [15,18,19]."

The claim regarding rRNA biogenesis is made without performing classical biochemical assays such as northern blots, which is surprising. The current presentation of rRNA biogenesis is very descriptive.

We respectfully note that we did not make claims about rRNA biogenesis, which comprises rRNA transcription, processing, and maturation. (We reviewed the manuscript for statements related to "rRNA biogenesis" and found none. We apologize if we missed any and we would be happy to revise the text if pointed to the right direction.)

Rather, we made claims on the broader process of formation and assembly of the ribosomes: ribosome biogenesis. These claims about ribosomal deficits are supported by four orthogonal lines of evidence: 1) *Ddx3x* haploinsufficient neurons have reduced ribosomal proteins from unbiased proteomics (Fig. 4), further validated by Western blot (Fig. S4B-C); 2) *Ddx3x* haploinsufficient neurons have reduced rRNAs levels in the cytoplasm (Fig. 5); 3) *Ddx3x* haploinsufficient neurons have smaller nucleoli (Fig. 5); and 4) human wild-type DDX3X is able to rescue the nucleolar phenotype, while an ATPase-deficient DDX3X is not (Fig. 6).

To make this point clear to the Reader, we have made two changes:

1) We have rephrased a sentence in the Results to read: "We reasoned that the *Ddx3x*-dependent female bias might reflect more complex aspects of ribosome formation, which undergoes sophisticated forms of regulation in neural cells [21,58]. Therefore, we measured the abundance and subcellular distribution of ribosomal RNA (rRNA)."
2) We have included a sentence in the Discussion: "Whether *Ddx3x* haploinsufficiency also impacts the processing and maturation of rRNAs remains to be examined".

We also felt it was important to reiterate the importance of studying protein synthesis in the cellular context that is pertinent to understand neurodevelopment and DDX3X syndrome. To this end, we included two new citations and the following sentence: "More broadly, our study adds on an ever-evolving landscape of mechanisms of protein synthesis in neurons, where ribosomes have distinct conformations [78], specific co-regulators [79], and non-canonical mechanisms of biogenesis [58]".