Temporal-specific roles of fragile X mental retardation protein in the development of the hindbrain auditory circuit

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AUTHORS: Xiaoyu Wang, Ayelet Kohl, Xiaoyan Yu, Diego Zorio, Avihu Klar, Dalit Sela-Donenfeld, and Yuan Wang

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The main question of this study is to determine roles of FMRP in embryonic development of the auditory brainstem. This is an intriguing question because most previous studies have focused on FMRP functions during postnatal development. In particular, to determine expression and roles of FMRP in the auditory nervous system during early developmental stage is critical to understand the mechanisms underlying auditory hypersensitivity in Frgile X syndrome.

Comments for the author

The main question of this study is to determine roles of FMRP in embryonic development of the auditory brainstem. This is an intriguing question because most previous studies have focused on FMRP functions during postnatal development. Authors well developed the system and carefully validated FMRP knockout and knockdown for studying this main question. Using chick embryo, authors identified axonal location of FMRP, specifically at presynaptic terminals, during embryonic development. They also found the key roles of FMRP in axonal projection and targeting of NM neurons to NL.

Authors should consider the following comments on the manuscript.
1. Expression of FMRP at presynaptic terminals. Authors showed Atoh1-mGFP and FMRP signals from distal axons. It is recommended to include immunostaining Atoh1-GFP distal axons with presynaptic marker (e.g. vGluT) and FMRP to show their location at presynaptic terminals in later embryonic stage
(E13-15) as well as E5.
2. Authors quantify the width of the GFP+ axonal bundle. Please clarify the methods used to quantify axonal width. It would be better to show how the axonal length and the mantle-ventricular width were measured. For example, indicate this measurement in Figure C-G or C'-G'. Also it needs to clarify how to measure the angle of individual axons (Supplementary 2).
3. Authors demonstrated morphological maturation of presynaptic terminals of NM neurons in Fig. 3.
How about the effect of FMRP knockout or knockdown on this morphological maturation?
4. FMFP knockouts showed disoriented and aberrant axons, which delayed crossing the midline. Glial guidance may be involved in this axonal growth and navigation. It would be better to discuss any effect of FMRP loss on astrocyte or microglia in this brain area, or the potential possibility of glial contribution to these axonal phenotypes.
5. FMRP loss impaired axonal targeting. Does this also affect tonotopic organization of this auditory system?

Minors.
Page 3, line 30. Check the typo.

Figure 4C-C''. Missing box in the figure.

Figure 5E. Indicate box area in the figure.

Reviewer 2

Advance summary and potential significance to field

In this paper, Wang and colleagues examine development of auditory hindbrain NM precursors. Overall this is an interesting study, employing chick embryo as in vivo model to study developmental features of axon maturation combined with state-of-the art genetic tools. After describing and presenting their model, the authors conduct immunostainings for FMRP and show FMRP granules in constitutive overexpression situation. The study then presents the foundational observation that FMRP is involved in axon branching and leads to aberrant projection in vivo upon FMRP genetic deletion and shRNA FMRP knockdown. This phenomenon is only transient, as at later

developmental stages axons cross the midline comparable to controls. Turning to in vitro, they corroborate the branching phenotype following Crispr-mediated FMRP deletion. Finally, there are data on late FMRP knockdown. The study, which includes per se interesting data, stays rather descriptive and there are some gaps that if filled in would make the overall study stronger and more compelling.

Comments for the author

1. While the introduction of the chick embryo is important, there is not a single quantification in the first five figures. To substantiate their qualitative arguments, the authors need quantifications. For example, in the discussion they mention known proteins colocalizing with FMRP (DSCAM). They could show colocalization with known markers here.
1a: Figure 3: to claim that these are mature terminals, a synaptic marker would be needed. Consider moving figure to supplemental material.
1b: Figure 4: the authors claim that there is 'heavy' immunostaining which is hard to see in the images. Why is not the whole cytoplasm in B labeled by Atoh1-mGFP Same is true for panels in B, where nuclear immunostaining is 'weaker' compared to cytoplasm. Panel C-C'': most of the FMRP-positive dots appear to be not in axons, is there a quantitative approach demonstrating that these granules are in axons (not only density)? In which cells are all the other FMRP granules? Astrocytes?
2. As their model is ideal to study development, a time course of expression of FMRP would help. As this could show, when FMRP starts localizing in distal axons of NM precursors.
3. Upon Crispr/Cas FMRP deletion the authors show axons growing mediolateral growth, but then conclude that there is only a delay as two days later most axons still were able to cross the midline. What about the aberrant axonal projections? Where do they project to or do they degenerate? What about the size of the axon bundles? Or is this phenotype exclusively transient and then neurons appear overall normal? Could the authors label single cells in vivo?
4. In figure 10 do the abnormal axon projections form synapses?

Statistics:
Student t-test not a very stringent test; are data normally distributed? A non-parametric test recommended

Additional minor comments
- Figure 5E where is the inset coming from? Please indicate this by a box.
- Some images are rather small, and a zoom in would be helpful.

First revision

Author response to reviewers' comments

We appreciate your review of our manuscript (DEVELOP/2020/188797) and the opportunity for revision. In the resubmission, we have addressed all comments and made the changes suggested by the editors and reviewers, following the strategic plan that you kindly approved on 5/5/2020. Please see below our response highlighted in blue to each comment.

In response to the Editor:

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript.
All changes and additions in the main text are highlighted in blue.

At this stage, we also ask you to ensure your manuscript complies with our formatting guidelines.
This resubmission has carefully followed all the formatting guidelines. The only remaining issue is that we are ~ 450 words over the 7000-word limit. This results from the multiple additions that were included in response to reviewer's comments (i.e., two new figures, additional panels in the other figures, new data description, additional discussions etc.). We have reduced the words as

much as we can without compromising the clarification of the manuscript and hope for your understanding.

In response to Reviewer #1:

1. Expression of FMRP at presynaptic terminals. Authors showed Atoh1-mGFP and FMRP signals from distal axons. It is recommended to include immunostaining Atoh1-GFP distal axons with presynaptic marker (e.g. vGluT) and FMRP to show their location at presynaptic terminals in later embryonic stage (E13-15) as well as E5.

Although FMRP plays an important role in axons, axonal FMRP is not necessary associated with synapses. In line with this statement, many FMRP targets, such as cytoskeletal proteins, cell adhesion proteins, and ion channels, are not synaptic proteins. Fragile X granules in mature axons as identified by others are often in axon bundles instead of the target areas (Akins et al., 2017; Chyung et al., 2018). In developing systems, as our data show, many FMRP puncta are not in the region where synapses are located. Moreover, a large part of the functional role of FMRP in our data focuses on E2.5-E6.5, stages when synapses are not developed yet. Thus, FMRP is most likely to exert its axonal functions that are identified in our study without being associated with synapses.

Akins MR, Berk-Rauch HE, Kwan KY, Mitchell ME, Shepard KA, Korsak LI, Stackpole EE, Warner-Schmidt JL, Sestan N, Cameron HA, Fallon JR. Axonal ribosomes and mRNAs associate with fragile X granules in adult rodent and human brains. Hum Mol Genet 2017, 26(1): 192-209.
Chyung E, LeBlanc HF, Fallon JR, Akins MR. Fragile X granules are a family of axonal ribonucleoprotein particles with circuit-dependent protein composition and mRNA cargos. J Comp Neurol 2018, 526(1): 96-108.

Nevertheless, we agree with the reviewer that determining the relationship of the subpopulation of FMRP puncta that are in the terminal region with synapses is important to the field. We have performed the immunostaining of FMRP with vGluT2 at both E4.5 and E15 as the reviewer suggested, and demonstrated their overlapping with NM axons. However, we are not able to determine the colocalization relationship with confidence at the level of light microscopy. Analyses at the level of electronic microscope would be needed to answer this question, for which we feel that this is beyond the scope of the current manuscript.

In the resubmission, we provide a new supplemental figure of the double labeling of FMRP and vGlut2 at E4.5 (Fig. S5).

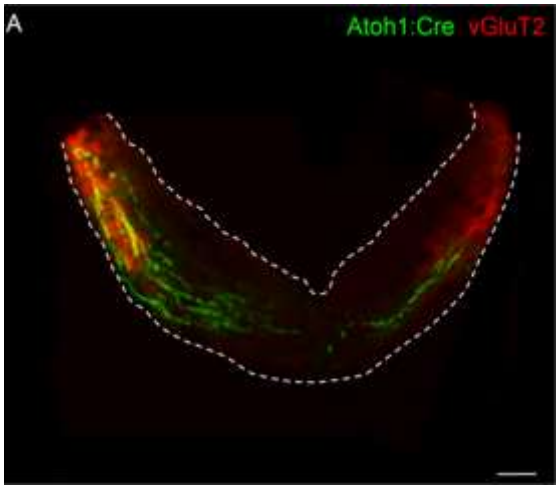


Figure S5. vGluT2 distribution in the axon course of Atoh1:cre transfected precursors. Transverse section of E4.5 hindbrain (r5) that was electroporated at E2.5 with Atho1:cre-cGFP plasmid and stained for the vesicular glutamate transporter 2(vGluT2) (n=7 embryos). Scale bars: 200 µm in A.

In the main text, we have further clarified this issue in the Discussion as following (Lin 415): “This

possibility is consistent with the localization of FMRP puncta in the distal axonal processes (Fig. 5). Although their exact relationship with synapses is yet to be determined, it is notable that many FMRP puncta are not in the region where synapses are located. Thus, FMRP is likely to exert its axonal functions that are identified in our study without being associated with synapses."

2. Authors quantify the width of the GFP+ axonal bundle. Please clarify the methods used to quantify axonal width. It would be better to show how the axonal length and the mantle-ventricular width were measured. For example, indicate this measurement in Figure C-G or C'-G'. Also it needs to clarify how to measure the angle of individual axons (Supplementary 2).
We have now added a schematic drawing that illustrates how these axonal parameters were measured. This drawing is shown as in Fig. 2D (below), as we have now quantified these parameters in Atoh1-mGFP labeled axons in addition to FMRP-manipulated axons in response to a comment of Reviewer 2.

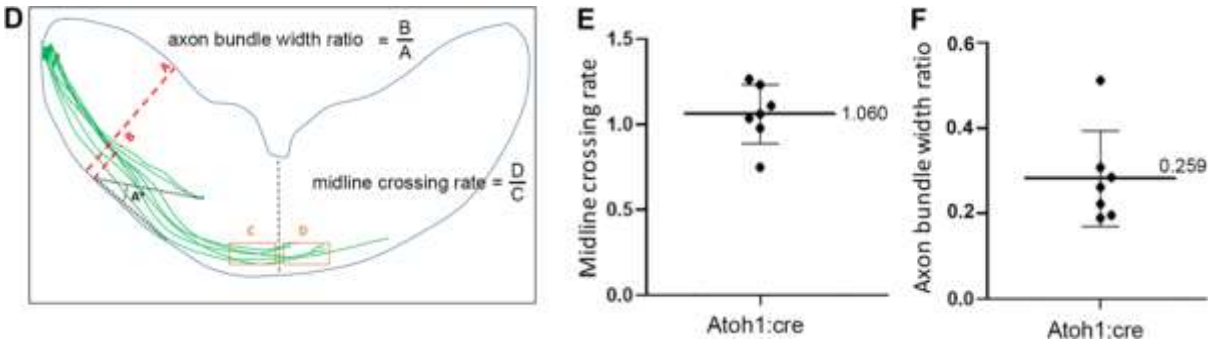


Figure 2. Axon development of NM precursors and neurons. D. An illustration describing the measurements used to quantify axonal growth patterns of NM precursors. Axon bundle width was calculated as the ratio between B (GFP$^+$ fascicule width) divided by A (mantle-ventricular width). Axonal midline crossing rate was calculated as D (area of GFP$^+$ contralateral axons) divided by C (area of GFP$^+$ ipsilateral axons). A° was the angle between the most medial GFP+ projecting axon and the mantle plate. **E-F.** Box plot analysis of the ratio of axonal midline crossing (E) and bundle width (F) of Atoh1:cre tagged axons at E4.5. Each data point represents one embryo (n=7).

3. Authors demonstrated morphological maturation of presynaptic terminals of NM neurons in Fig. 3. How about the effect of FMRP knockout or knockdown on this morphological maturation?
To address this question, we first quantified the morphological maturation of presynaptic terminals of NM neurons by measuring the number of filopodia per terminal during developmental stages. NM axonal terminals gradually retract their filopodia toward morphological maturity. We observed a significant reduction of filopodium numbers from E11-13 to E15.
Terminals with no filopodia are bouton like terminals, which are considered mature. Relatively immature terminals exhibit 1-5 filopodia. Terminals with a larger number of filopodia are relatively less mature. This new data is illustrated in the revised Fig. 3.

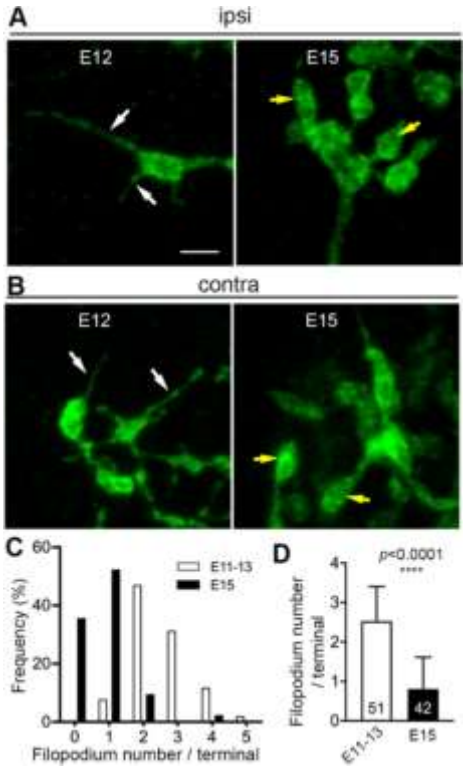


Figure 3: Morphological maturation of presynaptic terminals of NM neurons. Images were taken from embryos electroporated with Atoh1-mGFP at E2. **A-B.** NM axon terminals in the dorsal neuropil of the ipsilateral NL (A) and in the ventral neuropil of the contralateral NL (B) at E12 and E15. NM axons show a growth cone structure with filopodia (white arrows) at E12 and bouton-like terminals (yellow arrows) at E15. **C-D.** Frequency distribution (B) and population analysis (C) of the number of filopodia per terminal at E11-13 (n=51 terminals) and E15 (n=42 terminals). Additional images and data analyses are shown in Figs. S3, S4. Scale bars: 2 µm.

Additional data analyses were also included as a new supplemental figure (Fig. S4).

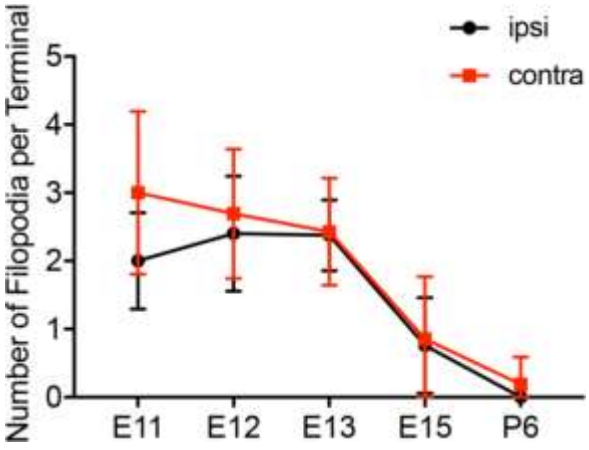


Figure S4. Quantification of terminal morphology at E11, E12, E13, E15 and P6.
Next, we determined the effect of FMRP knockdown on the number of filopodia per terminal at E15 by comparing between Atoh1-mGFP labeled terminals and *Fmr1*-shRNA transfected terminals. This analysis did not reveal a significant difference. The data is illustrated in a new figure (Fig. 12) and described in the Results (line 342).

"Finally, we examined whether FMRP knockdown altered the morphological maturation of NM

axonal terminals. In embryos expressing *Fmr1*-shRNA, the number of filopodia per EGFP$^+$ terminal is 0-2 at E15, similar to control as measured from Athoh1-mGFP labeled terminals (**Fig. 12**; p=0.5695)."

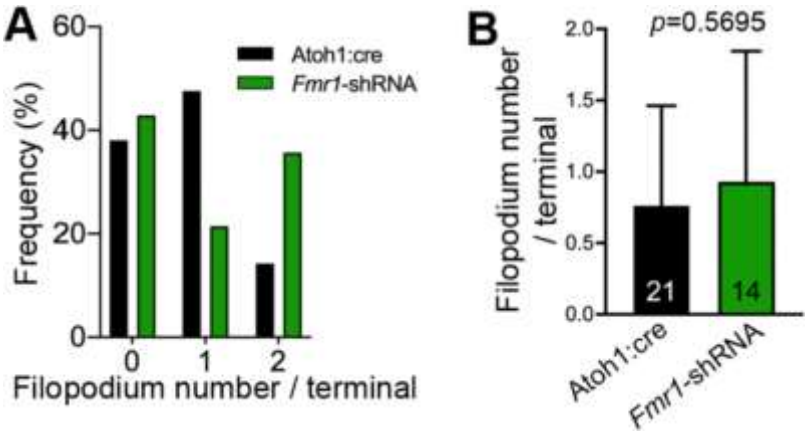


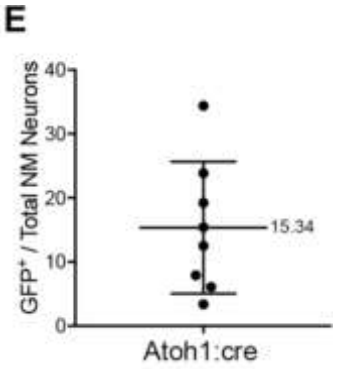
Figure 12. FMRP knockdown does not affect the morphological maturation of NM axonal terminals. A-B. Frequency distribution (A) and population analysis (B) of the number of filopodia per terminal following transfection with Atoh1:cre-mGFP (black bars; n=21 terminals) and Fmr1-shRNA (green bars; n=14 terminals). All terminals were measured form the ventral neuropil of the contralateral NL.

4. FMFP knockouts showed disoriented and aberrant axons, which delayed crossing the midline. Glial guidance may be involved in this axonal growth and navigation. It would be better to discuss any effect of FMRP loss on astrocyte or microglia in this brain area, or the potential possibility of glial contribution to these axonal phenotypes.
We appreciate the comment and have now expanded our discussion as following (Line 434):

" It is worth to note that axon-glia interactions may also contribute to FMRP regulation of axon events, given their well-established roles in axon guidance, fasciculation, and targeting (Rigby et al., 2020). Interestingly, some of the molecules that participate in a direct axon-glia contact, such as NCAM and Semaphorins-Plexins (Franceschini and Barnett, 1996; Goldberg et al., 2004; Keilhauer et al., 1985; Miragall et al., 1989; Moreau-Fauvarque et al., 2003; Neugebauer et al., 1988; Shim et al., 2012), are known as FMRP targets in neurons (Li et al., 2009; Liao et al., 2008; Menon and Mihailescu, 2007). Hence, it is possible that lack of FMRP in NM axons prevents their interaction with glial cells via these proteins that in turn, leads to aberrant axonal growth. Additionally, FMRP may control axonal targeting by regulating the formation of axon myelination (Doll et al., 2020; Pacey et al., 2013) which influences functional development of axon terminals (Berret et al., 2016; Xu et al., 2017)."

5. FMRP loss impaired axonal targeting. Does this also affect tonotopic organization of this auditory system?
While our approach provides a unique opportunity to determine effect of FMRP misexpression on individual or a small group of axons, the mosaic pattern of transfection of only from 3.4% to 34.4% neurons in the cell group is not expected to alter the tonotopic organization of the system. Particularly, transfected neurons are consistently located throughout NM along the tonotopic axis, preventing the ability of answering this question with certainty. A transgenic animal model in which FMRP in all or majority of neurons in a cell group is reduced will be an appropriate preparation to address this question, which will be the scope of a future study.

In the resubmission, we have provided the quantitative analyses of the transfection rate of our preparations (Fig. 1E). We have provided a discussion of the evidence that support a role of FMRP in tonotopic organization, as following (line 445):



"It remains unknown whether the tonotopic organization of NM axonal projection was affected by FMRP deficiency. Our manipulations affected only ~15% NM neurons that were often scattered throughout the cell group, thus unable to determine the effect on the tonotopic organization. Studies of Fmr1 knockout mice demonstrated a normal tonotopic frequency representation in the auditory cortex (Kim et al., 2013). However, FMRP loss diminishes the developmental plasticity of this representation (Kim et al., 2013), flattens the tonotopic organization of potassium channel Kv3.1b (Strumbos et al., 2010), and results in frequency- specific decreases in inhibitory presynaptic structures (McCullagh et al., 2017), suggesting a potential link of FMRP with specific features of tonotopic regulations."

Minors.
Page 3, line 30. Check the typo.
We appreciate the correction. This sentence is deleted from the revision for the concise of the manuscript.

Figure 4C-C''. Missing box in the figure.
A box is added to Fig. 4B-B'', 4C-C'' and D-D''.

Figure 5E. Indicate box area in the figure.
A box is added.

In response to Reviewer #2:

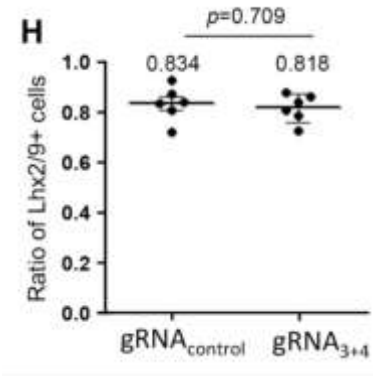
1. While the introduction of the chick embryo is important, there is not a single quantification in the first five figures. To substantiate their qualitative arguments, the authors need quantifications. For example, in the discussion they mention known proteins colocalizing with FMRP (DSCAM). They could show colocalization with known markers here.
We have provided the following quantifications in the revised manuscript:

- Figure 1: the percentage of Atoh1-mGFP transfected NM neurons out of all NM neurons. This quantification is important for interpreting the data using this approach.

- Figure 2: the axon bundle width and midline crossing rate of the Atoh1-mGFP electroporated cells. An illustration describing the measurements used to quantify these parameters is also added (in response to comment #2 of reviewer 1). These two axon parameters are the same that were used to determine the effects of FMRP knockout/knockdown on axon traveling (Figs 7, 8, and Fig. S8).

- Figure 3: the filopodium number per terminal as an indicator of terminal maturity. This quantification provides the foundation for determining the effects of FMRP knockdown on terminal morphology (new Fig. 12).

- Figure 6. Quantification of gRNA expressing dA1/NM precursors out of total dA1/NM precursor cells. This quantification (Fig. 6H) demonstrates the localized expression of Crispr/Cas plasmids in dA1/NM precursor cells in accordance with the qualitative data.



- Additionally, we have added a new supplemental figure showing the overlapping measures of Atoh1:cre transfected cells in relate to a general dA1 cell marker (Fig. S1). This quantification confirms that the majority of Atho1:cre transfected cells are dA1 cells, supporting our qualitative observations, as well as our previous study which utilized these plasmids for the first time (Kohl et al., J. Neuroscience 2012).

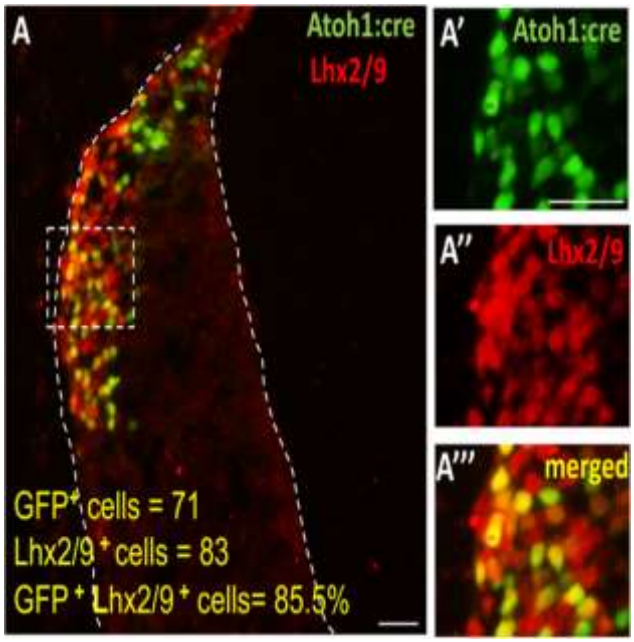


Figure S1. Atoh1:cre transfected cells are immunoreactive to Lhx2/9. Transverse section of E3.5 hindbrain (r5) that was electroplated at E2.5 with Atoh1:cre-nGFP plasmid and stained for Lhx2/9 to label endogenous dA1 cells. The dorsal half of the electroporated side of the neural tube is shown, demonstrating GFP$^+$ nuclei co-labeled with Lhx2/9 (n=7 embryos). Counting of cell nuclei expressing Atoh1:cre-GFP, Lhx2/9 or both in this section is provided in the lower left, demonstrating that 85.5% of electroporated cells co-express Lhx2/9. This ratio is in accordance with our previous publication (Kohl et al., 2012) that found 88% overlapping of Atoh1:cre with Lhx2/9 expressing cells. High-magnification views of the boxed area in (A) are represented in panels (A'-A''') in the different channels. Scale bars: 100um in A; 50um in A'.

Description of the added quantifications is provided in the Materials and Methods section.

1a: Figure 3: to claim that these are mature terminals, a synaptic marker would be needed. Consider moving figure to supplemental material.
We have moved most panels in Figure 3 to supplemental material (new Fig. S3). Figure 3 now contains images from two representative ages (E12 and E15) and the new quantification analyses.

A new supplemental figure (Fig. S4) is now added to provide quantitative measures at all examined ages (E11, E12, E13, E15, and P6). To verify NM terminals are mature at the ages older than E15, we have added images of NM axonal terminals with immunoreactivities of a synaptic marker (SNAP25) at E15 in Fig. S6.

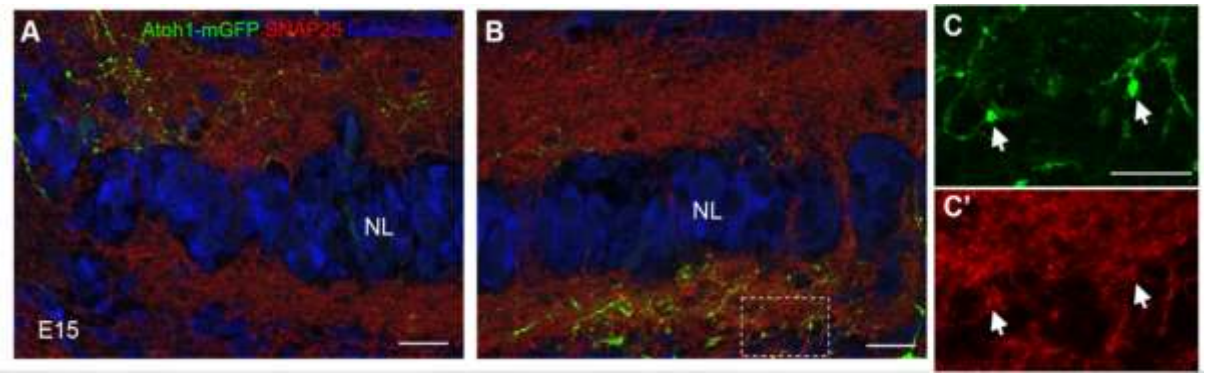
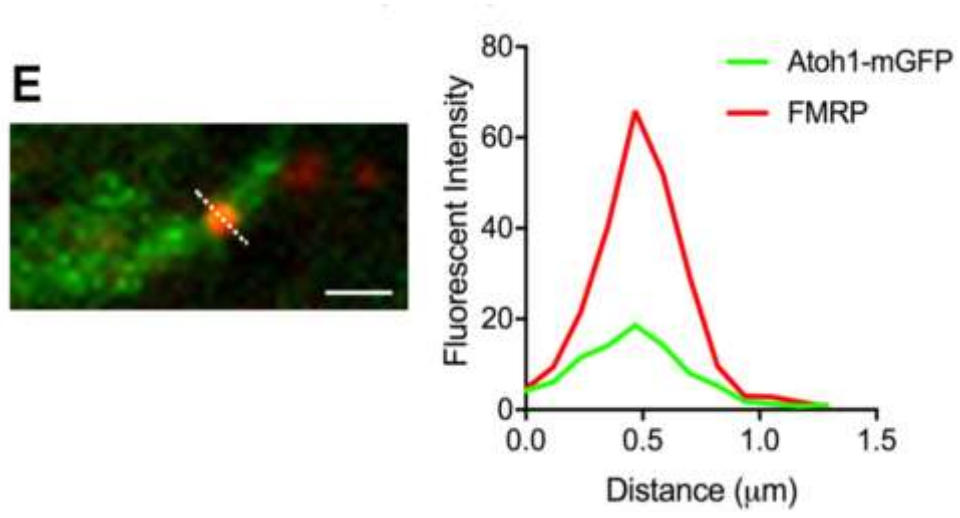


Figure S6. Atoh1-mGFP transfected axon terminals contain SNAP25. SNAP25 immunostaining was performed on the Atoh1-mGFP transfected E15 sections and counterstained with NeuroTrace. **A-B**: low magnification images of the ipsilateral (A) and contralateral (B) NL showing mGFP$^+$ axons in the dorsal and ventral NL neuropil regions, respectively. **C-C'**: High magnification images of the boxed area in B. mGFP$^+$ axon terminals (white arrows) are immunoreactive to SNAP25. Scale bars: 20 μm in A and B, 10 μm in C.

1b: Figure 4: the authors claim that there is 'heavy' immunostaining which is hard to see in the images. Why is not the whole cytoplasm in B labeled by Atoh1-mGFP Same is true for panels in B, where nuclear immunostaining is 'weaker' compared to cytoplasm. Panel C-C'': most of the FMRP-positive dots appear to be not in axons, is there a quantitative approach demonstrating that these granules are in axons (not only density)? In which cells are all the other FMRP granules? Astrocytes?

There is a misunderstanding. In this region of axonal terminals, there are very few cell bodies, which is confirmed by our DAPI nuclei staining, suggesting that the major contribution of these FMRP granules are from axons. Notably, we only labeled on average 15% NM neurons and their axons (please refer to the new Figure 1), thus the majority of FMRP puncta are not expected to overlap with EGFP+ axons. Non-overlapping FMRP granules are presumably axonal, but in non-transfected axons. To further exclude the other possibility, we performed colocalization analysis and confirmed the peaks of Atoh1-mGFP and FMRP staining intensity are overlapped, as shown below, demonstrating FMRP granules in the transfected NM axons.



In the revision, we have further clarified this idea in the figure legend as following:
"C-C''. High-magnification images of the box in B'' from the contralateral side (contra). A subset

of FMRP puncta are localized in mGFP+ axon processes (insets). FMRP puncta that are localized beyond mGFP+ axon processes are presumably in untransfected axons because this region contains no cell bodies as indicated with the lack of DAPI-labeled nuclei. E. Colocalization analysis of a representative FMRP punctum with Atoh1-mGFP+ labeled axon, confirming the axonal location of FMRP."

For the description of cytoplasmic FMRP immunoreactivity, we discarded the use of "heavy" to avoid confusion. FMRP immunoreactivity was detected throughout the cytoplasm (developing neurons have a relatively large nucleus during early development), as clearly illustrated in Fig. 4C. The reviewer is correct that FMRP level is weaker in the nuclei than in the cytoplasm, consist with reports in other systems (Feng et al., 1997; Bakker et al., 2000; Kim et al., 2009). However, the relative ratio of FMRP localization in nucleus vs. cytoplasm is not relevant to the idea expressed by this study, thus we choose not include additional analyses on this topic.

Bakker, C. E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A. T., Oostra, B. A. and Willemsen, R. (2000). Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Exp. Cell Res. 258, 162–170.
Feng, Y., Gutekunst, C. A., Eberhart, D. E., Yi, H., Warren, S. T. and Hersch, S. M. (1997). Fragile X mental retardation protein: nucleocytoplasmic shuttling and association with somatodendritic ribosomes. J. Neurosci. 17, 1539–1547.
Kim, M., Bellini, M. and Ceman, S. (2009). Fragile X mental retardation protein FMRP binds mRNAs in the nucleus. Mol. Cell. Biol. 29, 214–228.

2. As their model is ideal to study development, a time course of expression of FMRP would help. As this could show, when FMRP starts localizing in distal axons of NM precursors.

We have shown FMRP localization in distal axons of NM precursors as early as E4 and throughout the development into post hatch. NM cells are born at E2-2.5 and expression of plasmids takes 12-24 hours at least. Thus, the earliest time that we can visualize the axons of NM precursors is around E4. In a sperate study, we have confirmed FMRP expression in chicken hindbrain as early as E3, consistent with early-onset FMRP expression in humans and mice (Abitbol et al., 1993; Hinds et al., 1993).

Abitbol, M., Menini, C., Delezoide, A. L., Rhyner, T., Vekemans, M. and Mallet, J. (1993). Nucleus basalis magnocellularis and hippocampus are the major sites of FMR-1 expression in the human fetal brain. *Nat. Genet.* 4, 147–153.
Hinds, H. L., Ashley, C. T., Sutcliffe, J. S., Nelson, D. L., Warren, S. T., Housman, D. E. and Schalling, M. (1993). Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. *Nat. Genet.* 3, 36–43.

In the revision, we have added a discuss (line 354): "NM cells are born at E2-2.5 (Rubel et al., 1976). FMRP localization can be detected as early as E4 in developing axons of NM precursors, demonstrating that FMRP starts localizing in distal axons of NM precursors shortly after Fmr1 gene expression and axon genesis. This finding is consistent with FMRP localization in newly formed neurites of PC-12 cells and axon growth cones of cultured mammalian neurons (De Diego Otero et al., 2002; Antar et al., 2006; Hengst et al., 2006; Jain and Welshhans, 2016)."

3. Upon Crispr/Cas FMRP deletion the authors show axons growing mediolateral growth, but then conclude that there is only a delay as two days later most axons still were able to cross the midline. What about the aberrant axonal projections? Where do they project to or do they degenerate? What about the size of the axon bundles? Or is this phenotype exclusively transient and then neurons appear overall normal? Could the authors label single cells in vivo?
This is an important point and the reviewer is correct that in vivo tracking of a single axon over time will enable to answer this question. However, this methodology does not exist in the chicken embryonic system at present. Nevertheless, one scenario that can be raised based on our data is that the ratio of the wrong projections gradually decreases with time, together with the increase in the correct commissural projection (that we show in Figs. 7,8). We have re- examined our data to test this possibility and quantified the percentage of the wrong projections in E4.5 in comparison to E6.5. Our analyses demonstrated that the width of the axonal bundle is reduced at E6.5 in comparison to E4.5 in both Crisp/Cas and shRNA methods, suggesting partial recovery of

the axonal organization. We have added this data in the manuscript as Fig. S8B.C and in the Discussion. Nevertheless, we cannot rule out that a portion of the aberrant axons target other sites. Yet, figuring out the target sites along different hindbrain nuclei (or other CNS nuclei) will requires many more experiments at many different stages to identify targets which are currently unknown. We believe that the amount of work required for identifying unknown targets is not possible in the current situation and will be the scope of a future study.

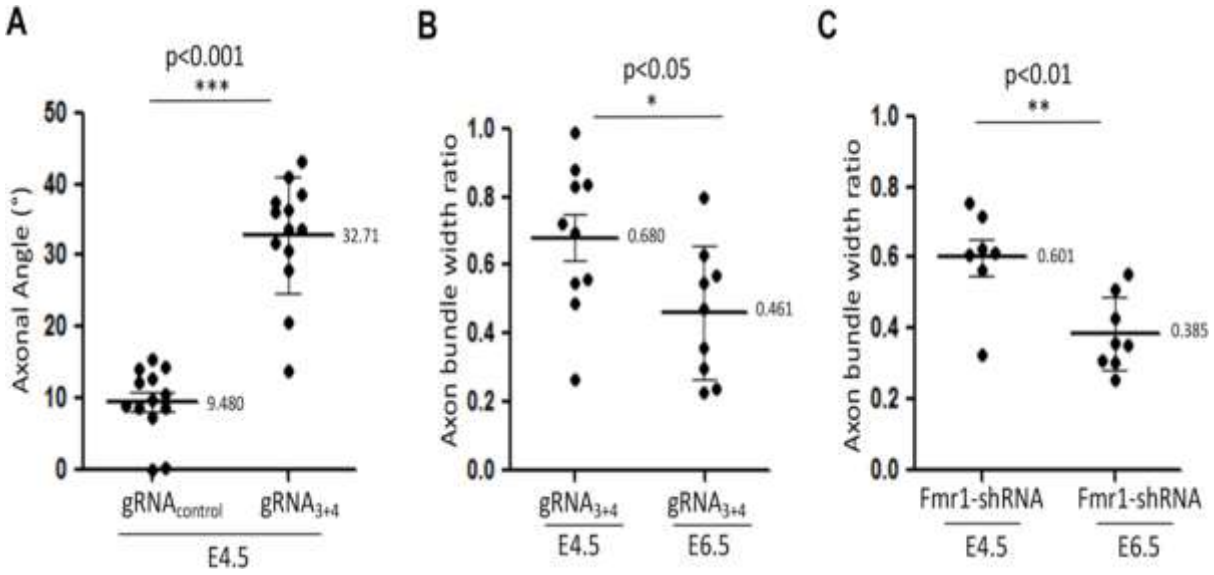


Figure S8. Additional data analyses in the Crispr-mediated FMRP knockout studies. A, Crispr-mediated FMRP knockout alters the angle of projecting axons. Box plot analysis of the angle measured between a projecting axon and the mantle zone plane from E4.5 embryos electroporated with gRNA$_{control}$ (n=7) or gRNA$_{3+4}$ using ImageJ software. Each data point represents a mean angle value of 8 projections. Measurements included 13 sections for each treatment from 5 different embryos. **B,** Box plot analysis of the width of the GFP^{+} axonal bundle measured in the circumferential axis in gRNA$_{3+4}$ electroporated embryos in E4.5 and E6.5. Each data point represents one embryo (n=10 at E4.5, n=9 at E6.5). The width of the axonal bundle is reduced at E6.5 in comparison to E4.5. **C**, Box plot analysis of the width of the GFP^{+} axonal bundle measured in the circumferential axis in Fmr1 shRNA electroporated embryos in E4.5 and E6.5. Each data point represents one embryo (n=7 at E4.5, n=8 at E6.5). The width of the axonal bundle is reduced at E6.5 in comparison to E4.5. For each plot, the mean value is indicated.

4. In figure 10 do the abnormal axon projections form synapses?

We have added two new analyses to address this question (line 337; Fig. 11) as following: “We next wanted to examine whether the aberrant NM axons form synapses. By dye-filling individual NL neurons, we found that EGFP+ axons were located immediately opposite to the dorsal dendrites of NL neurons (Fig. 11A-A’’). These EGFP+ axons were immunoreactive to synaptotagmin 2 (Syt2; Fig. 11B-B’’), a presynaptic vesicle calcium sensor for neurotransmitter release. Together, these observations demonstrate that the aberrant NM axons form synapses.”

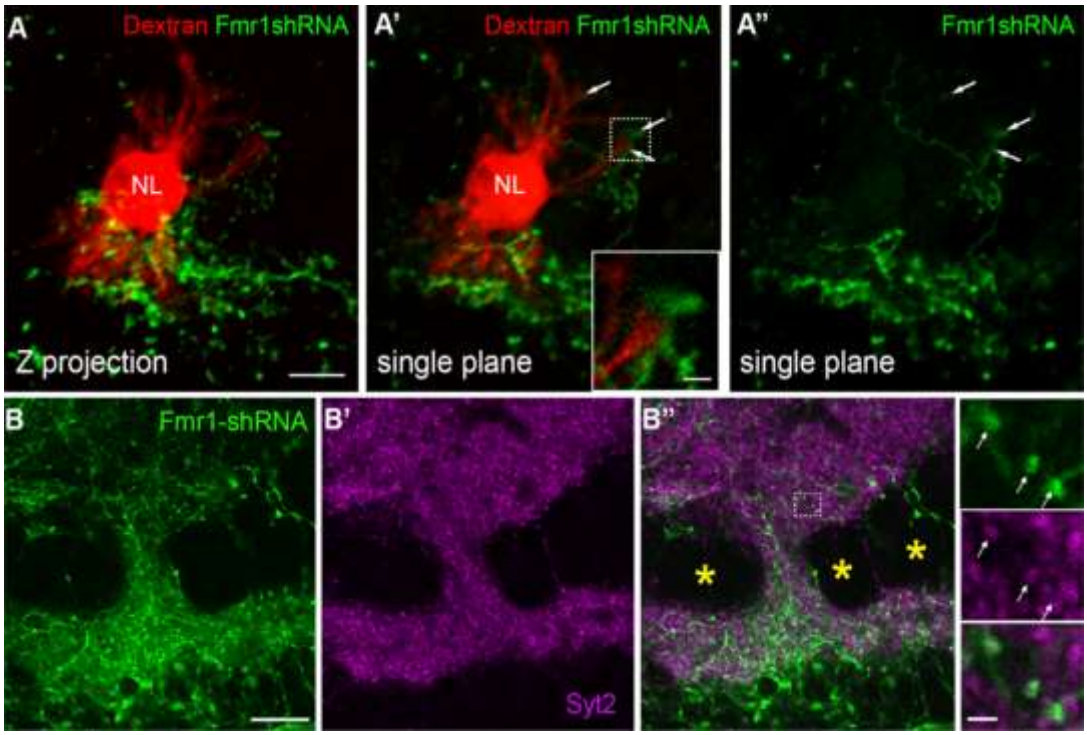


Figure 11. Aberrantly projected NM axons form synapses on NL dendrites. NM precursors were unilaterally transfected with Fmr1-shRNA-EGFP. Images were taken from the side contralateral to the transfection. **A-A''.** Images of a dye-filled NL neuron (red) whose dorsal and ventral dendrites are in close contact with EGFP+ NM axons (white arrows). **B-B''.** Double labeling of Syt2 immunoreactivity with EGFP+ NM axons. Stars indicate NL cell bodies in B''.
Higher-magnification views of the boxed area in I'' are represented in the right panels. EGFP+ axonal terminals (white arrows) are immunoreactive to Syt2. Scale bars: 10 µm in A, 2 µm in inset of A', 20 µm in B, and 2 µm in the right most column. Abbreviation: Syt 2, synaptotagmin 2.

Statistics: Student t-test not a very stringent test; are data normally distributed? A non-parametric test recommended.
We have now reanalyzed all comparisons using non-parametric tests (Mann-Whitney) and updated the results in the revision. These analyses did not change the conclusion.

Additional minor comments
-Figure 5E where is the inset coming from? Please indicate this by a box.
A box has been added to the figure.

-Some images are rather small, and a zoom in would be helpful.
We have resize and reorganize the figures to be compliant with the journal policy. We have reorganized a number of our figures and enlarged some of the images in response to this comment and following the *Formatting Guidelines* of the journal.

We hope that these explanations and modifications prove satisfactory, and wish to express our thanks to the editors and reviewers for their efforts.

Second decision letter

MS ID#: DEVELOP/2020/188797

MS TITLE: Temporal-specific roles of Fragile X mental retardation protein in the development of hindbrain auditory circuit

AUTHORS: Xiaoyu Wang, Ayelet Kohl, Xiaoyan Yu, Diego Zorio, Avihu Klar, Dalit Sela-Donenfeld, and Yuan Wang
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The main question of this study is to determine roles of FMRP in embryonic development of the auditory brainstem. This is an intriguing question because most previous studies have focused on FMRP functions during postnatal development. Authors well developed the system and carefully validated FMRP knockout and knockdown for studying this main question. Using chick embryo authors identified axonal location of FMRP, specifically at presynaptic terminals, during embryonic development. They also found the key roles of FMRP in axonal projection and targeting of NM neurons to NL. Understanding how FMRP functionally regulates axonal events in the developing brain is highly significant.

Comments for the author

In this revised manuscript, the authors well addressed reviewer's comments.

Reviewer 2

Advance summary and potential significance to field

The authors substantially improved the manuscript, provide adequate statistics and new quantifications. I see my concerns addressed and encourage publication.

Comments for the author

The authors substantially improved the manuscript, provide adequate statistics and new quantifications. I see my concerns addressed and encourage publication.