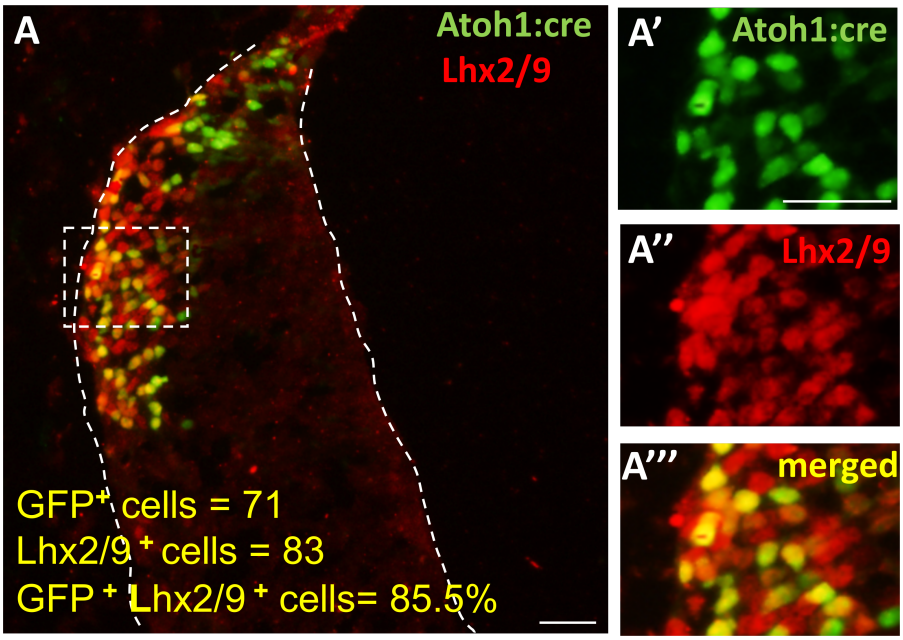


Figure S1. Atoh1:cre transfected cells are immunoreactive to Lhx2/9. Transverse section of E3.5 hindbrain (r5) that was electroplated at E2.5 with Atoh1:cre-nGFP plasmid and stained for Lhx2/9 to label endogenous dA1 cells. The dorsal half of the electroporated side of the neural tube is shown, demonstrating 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'.

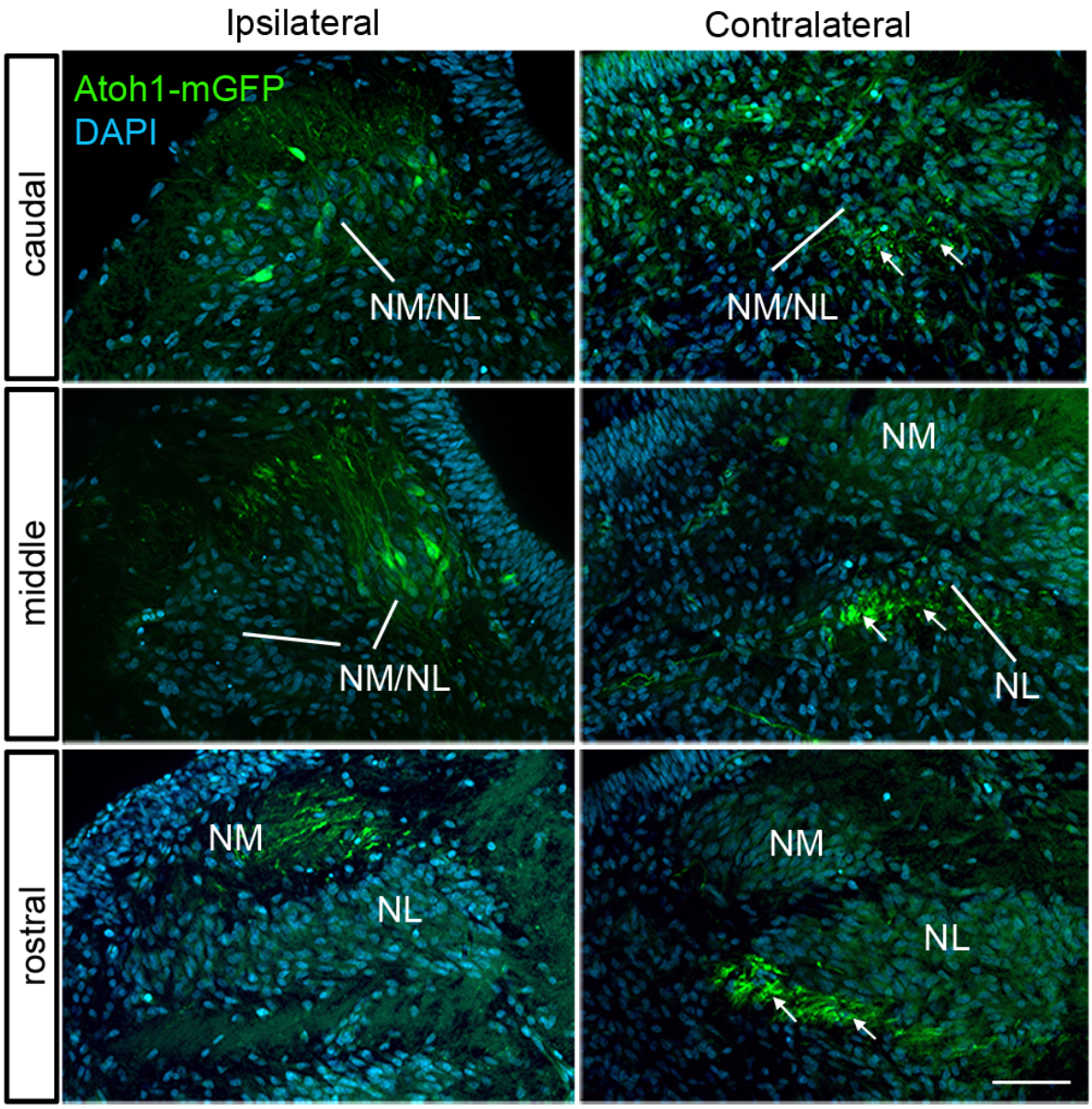


Figure S2. Separation of NM and NL at E7. Atoh1-mGFP labeling (green) at E7 on sections counterstained with DAPI (blue). At this stage, the NL and NM are migrating away from each other at the rostral level, while still merged at more caudal levels. White arrows point to the contralateral mGFP^{+} axons ventral to the NM/NL. Scale bars: 50 μm.

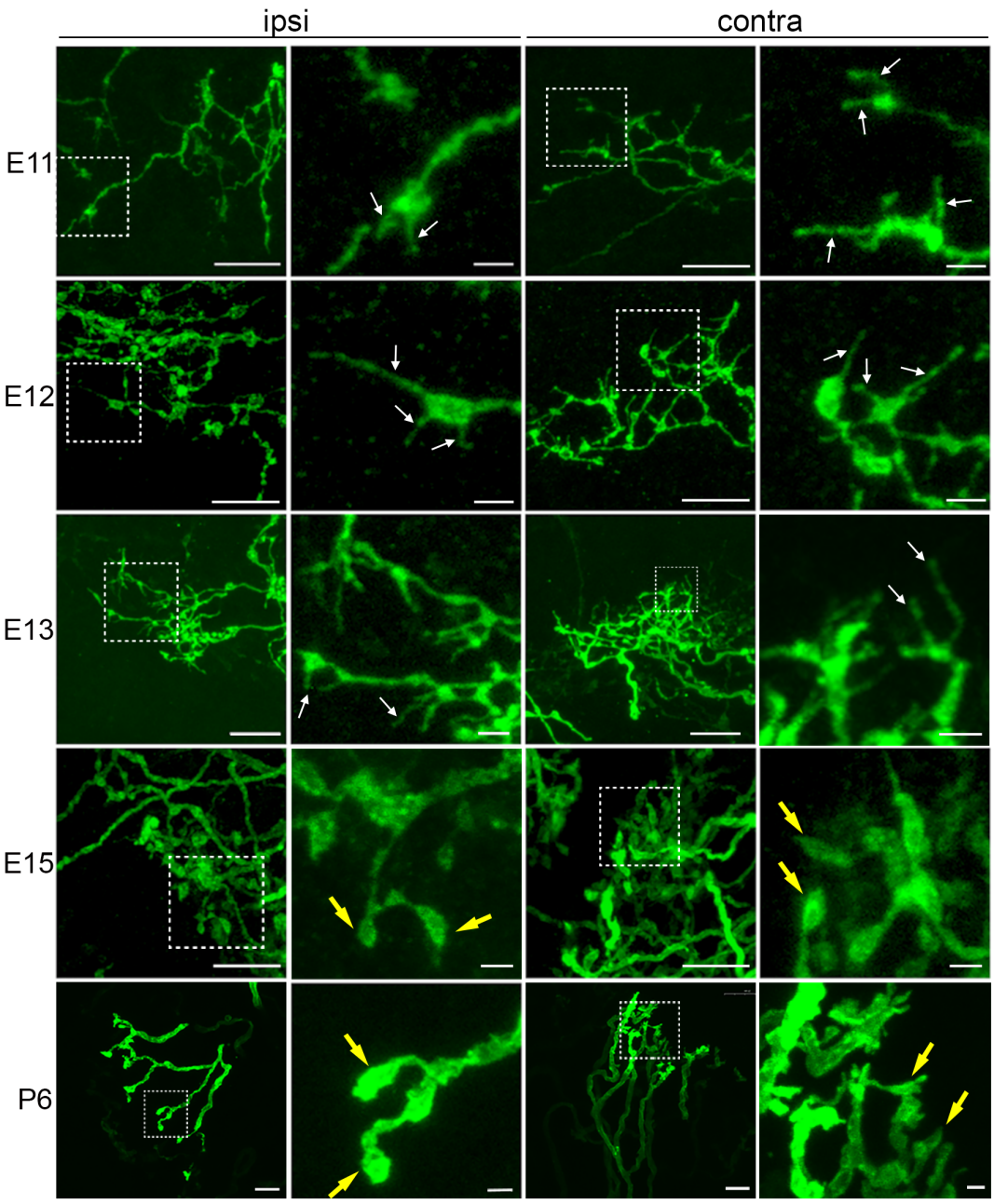


Figure S3. Morphological maturation of presynaptic terminals of NM neurons. Images were taken from embryos electroporated with Atoh1-mGFP at E2. The left two columns show NM axon terminals in the dorsal neuropil of the ipsilateral NL, while the right two columns show axons in the ventral neuropil of the contralateral NL. High-magnification views of the boxed areas appear in the right panel of each image. NM axons show a growth cone structure with filopodia (white arrows) at E11-13 and mature terminal morphology (yellow arrows) at E15 and later. Abbreviations: E, embryonic; P, post-hatch. Scale bars: 10 μm in lower-magnification images (columns 1, 3); 2 μm in higher-magnification images (columns 2, 4).

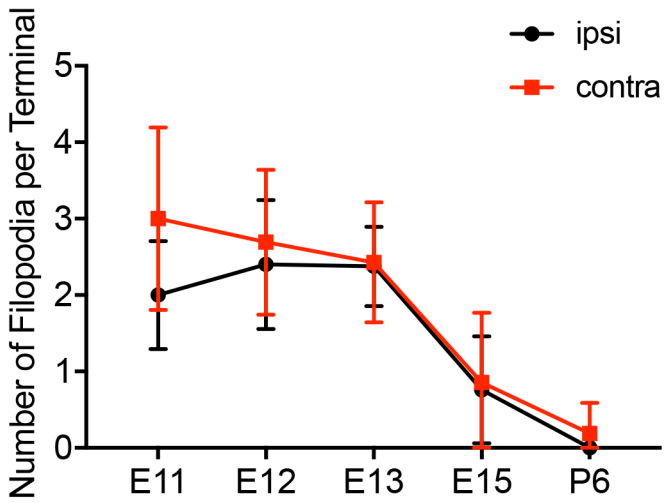


Figure S4. Quantification of terminal morphology at E11, E12, E13, E15 and P6.

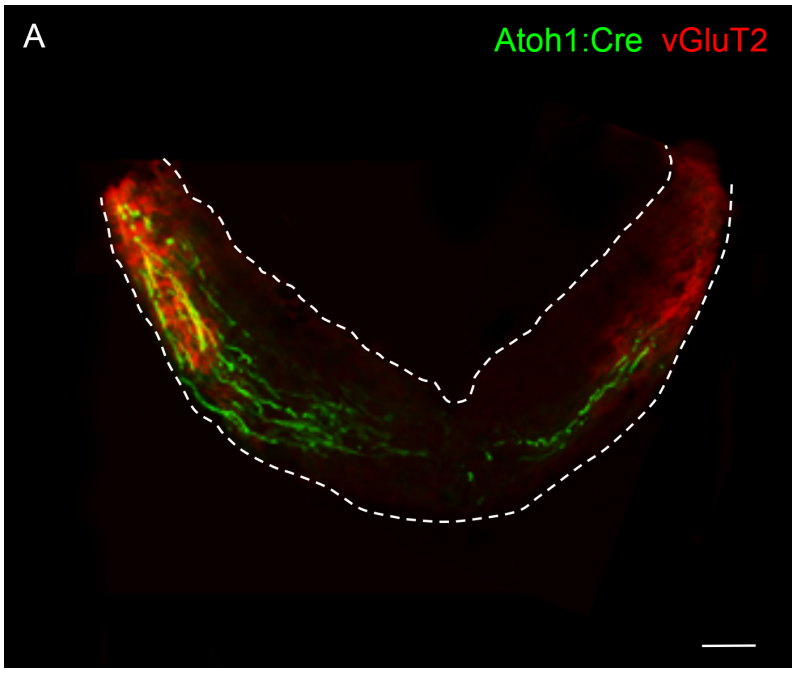


Figure S5. vGluT2 distribution in the axon course of Atoh1:cre transfected NM precursors. Transverse section of E4.5 hindbrain (r5) that was electroporated at E2.5 with Atoh1:cre-cGFP plasmid and stained for the vesicular glutamate transporter 2(vGluT2) (n=7 embryos). Scale bars: 200 μm in A.

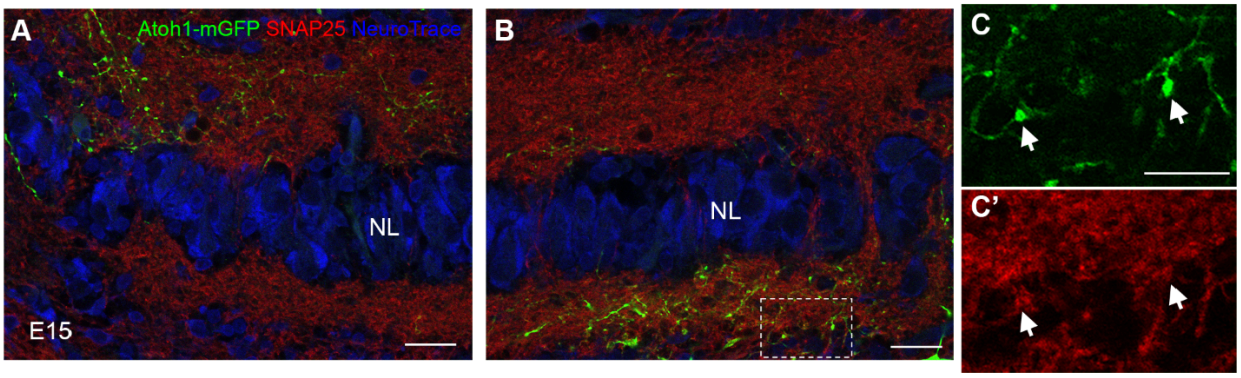


Figure S6. Atoh1-mGFP transfected axon terminals contain SNAP25. SNAP25 immunostaining was performed on the Atoh1-mGFP transfected E15 sections and counterstained with NeuroTrace. **A-B**: low magnification images of the ipsilateral (A) and contralateral (B) NL showing $mGFP^+$ axons in the dorsal and 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.

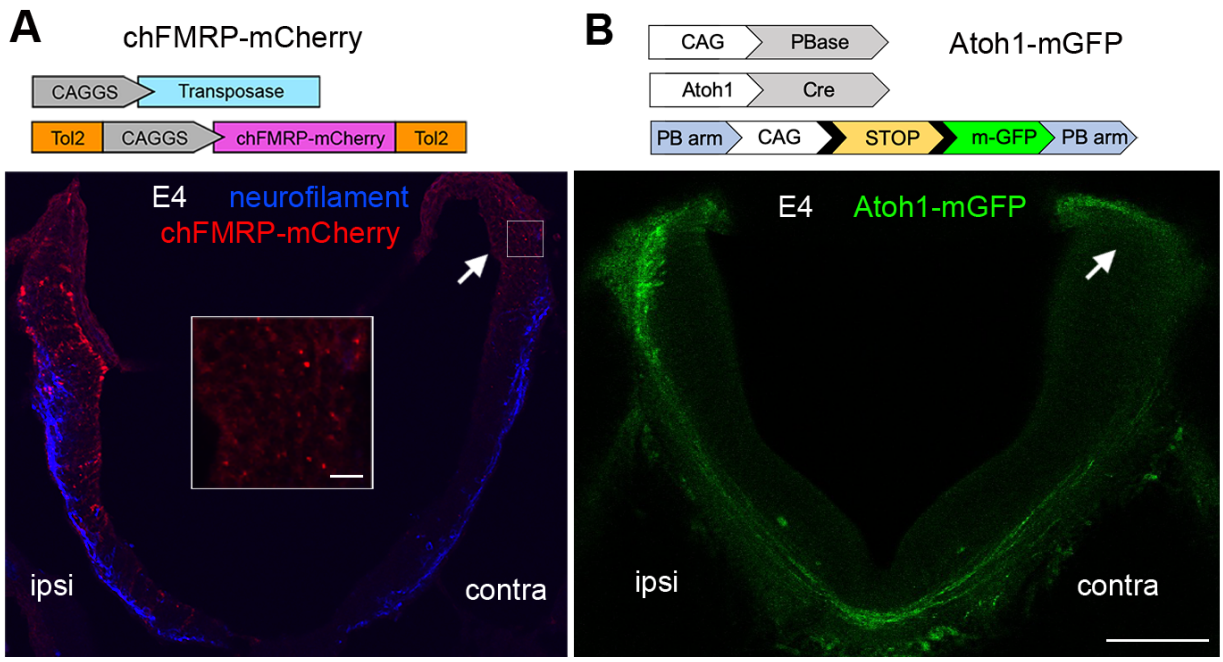


Figure S7. Axon localization of exogenous chicken FMRP (chFMRP) at E4. A. Transverse section from an E4 embryo electroporated with chFMRP-mCherry (red) at E2 and immunostained with neurofilament (blue), a marker for neuronal axons. mCherry^{+} puncta were detected in the dorsal hindbrain on the contralateral side (white arrow), where the axons of NM precursors terminate (see B). Inset shows an enlarged image of the boxed area. **B.** Transverse section from an E4 embryo electroporated with Atoh1-mGFP at E2 (green). mGFP^{+} axons extend contralaterally to the dorsal hindbrain, showing the approximate location of the auditory analogy (white arrow). Scale bars: 100 μm.

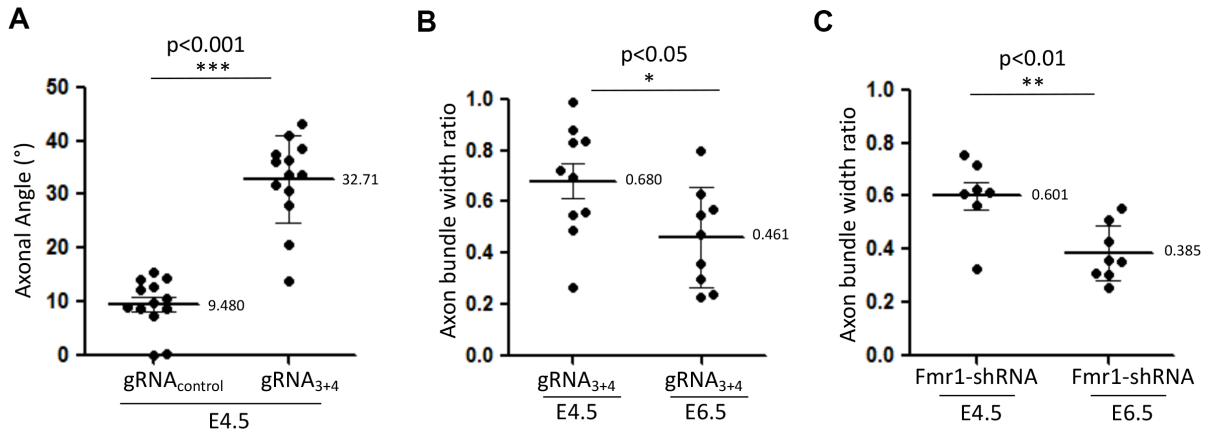


Figure S8. Additional data analyses in the Crispr-mediated FMRP knockout/knockdown studies. A, Crispr-mediated FMRP knockout alters the angle of projecting axons. Box plot analysis of the angle measured between a projecting axon and the mantle zone plane from E4.5 embryos electroporated with $gRNA_{control}$ (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.

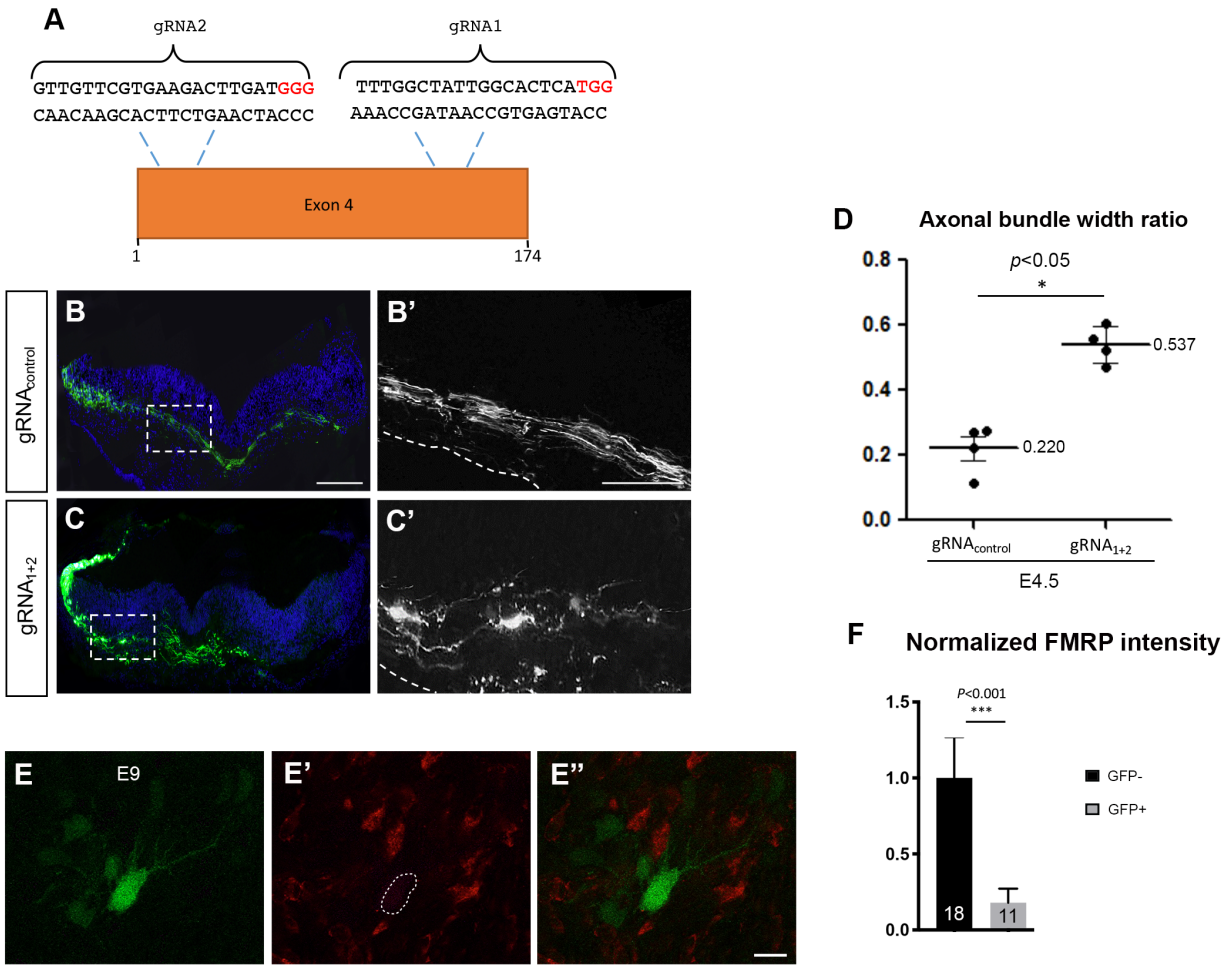
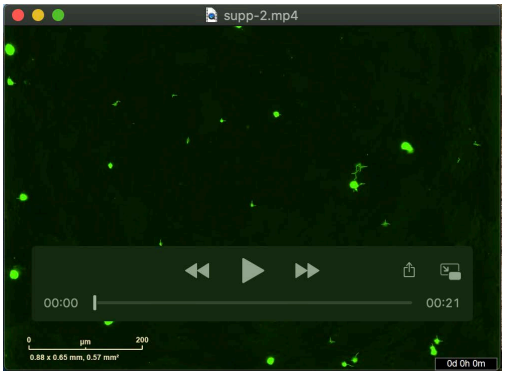


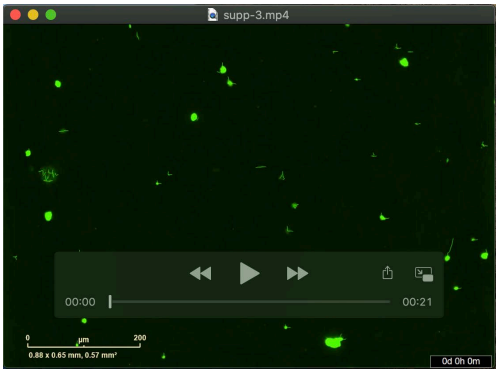
Figure S9. Crispr-mediated FMRP knockout induces axonal defasciculation. A. Crispr design of FMRP sequence in the region of exon 4. **B-C'.** Transverse sections of r5-6 level obtained from E4.5 embryos electroporated with $gRNA_{control}$ (B-B') or $gRNA_{1+2}$ (C-C') plasmids (n=4 embryos for each plasmid). Higher-magnification views of the boxed areas in the left panels (B,C) are represented in the right panels (B',C'). **D.** Box plot analysis of the width of the GFP^+ axonal bundle measured in the circumferential axis in $gRNA_{control}$ or $gRNA_{1+2}$ electroporated embryos. Each data point represents one section from one embryo. **E-E''.** Transverse sections from E9 embryos electroporated with $gRNA_{1+2}$ (green) and immunostained with FMRP antibody (red). The dashed circle surrounds to a transfected NM neuron that shows a loss of FMRP immunoreactivity as compared to neighboring non-transfected NM neurons. **F.** Bar graphs of normalized FMRP intensity, calculated as the corrected total cell fluorescence. Numbers on each bar indicate the number of neurons analyzed. Scale bars: 10 µm in B (applies to B and C), 50 µm in B' (applies to B' and C'), 10 µm in E'' (applies to E-E'').

Table S1. Plasmid sequences for gRNA production and validation.

	Exon of *Fmr1*	
G3	Exon8	GAGGTGGACCAACTACGTT
G4	Exon8	ACGTGGTCCAGGCTACGCTT
control		GGGTCTTCGAGAAGACCTG
Test-F3	Exon8	AGGTTGCTACCAGCTGTTGG
Test-F4	Exon8	TACTGCTATGAATAGCTCCTG
Test-R1	Exon8	GAAGCTATGTGCAAATATTAGCAG
Test-R2	Exon8	TTCTCATTGAACACTTGCATTTCC



Movie 1. Control cells



Movie 2. Manipulated cells

Movies 1 and 2. Time-lapse imaging of control and FMRP-knockout hindbrain cells in culture.

Time-lapse analysis of primary cell cultures prepared from entire hindbrains at E3.5. Hindbrains were electroporated at E2.5 with $gRNA_{control}$ plasmid (Movie 1) or $gRNA_{3+4}$ plasmid (Movie 2) to target the dA1 neuronal cell type. Cultures contained a mixture of electroporated (GFP^+) and non-electroporated cells. Cells were seeded in 48-well plates (n=6 wells for each treatment) and recorded every 6 hours for ~5 days. Time is indicated at the bottom right of the videos. In both movies, GFP^+ cells are clearly shown to extend neurites with time. Yet the neurite branching and extension rate is enhanced in Movie 2 compared to Movie 1. The corresponding still images from the time lapse imaging are presented in Fig. 9 of the main article.