© 2020. Published by The Company of Biologists Ltd | Development (2020) 147, dev188797. doi:10.1242/dev.188797

RESEARCH ARTICLE

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

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ABSTRACT

Fragile X mental retardation protein (FMRP) is an RNA-binding protein abundant in the nervous system. Functional loss of FMRP leads to sensory dysfunction and severe intellectual disabilities. In the auditory system, FMRP deficiency alters neuronal function and synaptic connectivity and results in perturbed processing of sound information. Nevertheless, roles of FMRP in embryonic development of the auditory hindbrain have not been identified. Here, we developed high-specificity approaches to genetically track and manipulate throughout development of the Atoh1$^+$ neuronal cell type, which is highly conserved in vertebrates, in the cochlear nucleus of chicken embryos. We identified distinct FMRP-containing granules in the growing axons of Atoh1$^+$ neurons and post-migrating NM cells. FMRP downregulation induced by CRISPR/Cas9 and shRNA techniques resulted in perturbed axonal pathfinding, delay in midline crossing, excess branching of neurites, and axonal targeting errors during the period of circuit development. Together, these results provide the first *in vivo* identification of FMRP localization and actions in developing axons of auditory neurons, and demonstrate the importance of investigating early embryonic alterations toward understanding the pathogenesis of neurodevelopmental disorders.

KEY WORDS: CRISPR-Cas9, Auditory circuit, Fragile X syndrome, Autism spectrum disorder, RNA-binding proteins, Axon development, Axon fasciculation, Axon targeting

INTRODUCTION

The fragile X mental retardation protein (FMRP; encoded by *FMR1*) is an RNA-binding protein that regulates many aspects of gene expression and protein function (Bagni and Greenough, 2005; Bassell and Warren, 2008; Davis and Broadie, 2017). Functional loss of FMRP during development leads to fragile X syndrome (FXS), an intellectual disability. Many FXS symptoms appear early in life, including increasing autism features and emerging sensory hyperarousal, anxiety and hyperactivity (Hagerman et al., 2017). These clinical observations, along with FMRP expression throughout gestation (Abitbol et al., 1993; Hinds et al., 1993), implicate a role of FMRP in embryonic and early postnatal brains. Although FMRP regulation of neurotransmission and synaptic plasticity plays important roles in relatively mature brains (Bagni and Zukin, 2019; Bear et al., 2004; Deng et al., 2013; Ferron et al., 2014), how FMRP regulates brain development during embryonic stages is largely unknown, except its involvement in cortical neurogenesis (Castrén, 2016).

Axon growth is a multi-event process of embryonic brain development, including axonogenesis, pathfinding, arborization, and establishment of terminals on appropriate postsynaptic structures (reviewed by Chédotal and Richards, 2010; Comer et al., 2019; Stoeckli, 2018). Multiple lines of evidence support an involvement of FMRP in axonal development. In the *Drosophila* mushroom body, FMRP limits axonal growth and controls axonal pruning (Bodaleo and Gonzalez-Billault, 2016; Pan et al., 2004; Tessier and Broadie, 2008). In vertebrates, FMRP knockout results in excessive axonal branches in zebrafish motor neurons (Shamay-Ramot et al., 2015) and abnormal projection patterns in the mouse forebrain (Bureau et al., 2008; Scharkowski et al., 2018). FMRP also associates with RNAs that encode proteins involved in axonogenesis and synaptogenesis, including the microtubule-associated protein MAP1b (Bodaleo and Gonzalez-Billault, 2016), cell adhesion molecule Dscam (Jain and Welshhans, 2016) and the axon guidance cue netrin (Kang et al., 2019). However, the exact *in vivo* functions of FMRP in distinct axonal events are unclear.

Here, we investigated the roles of FMRP in axonal development of the auditory brainstem using the chick embryo as a model system. The avian nucleus magnocellularis (NM) and nucleus laminaris (NL) are structurally and functionally similar to the mammalian anteroventral cochlear nucleus (AVCN) and medial superior olive (MSO), respectively. NM/AVCN neurons receive temporally precise excitation from the auditory nerve and, in turn, send bilaterally segregated signals to the NL/MSO. Bipolar neurons in the NL and MSO are specialized to compute interaural time differences (ITDs), time disparities in the arrival of signals between the two ears; these binaural cues are crucial for sound localization and segregation (Nothwang, 2016; Overholt et al., 1992; Vonderschen and Wagner, 2014). Clinical studies have revealed a tight association between FMRP level and temporal performance and have found impaired temporal processing of visual and auditory information in FXS (Farzin et al., 2011; Hall et al., 2009; Kéri and Benedek, 2011; Kogan et al., 2004; Rais et al., 2018). Cellular studies have further identified structural and physiological abnormalities in the AVCN and its target cell groups in FMRP knockout rodents (Brown et al., 2010; El-Hassar et al., 2019; Garcia-Pino et al., 2017; Lu, 2019; McCullagh et al., 2017; Rotschafer et al., 2015; Ruby et al., 2015; Strumbos et al., 2010; Wang et al., 2015a). Finally, the nucleotide and amino acid

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Handling Editor: Sally Dunwoodie
Received 30 January 2020; Accepted 29 June 2020

sequences of chicken FMRP are similar to human FMRP (Price et al., 1996; Wang et al., 2014). Thus, studying FMRP regulation of NM and NL neurons is functionally relevant for understanding FXS. Additionally, the stereotyped pattern of axonal projection from the NM to the NL (Fig. 1A) provides a suitable model for mechanistic studies of axonal circuitry development (Allen-Sharpley and Cramer, 2012; Cramer et al., 2004; Seidl et al., 2014).

To track specific cell types and neural circuits in complex vertebrate brains, we developed several genetic tools to selectively label and manipulate NM precursors and neurons in developing chicken embryos. We have identified an early-onset FMRP localization in axons of NM precursors and neurons, and discovered that FMRP is required for the orderly and timely development of multiple axon events. These findings provide insights into the potential contribution of compromised embryonic brain development to FXS pathogenesis.

RESULTS

Dissecting the axonal circuitry development of NM precursors and neurons

NM neurons project to the NL bilaterally (Fig. 1A). NL neurons are bipolar, with dendrites extending dorsally and ventrally from the soma to form two segregated dendritic domains. Cell bodies of NL neurons align into a single sheet, resulting in separate dorsal and ventral dendritic neuropil laminas. Individual NM axons bifurcate and project to the dorsal neuropil of the ipsilateral NL and the ventral neuropil of the contralateral NL. This segregated innervation pattern forms the anatomical substrate for ITD computation.

To label NM precursors and neurons selectively, we combined genetic markers with spatially controlled plasmid expression (Fig. 1B). The progenitor dA1 cells located along the dorsal-most region of the caudal rhombic lip express the basic helix-loop-helix transcription factor atonal homolog 1 (Atoh1), which gives rise to excitatory neurons in the auditory brainstem and precerebellar nuclei (Farago et al., 2006; Fujiyama et al., 2009; Helms et al., 2000; Machold and Fishell, 2005; Maricich et al., 2009). To enhance the specific labeling of the auditory neurons, we introduced a plasmid expressing the Atoh1-enhancer element upstream of Cre recombinase along with a Cre-dependent myristoylated-GFP (mGFP) reporter plasmid into rhombomeres 5-6 (r5-6), which contain NM and NL precursors, via *in ovo* electroporation (Avraham et al., 2009; Cramer et al., 2000; Helms et al., 2000; Kohl et al., 2012, 2013; Lipovsek and Wingate, 2018; Fig. S1). The electroporated Cre-conditional mGFP sequence was integrated into the chick genome by applying the PiggyBac transposition method (Wang et al., 2009), allowing prolonged expression of the reporter in the auditory neurons (Hadas et al., 2014; Lu et al., 2009). For more restricted NM labeling, we performed the electroporation at embryonic day (E) 2-2.5, before NL cells are born (Rubel et al., 1976). Following electroporation, mGFP$^+$ cell bodies exhibited a restricted distribution in anatomically defined NM on the transfected side when examined at later stages (Fig. 1C). Axons of mGFP$^+$ cells originated from the NM and projected to the NL bilaterally, exhibiting the characteristic pattern of NM-NL projection (Fig. 1C,D). The transfection rate, calculated as the percentage of mGFP$^+$ neurons among all neurons in the NM, was 15.3%±10.3% (mean±s.d.; *n*=8 embryos) ranging from 3.4% to 34.4% (Fig. 1E). No mGFP$^+$ cells were detected in the contralateral NM, NL, or surrounding brainstem regions. Thus, our genetic targeting of Atoh1-mGFP cells was predominantly the NM precursors, termed ‘Atoh1 precursors of NM’ henceforth, that establish the NM-NL circuit.

Next, we examined the development of the NM circuit stage by stage. We previously demonstrated that Atoh1/dA1 cells across r2-7 give rise to two contralateral axon projections (Kohl et al., 2012, 2015). One projection originated from the caudal hindbrain and elongated in a dorsal funiculus (DF), whereas the other arose from the more anterior hindbrain and formed a lateral funiculus (LF; Fig. 2A). The Atoh1 precursors of NM located at r5-6 extended their axons within the DF bundle (Fig. 2B). On transverse sections at E4.5, mGFP$^+$ axons had crossed the midline and arrived at the location where the NM and NL will form (Fig. 2C, yellow arrow), as indicated by a midline crossing rate of 1.060 (*n*=7 embryos; Fig. 2D,E). On the ipsilateral side, mGFP$^+$ axons formed a well-defined dorsal-to-ventral fascicule (Fig. 2C, white arrow), confirmed quantitatively by a small axonal bundle width ratio (0.259, *n*=7 embryos; Fig. 2D,F). At E7, the NL was separating from the NM with rostral-to-caudal progress (Fig. S2), consistent with a previous report (Hendricks et al., 2006). mGFP$^+$ axons arrived at the emerging NL on the contralateral side. In contrast, the ipsilateral projection was not visible, which is consistent with the results of individual axonal reconstructions that showed no ipsilateral projection until E8 (Young and Rubel, 1986). At E9 and later, the NM and NL were recognizable as individual nuclei. The ipsilateral projection of mGFP$^+$ cells to the dorsal neuropil of NL had formed, revealing the characteristic bilateral NM-NL

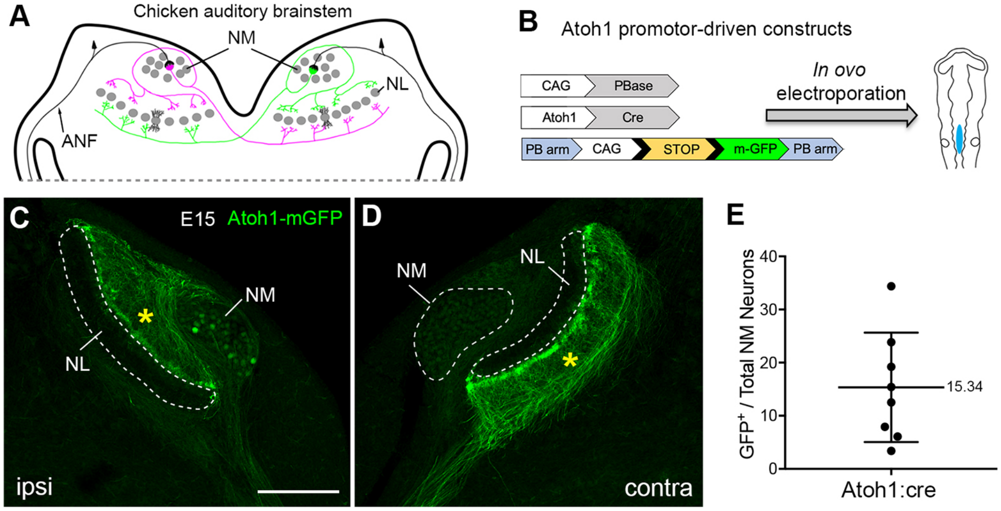

Fig. 1. High-specificity genetic labeling of NM precursors and neurons. (A) Schematic of the NM-NL circuit. (B) Plasmid design for Atoh1-mGFP. Electroporation was performed following plasmid injection into rhombomeres 5-6 (r5-6; blue). (C) E15 brainstem sections showing a restricted localization of mGFP$^+$ cell bodies in the NM on the transfection (ipsi) side. Yellow asterisks indicate bilateral NM axons to NL. (E) Proportion of transfected neurons in the NM. The mean value±s.d. are indicated for this and all plots in subsequent figures. ANF, auditory nerve fiber; contra, contralateral; ipsi, ipsilateral. Scale bar: 200 µm.

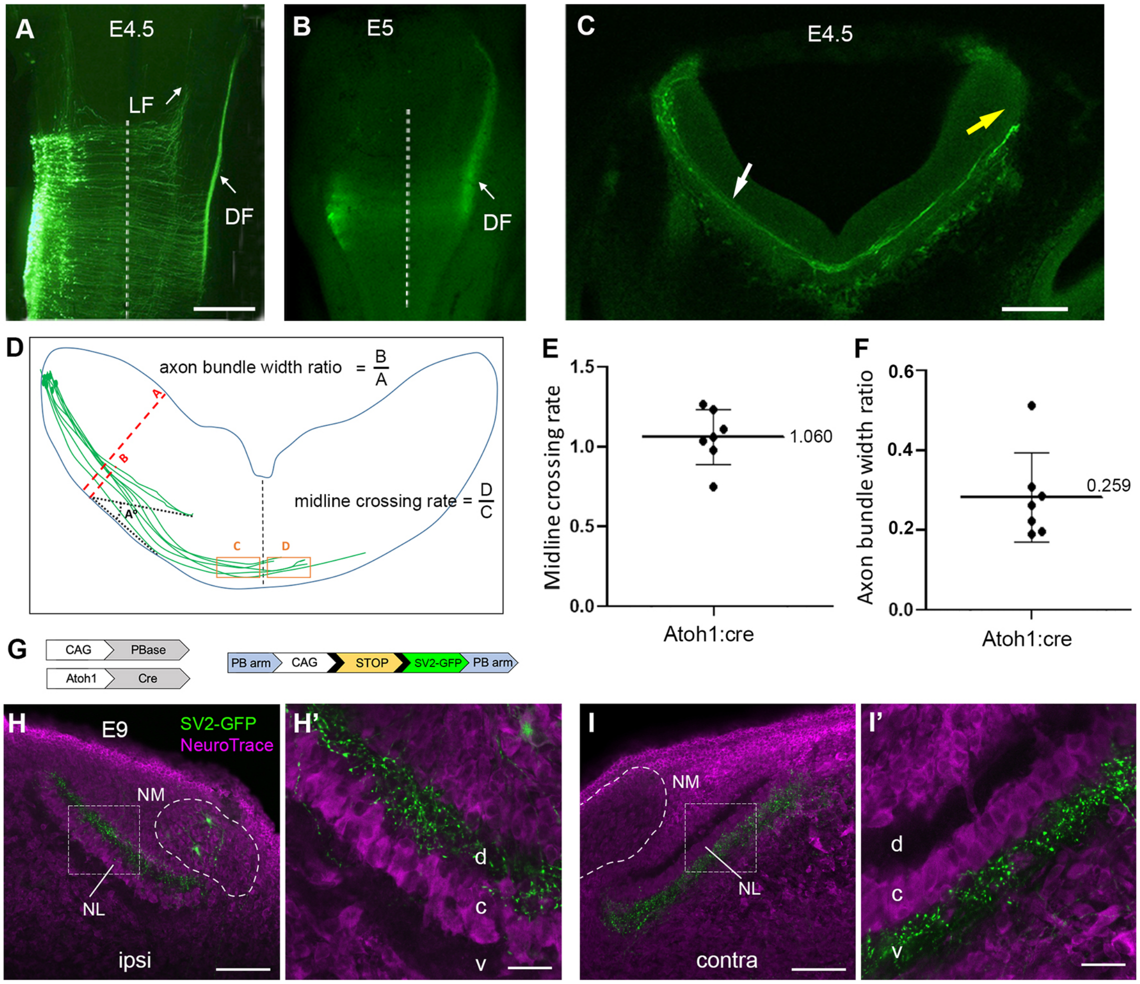

Fig. 2. Axon development of NM precursors and neurons. Images were taken from embryos electroporated with Atoh1-mGFP at E2-2.5. (A) Flat-mount view at E4.5 showing two contralateral projection bundles (LF and DF) of Atoh1/dA1 cells. (B) Top view at E5 showing that axons of Atoh1/dA1 cells at r5-6 join the DF bundle. Dashed lines indicate the midline in A and B. (C) Transverse section at E4.5 at the level of r5-6. mGFP$^+$ axons have crossed the midline and arrived in their contralateral target area (yellow arrow). White and yellow arrows indicate the axon bundle at the ipsilateral and contralateral sides, respectively. (D) Illustration describing the measurements used to quantify axonal growth patterns of NM precursors. Axon bundle width was calculated as the ratio of 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° is 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). (G) Plasmid design for SV2-GFP with Atoh1-enhancer and PiggyBac (PB) transposase. (H-I′) SV2-GFP (green) distribution in transverse sections counterstained with NeuroTrace (magenta) on the ipsilateral (H,H′) and contralateral (I,I′) sides. H′ and I′ are enlarged views of the boxes in H and I, respectively. NM is outlined by dashed circles. The cell body layer (c) as well as the dorsal (d) and ventral (v) dendrite domains of the NL are indicated. LF, lateral funiculus; DF, dorsal funiculus. Scale bars: 1 mm in A (applies to A,B) and C; 100 µm in H,I; 20 µm in H′,I′.

projection (Fig. S2). To confirm this connectivity at the synaptic level, E2 embryos were electroporated with SV2-GFP along with Atoh1 enhancers and the PiggyBac transposase (Fig. 2G), enabling the expression of GFP in presynaptic vesicles (Hadas et al., 2014; Kohl et al., 2012). SV2-GFP was detected in the dorsal NL ipsilaterally and ventral NL contralaterally at E9 (Fig. 2H-I′), confirming the segregated synaptic projection from Atoh1-NM neurons to the NL.

Closer examination of NM axons within NL revealed a stage-dependent terminal maturation (Fig. 3). At E11-13, the incoming NM axons ended with a typical growth cone morphology with one to five filopodia (Fig. 3, white arrows). These filopodia gradually disappeared and turned into bouton endings at E15 (Fig. 3, yellow arrows). By post-hatch day (P) 6, NM axons exhibited a mature terminal morphology (Figs S3 and S4). Immunostaining demonstrated a distribution of vesicular glutamate transporters (vGluT2) along the axon course of Atoh1 precursors of NM at E4.5 (Fig. S5). At E15, NM axonal terminals contain a presynaptic SNARE component, SNAP25 (Fig. S6), indicating functional synapses. The time frame of the terminal morphological change was similar between the ipsilateral and contralateral projections of NM neurons, which indicates that the maturation of presynaptic terminals from the two NM inputs to NL neurons is temporally synchronized, although the two inputs differ in their time of arrival at the target area.

Axonal localization of FMRP in NM precursors and neurons

FMRP is strongly expressed in hindbrain (Fig. 4A). It is not known whether FMRP is localized in NM axons, and if so, when this localization emerges during development. Here, we addressed this question by immunostaining endogenous FMRP and localizing exogenous FMRP. Embryos were electroporated with Atoh1-mGFP at E2. At E4-5 (*n*=5 embryos), mGFP$^+$ cells consistently showed somatic FMRP immunoreactivity (Fig. 4B-C″). Contralaterally,

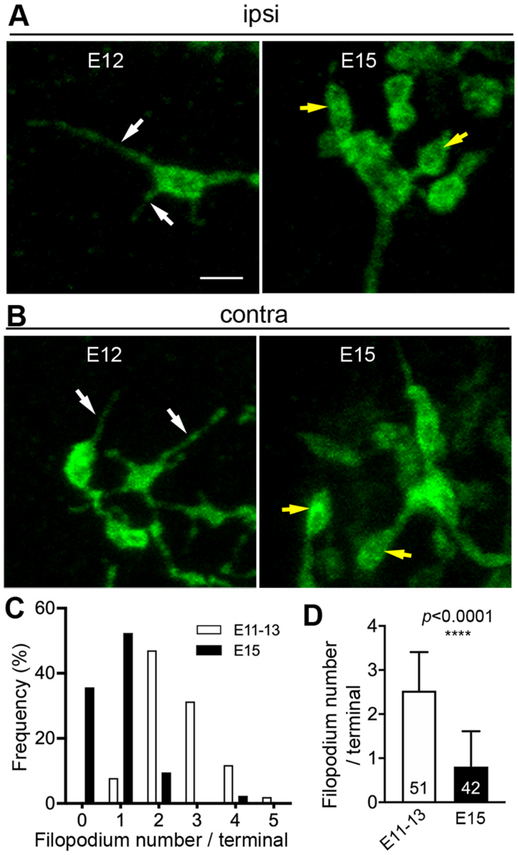

Fig. 3. Morphological maturation of presynaptic terminals of NM neurons. Images were taken from embryos electroporated with Atoh1-mGFP at E2. (A,B) NM axon terminals in the dorsal neuropil of the ipsilateral NL (A) and in the ventral neuropil of the contralateral NL (B) at E12 and E15. NM axons show a growth cone structure with filopodia (white arrows) at E12 and bouton-like terminals (yellow arrows) at E15. (C,D) Frequency distribution (C) and population analysis (D) 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 and S4. Scale bar: 2 µm.

mGFP$^+$ axons terminated in a cell-free region where FMRP staining was generally low (Fig. 4B-B″, yellow arrows). Closer observation demonstrated distinct FMRP puncta in this region (Fig. 4D′). These puncta were 0.2-0.7 µm in diameter, with an average density of 4.3 puncta per 100 µm^2 (28 sections from 5 embryos). A subset of FMRP puncta overlapped with mGFP$^+$ axon processes (Fig. 4D-E), confirming FMRP localization in distal axons of NM precursors.

We next determined whether FMRP is localized in NM axons at late embryonic stages when they have formed synaptic connectivity with NL neurons. During this time window (E9 to E19), the neuropil regions of NL contain a mixture of NM axons, NL dendrites, and astrocyte processes. We developed a transposon-based vector system expressing chick FMRP (chFMRP) fused with mCherry (Fig. 5A) for constitutive expression (Schecterson et al., 2012). At E4, mCherry$^+$ puncta were identified in the fibrous area where contralateral axons of NM precursors terminate (Fig. S7), consistent with the localization of endogenous FMRP puncta shown in Fig. 4. We co-electroporated E2 embryos with chFMRP-mCherry and Atoh1-mGFP (Fig. 5B) and harvested brainstem sections between E9 and E19 (n=13 embryos). A substantial number of NM cells expressed chFMRP-mCherry on the transfection side (Fig. 5C, left column). In addition, mCherry$^+$ NL neurons were seen on the same side in some cases. To avoid this confounding factor, further analyses were performed in the contralateral NL in which mCherry labeling was exclusively derived from transfected NM axons. Across all cases, mCherry$^+$ puncta were identified in the fiber region between the NL and the ventral brainstem, which contains incoming NM axons, as well as within the ventral neuropil domain of the NL (Fig. 5C, right column). This localization pattern indicates that the introduced chicken FMRP is localized in the distal portions of NM axons. This was further confirmed by the presence of mCherry$^+$ puncta in Atoh1-mGFP expressing axons (Fig. 5D). Next, we replaced chFMRP-mCherry with human FMRP (hFMRP)-EGFP in the plasmid (Fig. 5A) and identified a similar pattern of FMRP distribution (Fig. 5E). This result indicates that the sequence of FMRP coding for its axon localization in NM axons is conserved between birds and humans.

FMRP deficiency affects axonal growth pattern of NM precursors

In vitro studies implicate FMRP regulation in neurite outgrowth (Doers et al., 2014), axon elongation (Wang et al., 2015b), and branching (Zimmer et al., 2017). Together with our finding that Atoh1 precursors of NM contain FMRP in distal axons (Fig. 4), these studies raise the possibility that FMRP regulates axonal growth and pathfinding of NM precursors *in vivo*. We examined this possibility by determining the effects of downregulating FMRP on axon development of Atoh1 precursors of NM.

CRISPR-mediated FMRP knockout

We first downregulated FMRP in Atoh1$^+$ neurons using the CRISPR/Cas9 system (Cong et al., 2013; Hille and Charpentier, 2016). Two guide RNAs (gRNA$_3$ and gRNA$_4$) were designed to target exon 8 of the FMRP coding sequence to cause a deletion of ∼260 bp (Fig. 6A; Table S1). To verify this deletion, gRNA$_{3+4}$ plasmids, which contain Cas9 and GFP on the same pCAG-construct, were co-electroporated into the dorsal-most region of E2.5 embryos. Control embryos were electroporated with a control-gRNA construct (gRNA$_{\text{control}}$; Table S1). Both gRNA$_{\text{control}}$ and gRNA$_{3+4}$ electroporated embryos demonstrated a 459 bp fragment of the size of the intact *Fmr1* sequence, but gRNA$_{3+4}$ embryos also presented a lower-size band of 260 bp (Fig. 6B, red arrow), which reflects the deletion of ∼200 bp in electroporated cells. Next, we confirmed that this deletion prevents FMRP synthesis. At E6.5 (n=7 embryos), the majority of GFP$^+$ cells (80%) were FMRP immunoreactive in embryos electroporated with gRNA$_{\text{control}}$ (Fig. 6C-C″, arrows). In contrast, only 10% of GFP$^+$ cells expressed FMRP following gRNA$_{3+4}$ expression (Fig. 6D-D″, arrowheads; Fig. 6E). Finally, we confirmed that expression of gRNA$_{\text{control}}$ and gRNA$_{3+4}$ plasmids was confined to dA1 neurons, showing the overlapping expression of GFP with Lhx2/9 (Fig. 6F-H), a specific marker for dA1/Atoh1$^+$ interneurons (Bermingham et al., 2001; Gray, 2013; Kohl et al., 2012).

FMRP knockout induces axon growth defects

To examine whether FMRP knockout affects dA1 axonal projections, embryos were electroporated with RNA$_{\text{control}}$ or gRNA$_{3+4}$ CAG plasmids at E2.5 and harvested at E4.5 (n=7-10 embryos for each plasmid) and E6.5 (n=6-9 embryos for each plasmid). These time points encompass the period during which dA1 interneurons extend their axons along a well-defined dorsal-to-ventral fascicule, cross the midline, and project in a parallel ventral-to-dorsal trajectory until reaching the contralateral auditory nuclei anlage (Fig. 2C; Kohl et al., 2012). As expected, flat-mount views of E4.5 control embryos exhibited this typical trajectory of dA1 axons that cross the midline (Fig. 7A,A′, arrows), indicating

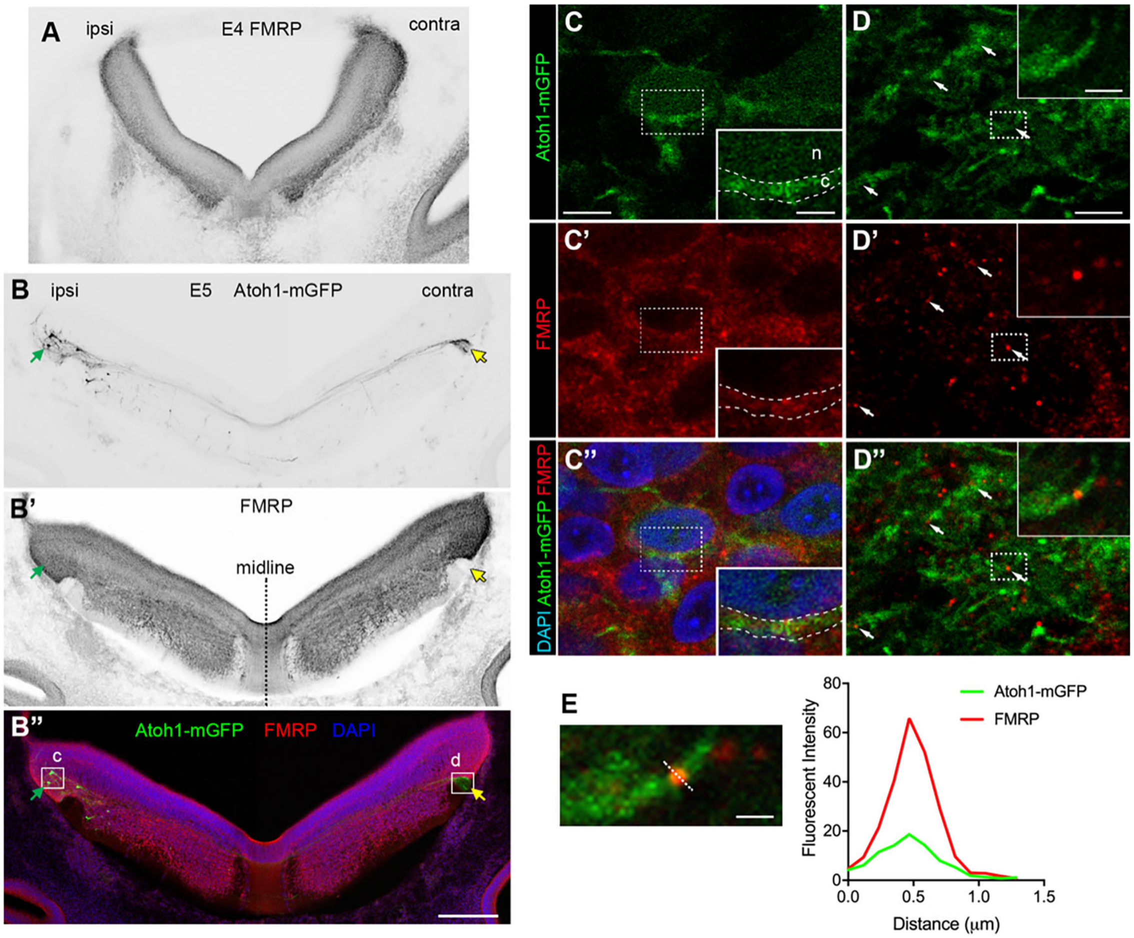

Fig. 4. Endogenous FMRP is localized in distal axons of NM precursors. (A) FMRP immunostaining of an E4 embryo. (B-B″) FMRP immunostaining (B′) of an E5 embryo transfected with Atoh1-mGFP (B). B″ is the merged image. Two photomicrographs from the left and right halves of the section were manually tiled for the whole view in B-B″. Note the FMRP immunostaining in the region where transfected cell bodies are located (green arrows). The terminal region on the contralateral side (yellow arrows) is low in FMRP immunoreactivity. (C-C″) High-magnification images of the box in B″ from the transfection (ipsi) side. Transfected cells (green) contain FMRP immunoreactivity (red) in the cytoplasm (c in insets) and a weaker staining in the nuclear (n in insets). (D-D″) High-magnification images of the box in B″ from the contralateral side (contra). A subset of FMRP puncta (arrows) 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. Dashed line indicates the region of interest for colocalization analysis. Scale bars: 100 μm in A; 200 μm in B″ (applies to B-B″); 5 μm in C,D (applies to C-D″); 2 μm in insets; 1 μm in E.

that gRNA$_{\text{control}}$ expression did not affect axonal growth. Observations from transverse sections further demonstrated that these axons projected in a fasciculated lateral bundle in the ipsilateral route and projected to the contralateral side (Fig. 7C,C′, arrows). Strikingly, many gRNA$_{3+4}$-expressing axons did not extend toward the floor plate and showed disorganized ipsilateral routes (Fig. 7B,B′, dashed arrows). Observations from transverse sections confirmed that axons projected ventrally in a broad mediolateral pattern rather than in a directional ventrolateral route, as well as extending medially toward the ventricle (Fig. 7D-E′, arrowheads). Quantitative analyses (as illustrated in Fig. 2D) revealed that the width of the GFP$^+$ axonal bundle, measured in the circumferential axis, was significantly greater in gRNA$_{3+4}$-electroporated embryos than the control embryos (Fig. 7H; non-parametric $P<0.001$; Mann–Whitney U-test for this and all following comparisons). In addition, the angle of individual axons in relation to the mantle zone angle of the neural tube (Fig. 2D) was significantly increased following FMRP knockout ($P<0.0001$; Fig. S8A). This randomized axonal growth phenotype persisted in E6.5 embryos (Fig. 7G,G′, arrowheads) as opposed to control embryos (Fig. 7F-F′, arrows, 7I; $P<0.001$), but at a significantly reduced degree compared with E4.5 (Fig. S8B; $P<0.05$). To further validate the effect of FMRP knockout using the CRISPR/Cas approach, we designed an additional set of guide RNAs (gRNA$_1$ and gRNA$_2$) to target exon 4 of FMRP (Fig. S9A). Electroporation of gRNA$_{1+2}$ plasmids demonstrated significant disorganized growth of NM-GFP$^+$ axons ($P<0.05$; Fig. S9B-D) as well as loss of FMRP immunoreactivity in the electroporated cells (Fig. S9E-F). Together, these results indicate that FMRP is required for the directed growth of NM precursor axons in a tight dorsal-to-ventral fascicule.

In addition to the disoriented pattern of axonal growth, possibly due to axon defasciculation, fewer axons crossed and progressed to the contralateral side following FMRP knockout on flat-mount views of E4.5 embryos (Fig. 7B,B′). Observations from transverse sections confirmed that fewer axons reached the level of the floor plate (Fig. 7E, arrows). We evaluated the rate of midline crossing by calculating the ipsilateral/contralateral ratio of GFP$^+$ axons of the same transverse section, as described in Fig. 2D. At E4.5, the majority of GFP$^+$ axons crossed the midline in control embryos, whereas less than half extended contralaterally following FMRP knockout (Fig. 7J; $P<0.01$). Yet, 2 days later at E6.5, the majority of

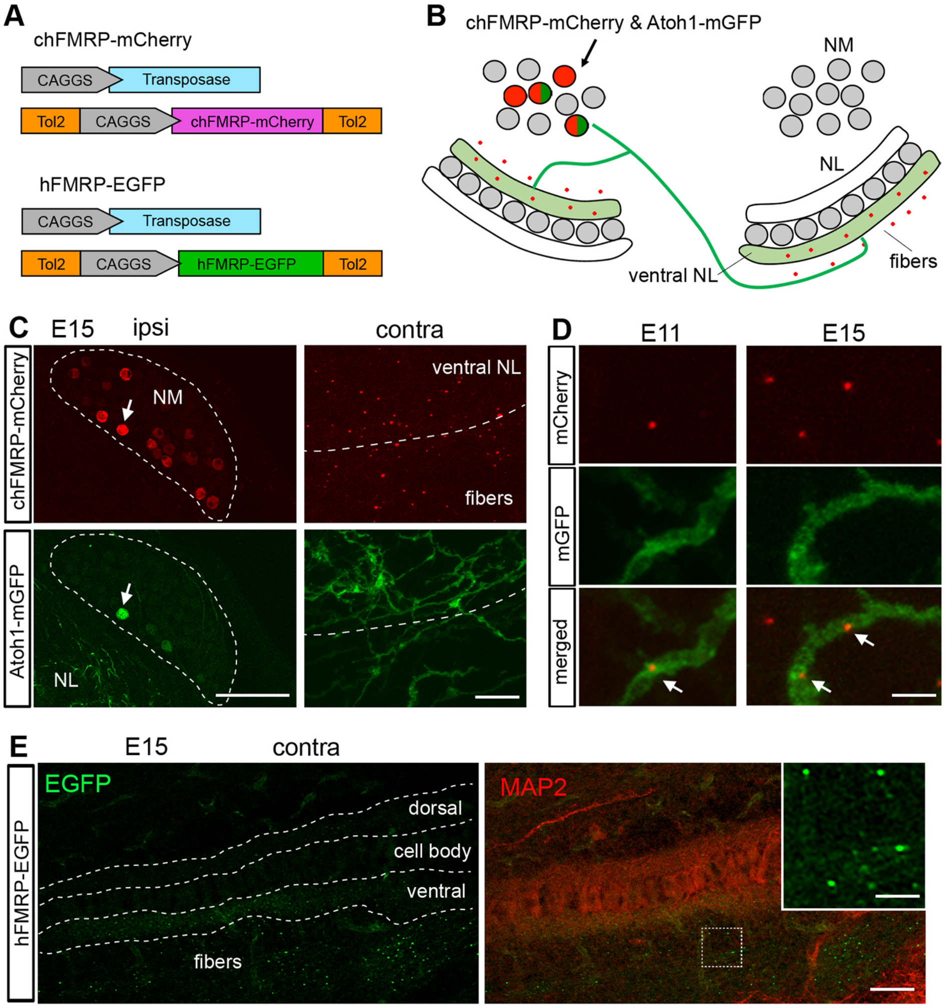

Fig. 5. Axon localization of FMRP in NM neurons. (A) Plasmid designs for constitutive expression of chicken and human FMRP (chFMRP and hFMRP). (B) Schematic of the co-transfection protocol for chFMRP-mCherry and Atoh1-mGFP. NM cells are transfected either with chFMRP-mCherry only (red circles) or with both plasmids (half red and half green circles). This co-transfection protocol yields very few NM cells transfected with Atoh1-mGFP only. Grey circles indicate nontransfected neurons in NM and NL. Green lines indicate axons of Atoh1-mGFP-labeled NM cells on the dorsal NL ipsilaterally and ventral NL contralaterally (green). chFMRP-mCherry-labeled puncta are indicated as small red points in these two NL neuropil regions. mCherry-labeled puncta are also located in the fiber regions adjacent to NL neuropil regions. (C) Transverse sections at E15 showing transfected cell bodies in the NM (left column) and their contralateral projection in the NL (right column), following the co-transfection shown in B. White arrows indicate a co-transfected NM neuron. On the contralateral side, chFMRP-mCherry puncta are detected within the ventral neuropil domain of NL as well as the fiber region containing incoming NM axons. (D) High-magnification images of the ventral neuropil of the contralateral NL at E11 and E15. A subset of FMRP-mCherry puncta are located in Atoh1-mGFP$^+$ NM axons (arrows). (E) Images of the contralateral NL at E15 following transfection with hFMRP-EGFP and MAP2 counterstaining (red), a somatodendritic marker. hFMRP puncta are distributed in the ventral fiber region and the ventral NL neuropil. Inset shows a high magnification of the boxed area. MAP2, microtubule-associated protein 2. Scale bars: 100 µm in C, left column; 20 µm in C, right column; 5 µm in D; 50 µm in E; 7.5 µm in inset.

GFP$^+$ axons had crossed the midline in gRNA$_{3+4}$-electroporated embryos (Fig. 7G, arrows), similar to control embryos (Fig. 7F, arrows; Fig. 7K; P=0.645). This observation demonstrates that FMRP knockout induces a delay in axons reaching the floor plate but axons maintain the ability to cross the midline.

FMRP knockdown induces axon growth defects

We next examined whether a partial reduction in FMRP expression affects the axonal growth pattern using a shRNA method. *Fmr1* and control (scrambled) shRNAs were cloned into a transposon-based vector system with a *Tol2* vector containing doxycycline (Dox) regulatory components and an EGFP reporter (Wang et al., 2018), enabling Dox-dependent temporal control of gene expression. We electroporated *Fmr1* and scrambled shRNA plasmids into E2.5 hindbrains, triggered shRNA expression with Dox treatment immediately following the electroporation, and fixed embryos at E4.5 and E6.5 (n=6-8 embryos for each plasmid at each stage). As expected, the scrambled-shRNA group exhibited the typical dA1 projecting pattern (Fig. 8A,A′,C,C′,F,F′, arrows). Embryos expressing *Fmr1*-shRNA, however, showed profoundly aberrant axons (Fig. 8B,B′, dashed arrows), similar to the effect of FMRP knockout. Transverse section views confirmed that many *Fmr1*-shRNA-EGFP$^+$ axons projected randomly toward the ventricular zone or toward the midline in a disorganized manner (Fig. 8D,D′,E,E′,G,G′, arrowheads), in stark contrast to the organized and directional pattern in control embryos (Fig. 8C,C′,F,F′). The width of *Fmr1*-shRNA-GFP$^+$ axons was significantly larger than that of control axons at both E4.5 (Fig. 8H; P<0.01) and E6.5 (Fig. 8I; P<0.05). Nevertheless, similar to the effect of gRNA$_{3+4}$ expression, the axonal bundle width at E6.5 was reduced compared with E4.5

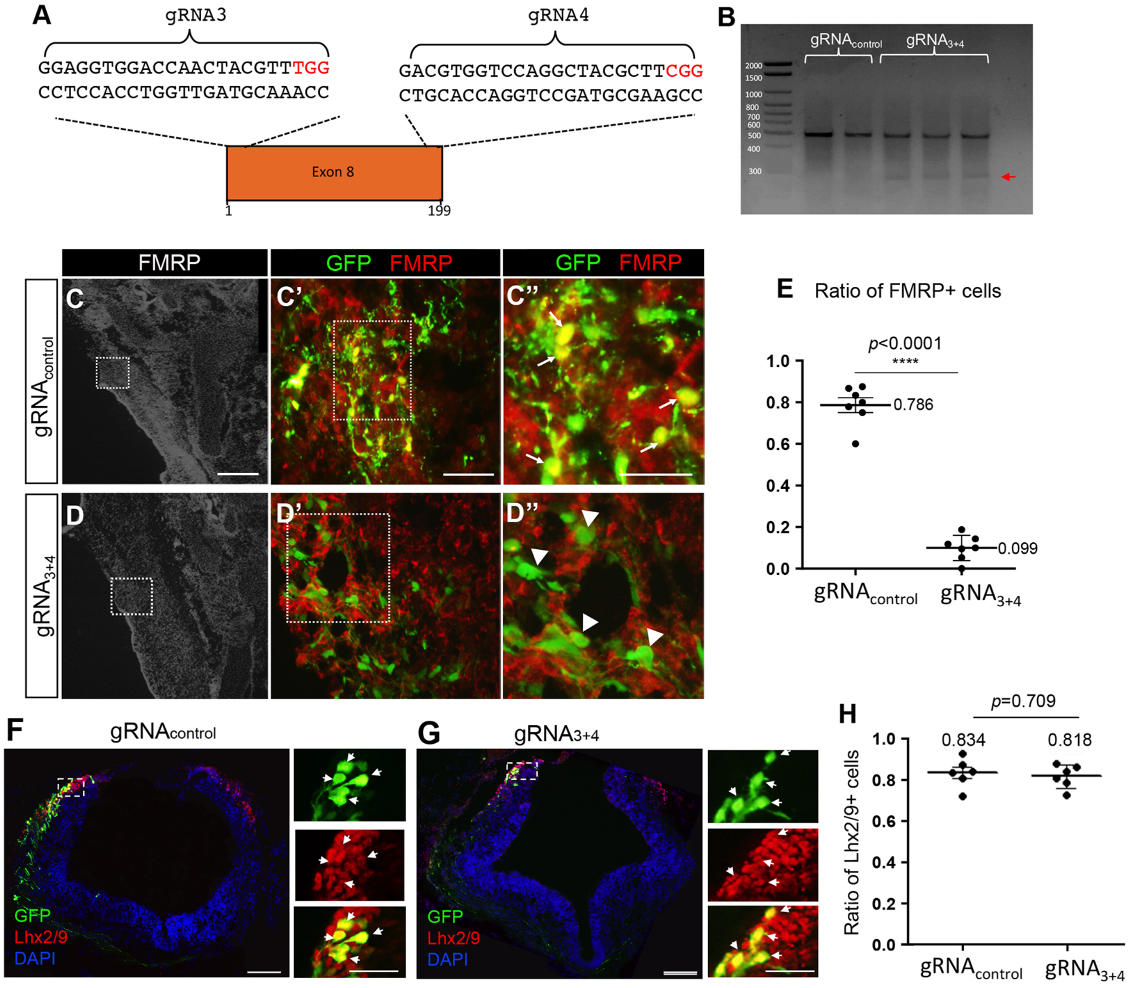

Fig. 6. FMRP knockout with CRISPR/Cas9 strategy. (A) CRISPR design of FMRP sequence in exon 8. (B) Gel electrophoresis of PCR products from hindbrains electroporated with gRNA$_{control}$ and gRNA$_{3+4}$ plasmids. Red arrow points to a ∼260 bp fragment obtained by Cas9 deletion. (C-D″) Sagittal-section views of E6.5 brainstems expressing gRNA$_{control}$ (C-C″) or gRNA$_{3+4}$ (D-D″) plasmids (green) and stained for FMRP antibody (red). High-magnification views of the boxed areas in C,D and C′,D′ appear to the right of each image. Arrows and arrowheads point to FMRP$^+$ and FMRP$^−$ cells, respectively. (E) Box plot quantification of FMRP-immunoreactive cells out of total GFP$^+$ cells. Each data point represents one embryo (*n*=7 embryos for each group). (F,G) Cross-section views of E4.5 hindbrains obtained from embryos that were electroporated with gRNA$_{control}$ or gRNA$_{3+4}$ (green) and stained with Lhx2/9 antibody (red). Higher-magnification views of the boxed areas in F and G are represented in the right of each panel in different channels. Arrows indicate the same cells in all channels. (H) Box plot quantification of Lhx2/9-immunoreactive cells out of total GFP$^+$ cells. Each data point represents one section (*n*=3 embryos for each group). Scale bars: 100 µm in C (applies to C,D); 100 µm in F,G (main panels); 50 µm in C′ (applies to C′,D′) and F,G (right panels); 20 µm in C″ (applies to C″,D″).

(Fig. S8C; *P*<0.05). Two-way ANOVA analyses did not reveal a significant effect of either the type of FMRP manipulation [*F*(1,29)=4.127; *P*=0.052] or the developmental stage [*F*(1,29)=1.176; *P*=0.287] on the degree of FMRP deficiency-induced changes in the width of the axon bundle. In contrast to the FMRP knockout, the majority of axons following shRNA-induced FMRP knockdown appeared to cross the midline normally at E4.5 (Fig. 8D,E). The rate of midline crossing was not significantly different between the groups at either developmental stage (Fig. 8J,K; E4.5: *P*=0.2403; E6.5: *P*=0.7209). Altogether, using two loss-of-function strategies we confirmed that FMRP expression in dA1 axons is required for directional axonal growth in a defined fascicule while navigating through developing brains.

To further determine whether loss of FMRP impairs the organized axonal growth of NM precursor axons, we analyzed its effect *in vitro*. Following electroporation of gRNA$_{control}$ or gRNA$_{3+4}$ plasmids at E2.5 (*n*=12 embryos for each plasmid), hindbrains were isolated at E3.5, suspended into single cells, and incubated for 5 days. The cultures contained GFP$^+$ cells along with non-transfected hindbrain cells (Fig. 9). To monitor the dynamics of neurite outgrowth, cultures were traced by live imaging every 6 h. Cells expressing gRNA$_{control}$ plasmid demonstrated a gradual extension and elongation of neurites (Fig. 9A,C,E,G; Movie 1). Strikingly, cells expressing gRNA$_{3+4}$ plasmid demonstrated neurite overgrowth accompanied by aberrant turning of axons and enhanced branching along the neurites and in their terminals (Fig. 9B,D,F,H,I-L; Movie 2). Quantification of the results (*n*=6 wells for each plasmid) confirmed a gradual increase in neurite branch point (*P*<0.01) and length (*P*<0.001) over time in both treatments (Fig. 9M,N). However, the values differ greatly between the groups, as indicated for instance by the ∼3.5-fold increase in neurite branch points and length in cells expressing gRNA$_{3+4}$ plasmid compared with control cells at day 4. These *in vitro* results demonstrate that axons tend to spread and branch more extensively in the absence of FMRP, further verifying that FMRP is required to control the axonal growth behavior of NM precursors.

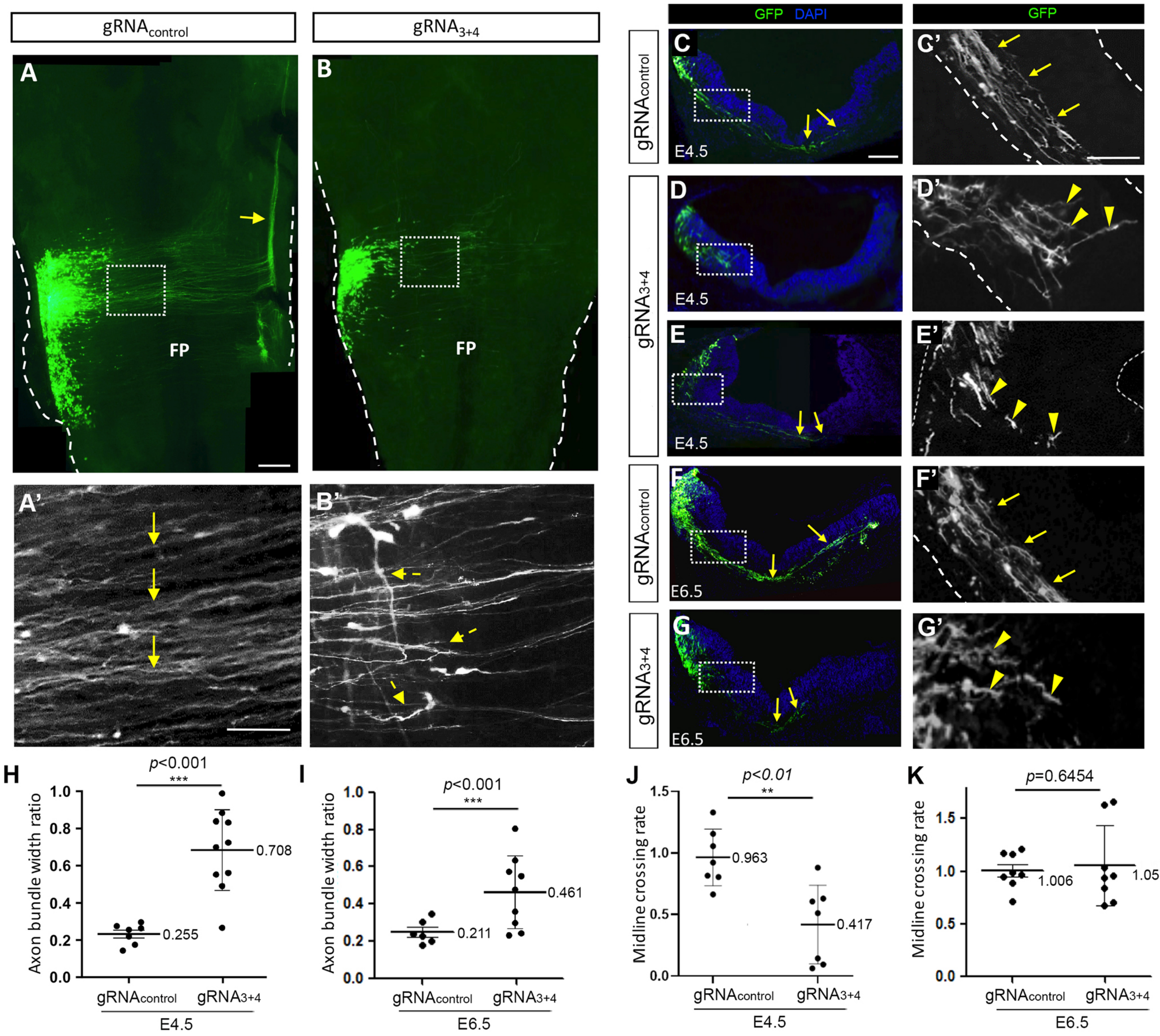

Fig. 7. CRISPR-mediated FMRP knockout induces disoriented axonal growth. (A-B′) E4.5 flat-mounted hindbrains from embryos electroporated at E2.5 with $\text{gRNA}_{\text{control}}$ (A) or gRNA_{3+4} (B). Higher-magnification views of the boxed areas in A,B are represented in A′,B′. Arrows point to organized axons in A,A′. Dashed arrows in B′ indicate disoriented axons. (C-G′) Transverse sections of r5-6 level at E4.5 (C-E′) and E6.5 (F-G′) from embryos electroporated with $\text{gRNA}_{\text{control}}$ (C,C′,F,F′) or gRNA_{3+4} (D-E′,G,G′) plasmids. Higher-magnification views of the boxed areas in C-G are represented in C′-G′. Arrows in the left panels indicate axons that crossed the midline. Arrows and arrowheads in the right panels point to organized and disorganized axons, respectively. (H,I) Box plot analysis of the width of the GFP^+ axonal bundle at E4.5 (H) and E6.5 (I). (J,K) Box plot analysis of the axonal midline crossing rate at E4.5 (J) and E6.5 (K). Each data point represents one embryo. FP, floor plate. Dashed lines outline the border of embryos and sections. Scale bars: 100 µm in A (applies to A,B) and C (applies to C-G); 50 µm in A′ (applies to A′,B′) and C′ (applies to C′-G′).

FMRP deficiency induces synaptic projection errors of NM axons in NL

We next determined whether FMRP is required for presynaptic targeting by assessing the effects of FMRP downregulation on the pattern of synaptic connectivity of NM axons within NL. We electroporated E2 embryos with *Fmr1*-shRNA or control (scrambled) shRNA into NM precursors and triggered shRNA expression with Dox treatment at E8 (Fig. 10A). This late-onset expression preserved earlier developmental events of NM axons before NL neurons reach their final destination. During this time window, FMRP immunoreactivity was reduced by 40-60% in NM cell bodies as we measured previously (Wang et al., 2018).

We first examined embryos at E15 (*n*=8 embryos for scrambled-shRNA and 9 for *Fmr1*-shRNA). A typical projection pattern of NM axons was seen in both groups: EGFP^+ axons arising from the NM extended to both the ipsilateral and contralateral NL. In embryos expressing scrambled-shRNA, NM axons were restricted to the dorsal NL ipsilaterally and ventral NL contralaterally (Fig. 10B,D). In contrast, embryos expressing *Fmr1*-shRNA demonstrated EGFP^+ axons that projected beyond their assigned neuropil domain, extended through the cell body layer, and terminated within the other domain (Fig. 10C,E). We measured the area containing EGFP^+ axons in each neuropil domain of the contralateral NL and calculated the dorsal/ventral ratio of this measure. This ratio was low in embryos expressing scrambled-

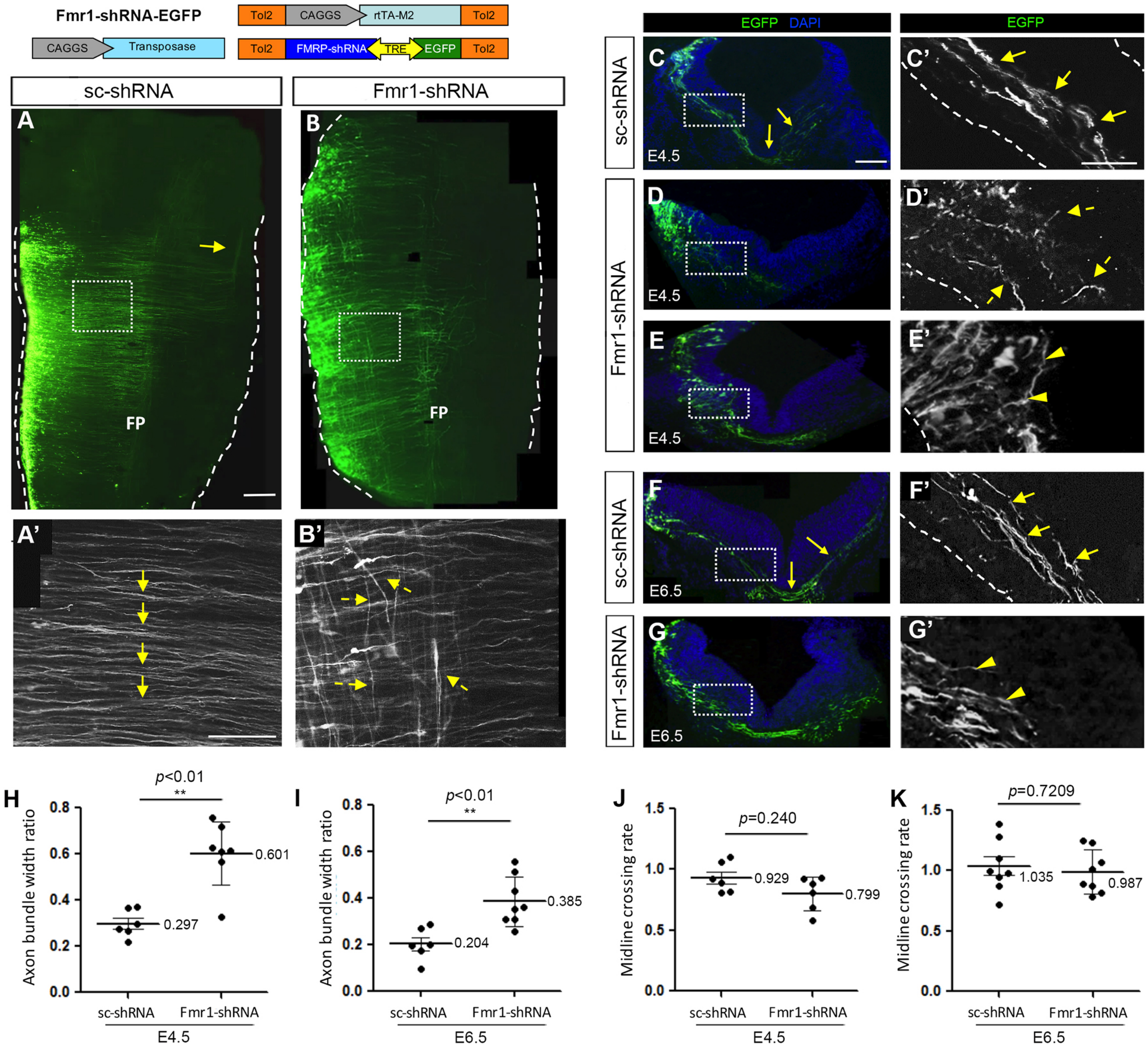

Fig. 8. shRNA-mediated FMRP knockdown induces axonal disorganization. (A-B′) E4.5 flat-mounted hindbrains from embryos that were electroporated at E2.5 with scrambled-shRNA-EGFP (sc-shRNA; A) or *Fmr1*-shRNA-EGFP (B). Higher-magnification views of the boxed areas in A,B are represented in A′,B′. Plasmid design for *Fmr1*-shRNA is illustrated on the top. Arrows and dashed arrows represent organized and disoriented axons, respectively. (C-G′) Transverse sections of r5-r6 level at E4.5 (C-E′) and E6.5 (F-G′) from embryos electroporated with sc-shRNA (C,C′,F,F′) or *Fmr1*-shRNA (D-E′,G,G′) plasmids. Higher-magnification views of the boxed areas in C-G are represented in C′-G′. Arrows in left panels indicate axons that crossed the midline. Arrows and arrowheads in the right panels point to organized and disorganized axons, respectively. (H,I) Box plot analysis of the width of the GFP$^+$ axonal bundle at E4.5 (H) and E6.5 (I). (J,K) Box plot analysis of the axonal midline crossing rate at E4.5 (J) and E6.5 (K). Each data point presents one embryo. FP, floor plate. Dashed lines outline the border of embryos and sections. Scale bars: 100 µm in A (applies to A,B) and C (applies to C-G); 50 µm in A′ (applies to A′,B′) and C′ (applies to C′-G′).

shRNA, indicating a strong preference for ventral localization, and was significantly enhanced following *Fmr1*-shRNA transfection (P=0.0079; Fig. 10G), demonstrating abnormal axonal overshoot. This phenotype was not observed at E19 (n=5 embryos; Fig. 10F,G), indicating that the effect of FMRP deficiency on axon targeting is stage dependent.

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 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.

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 was zero, one or two at E15, similar to the control group as measured from Atoh1-mGFP labeled terminals (Fig. 12; P=0.5695).

DISCUSSION

Using high-specificity genetic tools in chicken embryos, we uncovered an early onset of FMRP localization in developing axons of auditory neurons and demonstrated that cell-autonomous

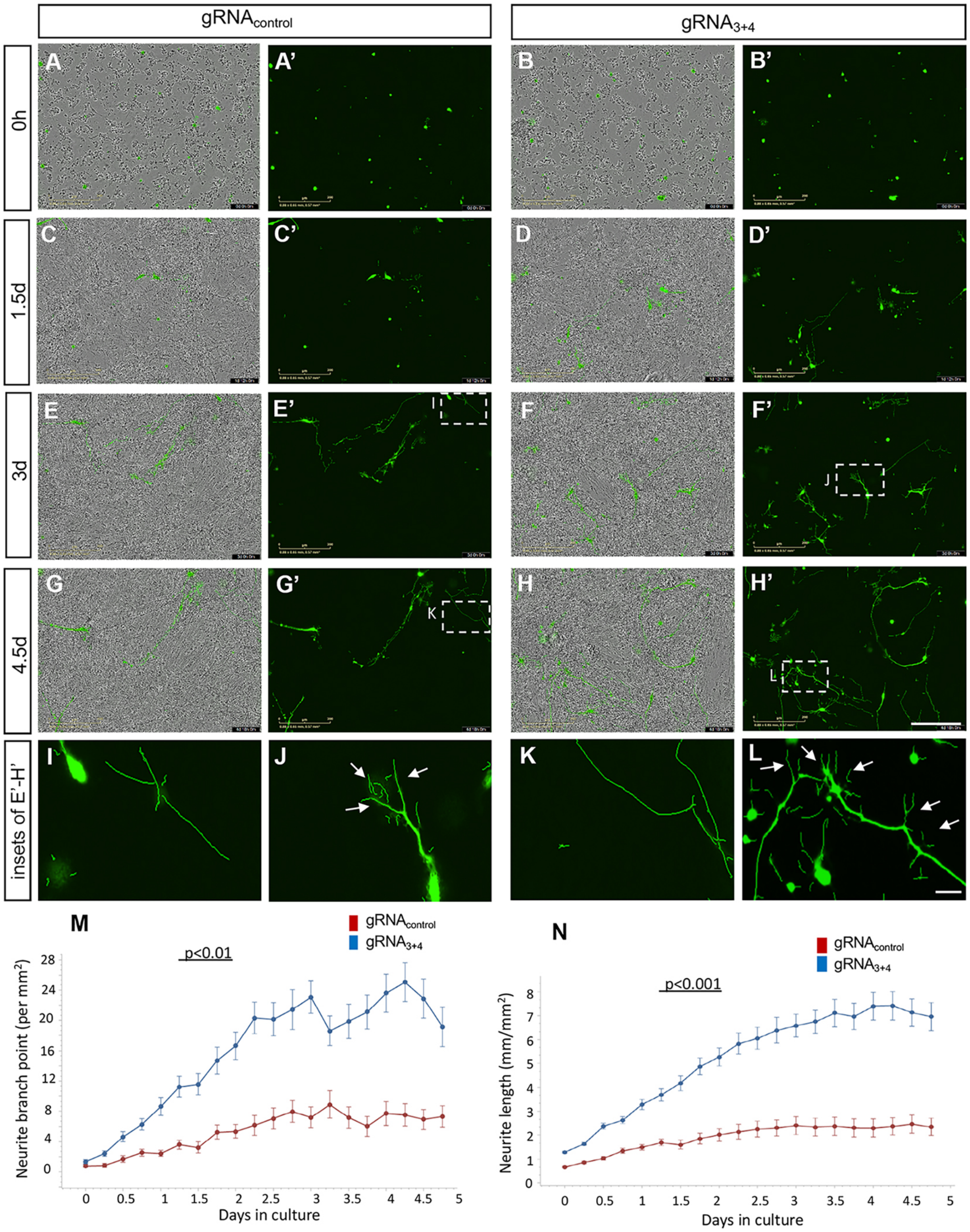

Fig. 9. CRISPR-mediated FMRP knockout induces neurite overgrowth and overbranching in hindbrain culture. (A-H′) Time-lapse analysis of cell cultures obtained from E3.5 hindbrains that were electroporated at E2.5 with gRNA$_{control}$ (A,C,E,G) and gRNA$_{3+4}$ (B,D,F,H) plasmids. Cells were documented every 6 h for 5 days. Representative phase (A-H) and green fluorescence (A′-H′) images in different time points are shown. GFP$^+$ neurites are evident in all images. (I-L) Higher-magnification views of the boxed areas in E′-H′. Arrows in J,L show overbranching along the neurite up to its terminal. (M,N) Quantification of neurite branch point (M) and neurite length (N) along 5 days using NeuroTrack analysis. Each data point represents six different wells of a 48-well plate. Scale bars: 200 µm in H′ (applies to A-H′); 50 µm in L (applies to I-L).

FMRP expression is required for orderly and timely axonal navigation and synaptic targeting *in vivo* during discrete episodes of axon and circuit development.

FMRP in axon navigation

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 (De Diego Otero et al., 2002) and axon growth cones of cultured mammalian neurons (Antar et al., 2006; Hengst et al., 2006; Jain and Welshhans, 2016). FMRP has also been identified in relatively mature axons as a component of fragile X granules (FXGs) in postnatal mammalian brains (Christie et al., 2009; Chyung et al., 2018; Korsak et al., 2017; Shepard et al.,

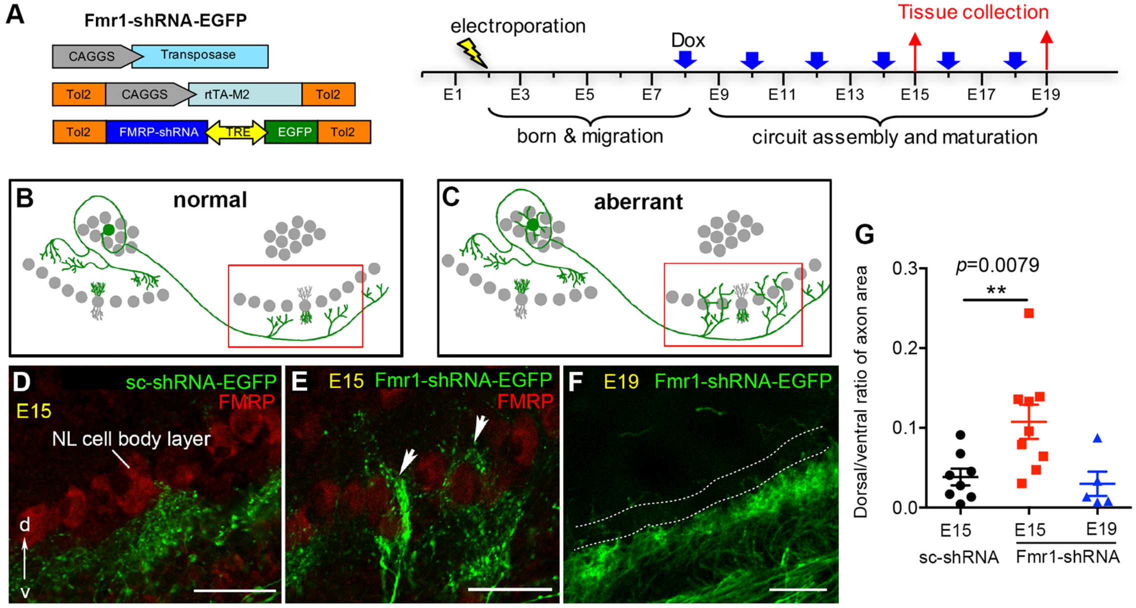

Fig. 10. FMRP knockdown leads to axon projection errors in NL. (A) Transfection protocol for late-onset shRNA expression. Blue arrows indicate the days for Dox treatment. (B,C) Schematics of normal (B) and aberrant (C) axon targeting of NM neurons in the contralateral NL. (D,E) Photomicrographs of NM axons in the contralateral NL at E15 following scrambled-shRNA (D) and *Fmr1*-shRNA (E) expression. Arrows point to abnormally projected NM axons through the cell body layer into the dorsal neuropil. (F) NM axons in the contralateral NL at E19 following *Fmr1*-shRNA expression. The axons are predominantly distributed in the ventral neuropil, similar to the control. Dashed lines indicate the cell body layer. (G) Quantification of the dorsal/ventral ratio of axon area. This ratio is significantly increased in *Fmr1*-shRNA transfected embryos at E15 (red squares) but not E19 (blue triangles), compared with control embryos (black circles). d, dorsal; v, ventral. Scale bar: 50 µm.

2020). FMRP puncta found in developing NM axons resemble these FXGs in size and density (Christie et al., 2009). However, the majority of FXGs in postnatal mouse brainstems contain the fragile X-related proteins FXR1P and FXR2P (FXR1 and FXR2, respectively) but not FMRP (Chyung et al., 2018). Whether this difference reflects interspecies variation or developmental stage dependency is yet to be determined.

Consistent with axon localization of FMRP during early development, FMRP deficiency in Atoh1/NM precursors results in widened axonal bundles due to randomized axonal growth instead of directional growing in a defined fascicule. It is known that axon fasciculation can be controlled at the level of axonal growth cones (Honig et al., 1998) and/or regulated by axon tension through shaft-shaft interactions (Šmít et al., 2017). Our *in vitro* results support a likely involvement of growth cone behaviors as the absence of FMRP in NM precursor axons leads to excessively branched growth cones together with axonal overgrowth. Indeed, previous studies showed that FMRP loss enhances growth cone filopodia and attenuates growth cone collapse *in vitro* (Antar et al., 2006; Doers et al., 2014; Li et al., 2009), and these actions may involve FMRP regulation of cell adhesion and axon guidance cues. For example, FMRP colocalizes with Dscam mRNAs in cortical axons (Jain and Welshhans, 2016) and Dscam promotes axon fasciculation in the developing optic fiber (Bruce et al., 2017). Netrin mRNAs are associated with FMRP in HEK293 cells and was linked to axon extension phenotype in *Fmr1* knockout *Drosophila* (Kang et al., 2019). Notably, netrin has a profound role in navigating commissural axons in the hindbrain and spinal cord in a tight bundle toward the midline (Moreno-Bravo et al., 2019; Serafini et al., 1996; Varadarajan et al., 2017; Yung et al., 2018). Notably, the degree of the aberrant projections decreases as development proceeds. The partial recovery in the axonal directionality may suggest that FMRP-deficient axons are capable of correcting their growth pattern with time, as shown for instance in an ascending projection connecting specific cortical layers in *Fmr1* knockout mice (Bureau et al., 2008). Yet, to fully decipher the fate of FMRP-deficient axons, advanced *in vivo* live-imaging techniques will be needed to trace the behavior of individual axons.

The second phenotype we identified was a delay in axonal midline crossing. In control embryos, axons of Atoh1/NM precursors crossed the midline at E4.5. Following FMRP knockout, the axon crossing was not complete until 2 days later at E6.5. This phenotype may be caused by a general slowing down of axon growth *in vivo*. For example, FXS neurons derived from human pluripotent stem cells show reduced neurite outgrowth (Doers et al., 2014). FMRP knockdown significantly reduces axonal growth of cultured mouse neurons in response to nerve growth factor (Wang et al., 2015b). This slowed growth may be partially associated with FMRP regulation of microtubule signaling and dynamics (Bodaleo and Gonzalez-Billault, 2016; Wang et al., 2015b). Alternatively, a delay in midline crossing could be secondary to axon defasciculation. In the zebrafish forebrain, axon-axon interaction (likely axon fasciculation) shapes the midline kinetics of commissural axons (Bak and Fraser, 2003). Moreover, overgrowth and overbranching of axons in brains of *Drosophila* FMRP mutants have been reported (Pan et al., 2004), consistent with our *in vitro* data in which, rather than attenuation in axonal growth, we observed extensive neurite growth and enhanced branching points upon FMRP knockout. Reduced axon fasciculation thus may negatively affect midline crossing in auditory neurons. However, a delay in midline crossing was not detected following FMRP knockdown, although FMRP knockdown resulted in similar degrees of axon defasciculation as did knockout of FMRP. This, then, suggests that FMRP regulates multiple factors in controlling the speed of axon crossing. Additional mechanisms may include

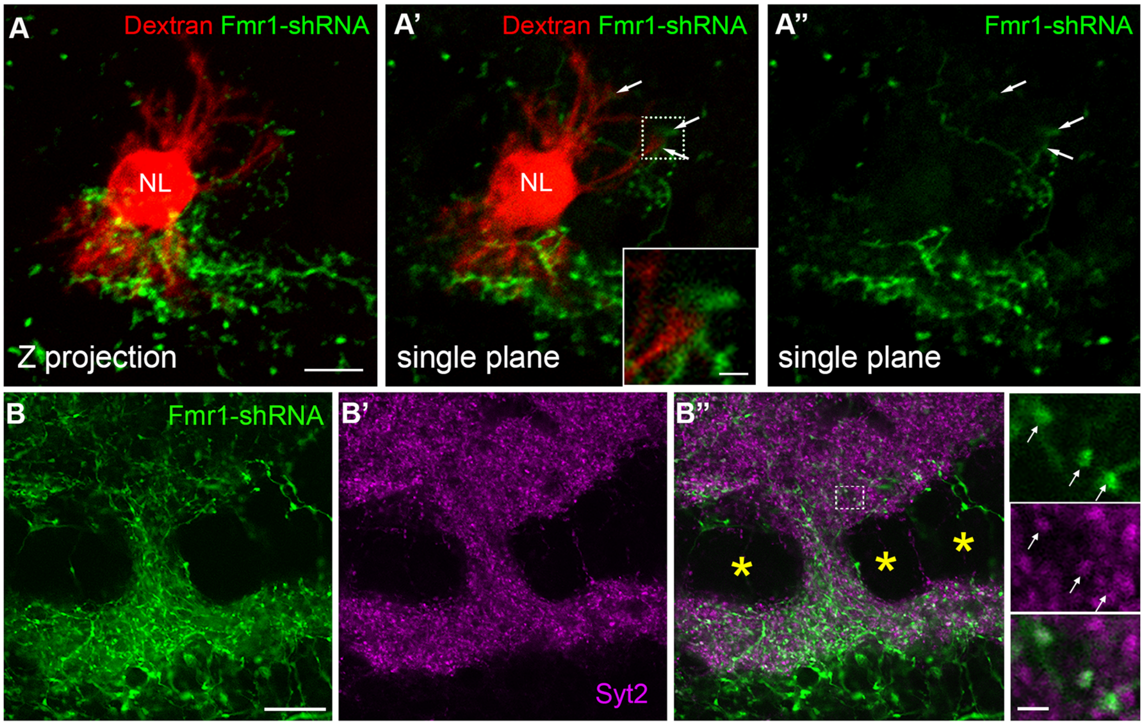

Fig. 11. Aberrantly projected NM axons form synapses on NL dendrites. NM precursors were unilaterally transfected with *Fmr1*-shRNA-EGFP. Images were taken from the side contralateral to the transfection. (A-A″) Images of a dye-filled NL neuron (red) the dorsal and ventral dendrites of which are in close contact with EGFP$^+$ NM axons (white arrows).Inset in A′ shows higher magnification of the boxed area. (B-B″) Double labeling of Syt2 immunoreactivity with EGFP$^+$ NM axons. Asterisks indicate NL cell bodies in B″. Higher-magnification views of the boxed area in B″ are represented to the right. EGFP$^+$ axonal terminals (white arrows) contain Syt2 immunoreactivity. Scale bars: 10 µm in A; 2 µm in inset in A′ and in right-hand panels in B; 20 µm in B. Syt 2, synaptotagmin 2.

suppressed expression of axon guidance genes and compromised neuronal response to guidance cues following FMRP loss (Halevy et al., 2015; Li et al., 2009).

FMRP in synaptic targeting

In addition to controlling axon pathfinding, FMRP is also involved in determining the pattern of local axon projection in their target area. Following acute FMRP deficiency, NM axons terminate, and likely form functional synapses, on both the dorsal and ventral dendrites of the same NL neurons. This projection pattern is expected to negatively affect the accuracy of coincidence detection of NL neurons. This change can be interpreted as a compromised ability of developmental axon pruning, as seen in *Drosophila* FMRP mutants (Pan et al., 2004; Tessier and Broadie, 2008). Defective synaptic elimination and dendritic pruning have also been observed in brains of FXS individuals and FMRP knockout mice (Comery et al., 1997; Ivanco and Greenough, 2002; Jawaid et al., 2018) as well as in FMRP-reduced NM neurons (Wang et al., 2018). However, there is no evidence that under normal circumstances NM axons project to both dendritic domains of the same NL neurons and subsequently retract from one domain (Young and Rubel, 1986; Rubel and Fritzsch, 2002). It is therefore likely that the aberrant axon projection following FMRP knockdown reflects errors in axon targeting. NM axons with less FMRP may become less sensitive to guiding cues from NL neurons or local astrocytes that control the pattern of synaptic distribution (Allen-Sharpley and Cramer, 2012; Korn et al., 2012; Rotschafer et al., 2016). 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 the axonal functions that have been identified in our study without being associated with synapses.

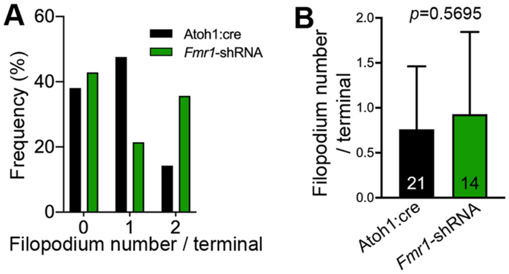

Fig. 12. FMRP knockdown does not affect the morphological maturation of NM axonal terminals. (A,B) Frequency distribution (A) and population analysis (B) of the number of filopodia per terminal following transfection with Atoh1:cre-mGFP (black bars; *n*=21 terminals) or *Fmr1*-shRNA (green bars; *n*=14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

Additional lines of evidence in support of FMRP regulation of axonal targeting via growth cone dynamics include the presence of abnormal protein patterns only during the period when NM axons exhibit dynamic growth cones with filopodia and the normal maturation of axonal endings from growth cones to bouton-like terminals independent of FMRP expression.

It is worth noting 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, which, 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).

It remains unknown whether the tonotopic organization of NM axonal projection was affected by FMRP deficiency. Our manipulations affected only ∼15% NM neurons, which were often scattered throughout the cell group, thus it was not possible 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.

New insights in FXS pathogenesis

Our results enhance the current understanding of FXS pathogenesis in three aspects. First, we strengthen the concept that FXS neuropathology involves sensory systems. FMRP is strongly expressed in the auditory system (Zorio et al., 2017) and FMRP loss alters cellular properties of auditory neurons and auditory processing (reviewed by McCullagh et al., 2020). Our current and previous studies (Wang et al., 2018) further demonstrate a role of FMRP in the proper development of auditory connectivity. Second, we reveal a cell-autonomous regulation of FMRP in axon navigation. Early-onset axon localization of FMRP suggests that this regulation occurs locally in axons, supporting axonal mechanisms of FXS pathology. For example, diffusion tensor imaging in FXS females revealed morphological changes in white matter tracts that may reflect alterations in axon density or coherence (Barnea-Goraly et al., 2003). Thus, FMRP loss-induced axon defasciculation may be a mechanism that underlies this clinical phenotype. Lastly, our results add to the existing literature that FMRP loss leads to substantial alterations in developing brains that may be undetectable later in life. FMRP knockout mouse cortex shows alterations in connection probability, axon shape and dendritic spine length at early, but not late, postnatal ages (Bureau et al., 2008; Galvez and Greenough, 2005; Nimchinsky et al., 2001). Our current and previous studies further show developmentally restricted dendritic and axonal alterations in auditory neurons (Wang et al., 2018). The significance of these early-onset and transient changes was recently highlighted in *Drosophila*, in which the requirement of FMRP for normal brain function and behaviors is tightly restricted to an early developmental period (Doll and Broadie, 2015; Sears and Broadie, 2018). If this holds true in vertebrates, it would suggest that early axon deficits following FMRP loss may be responsible for life-long behavioral deficits in FXS. Although challenging, identifying FMRP regulation of early developmental events and determining how this regulation influences later circuit properties may be the beginning of a deeper understanding of FXS neuropathology. The auditory brainstem circuits characterized and the novel genetic tools developed in this study provide a strategy that contributes to this effort.

MATERIALS AND METHODS

Animals and *in ovo* electroporation

Fertilized White Leghorn and Loman Broiler chicken eggs (*Gallus gallus domesticus*) were obtained from Charles River Laboratories (Wilmington, MA, USA) and Gil-Guy Farm (Orot, Israel), respectively. Eggs were incubated for 2-2.5 days at 38°C until Hamburger-Hamilton stage 12-15. *In ovo* electroporation was performed as described previously (Kohl et al., 2012; Wang et al., 2018). Briefly, DNA constructs (4-5 µg/µl, diluted in PBS) were injected into the lumen of neural tubes at the rhombomere 5-6 level. Electroporation was performed with a platinum bipolar electrode or bent L-shaped gold electrodes that were placed on the two sides of the hindbrain to gain unilateral transfection. Embryos underwent four electrical pulses of 20-25 V 30-45 ms in duration using a BTX 3000 (Harvard Apparatus) or a Grass SD9 electroporator (Grass instruments). Following electroporation, the eggs were re-incubated until dissection at the desired developmental stages. Embryos electroporated with drug-inducible constructs (see below) were treated by adding 50 µl of doxycycline (1 mg/ml in sterile PBS; MilliporeSigma) onto the chorioallantoic membrane to trigger transgene transcription. Following the first Dox administration, embryos were treated again every other day to maintain gene expression before tissue dissection.

Hindbrain primary cultures and time-lapse analysis

Hindbrains from electroporated embryos were dissected at E3.5 and incubated for 10 min at 37°C with TrypLE Express (Gibco, Thermo Fisher Scientific) to dissociate the tissue into single cells, as previously described (Peretz et al., 2016, 2018). TrypLE was neutralized with embryonic stem cell media containing DMEM/F-12 1:1, 20% KnockOut serum replacement, 2 mM GlutaMax L-alanyl-L-glutamine, 0.1 mM nonessential amino acids and 1:50 penicillin-streptomycin (all from Gibco, Thermo Fisher Scientific), together with 0.1 mM β-mercaptoethanol and amphotericin B (1:400) (both from MilliporeSigma). Cells were passed through a 100 µm mesh strainer, centrifuged at 600 ***g*** for 10 min, seeded in 48-well plates (∼2×10^5 cells/well) (Nunclon Delta Surface, Thermo Fisher Scientific), and incubated at 37°C in 5% CO_2. For live imaging, well plates were imaged every 6 h in the IncuCyte S3 Zoom HD/2CLR time-lapse microscopy system equipped with ×20 Plan Fluorobjective (Sartorius). Time-lapse movies were generated by capturing phase and green fluorescence images of cells in wells for up to 5 days. Stacks of images were exported in TIF format using the IncuCyte graph/export menu. Videos were assembled by exporting into MP4 format.

Plasmid construction

For genetic targeting of Atoh1-expressing neurons, an *Atoh1* enhancer element (Helms et al., 2000; Pennacchio et al., 2006) was cloned upstream of a Cre-recombinase sequence (Atoh1-Cre) and electroporated along with a conditional reporter plasmid containing a floxed STOP cassette in between the CAGG enhancer/promoter module and nuclear (n) or membranal (m) *GFP* gene (pCAGG-LoxP-STOP-LoxP-n/mGFP), as previously reported (Avraham et al., 2009; Kohl et al., 2012; Lumpkin et al., 2003; Reeber et al., 2008). For plasmid integration into the genome, the conditional reporter cassette was cloned between two PiggyBac (PB) arms (PB-CAGG-LoxP-STOPLoxPSTOP-GFP-PB) and electroporated along with the Atoh1-Cre and Pbase transposase plasmids (Hadas et al., 2014; Kohl et al., 2012; Lu et al., 2009; Wang et al., 2010). For tracing pre-synaptic connections, a reporter plasmid containing the synaptic tracer SV2-GFP (PB-CAG-LoxP-STOP-LoxP-SV2-GFP-PB) (Hadas et al., 2014; Kohl et al., 2012) was electroporated along with the *Atoh1* enhancer and the Pbase transposase.

For constitutive expression of chicken or human *Fmr1*, mCherry-*Fmr1* fused coding sequence was chemically synthesized (GenScript) and sub-cloned into the pT2K-CAGGS vector. For electroporation, the two plasmids (pT2K-CAGGS-mCherry-chFMRP and pCAGGS-T2TP) were concentrated at 4-5 µg/µl and mixed in equal amounts.

For shRNA targeting of FMRP, five shRNAs directed against specific sequences of chicken *Fmr1* were designed using siRNA Wizard v3.1 (InvivoGen) and the siDESIGN Center (Thermo Fisher Scientific). Plasmids were chemically synthesized (GENEWIZ) and EndoFree DNA

Maxi Preps were performed (Qiagen). The most effective shRNA (gaggatcaagatgcagtgaaata; nucleotides 951-973 of chicken *Fmr1*) was determined based on its knockdown effect in the developing brainstem (Wang et al., 2018) and used for subsequent experiments. A scrambled shRNA (attagaataagtgcgagagaata) was designed using the Genscript algorithm and confirmed by blasting this shRNA sequence against the chicken genome. *Fmr1* and scrambled shRNAs were cloned into a transposon-based vector system with a *Tol2* vector containing doxycycline regulatory components (Schecterson et al., 2012; Wang et al., 2018). Tol2 transposable element sequences enable stable integration of the transposon into the chick genome, whereas doxycycline regulatory elements allow temporal control of gene expression.

For CRISPR/Cas9 targeting of FMRP, we used the Genome Engineering Toolbox that was designed by the Zhang lab (Cong et al., 2013). The pX330 plasmid (#42230, Addgene) (Sakuma et al., 2014) was modified by adding a T2A-EGFP cassette at the carboxyl terminus of Cas9. gRNAs for *Fmr1* were designed utilizing the chopchop design tool (https://chopchop.cbu.uib.no/). gRNAs targeting exon 8 were cloned into the modified pX330 plasmid (Table S1). For testing the efficiency of the gRNA, the targeting plasmids were electroporated into the hindbrain at E2.5. Hindbrains were dissected 48 h following electroporation, and a 2 mm piece of hindbrain tissue was processed for DNA extraction, using a previously published 'tail digestion and DNA extraction' protocol (Wang and Storm, 2006). Genomic DNA was analyzed by polymerase chain reaction (PCR) using primers specific to sequences up- and downstream of the FMRP-gRNA$_{3+4}$ target sites. Nested PCR was used to amplify the targeted region. For exon 8 targeting, Test-F3 and Test-R1 were used for the first round of PCR, followed by Test-F2 and Test-R2 for the second round.

Staining and immunocytochemistry

Brainstem was dissected at various stages and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight at 4°C. For whole-mount preparation, hindbrains were cut open along the roof plate, after which the tissue was spread open on slides to produce flat-mount preparations (Kayam et al., 2013; Weisinger et al., 2012). For transverse sections, brainstems were transferred to 30% sucrose in PB until settling, followed by their sectioning in the coronal plane at 30 µm. Alternate sections were immunohistochemically stained by incubation with primary antibody solutions diluted in PBS with 0.3% Triton X-100 overnight at 4°C, followed by Alexa Fluor secondary antibodies (goat anti-rabbit or anti-mouse; Thermo Fisher, A11019 and A11036, or A11012) at 1:1000 overnight at 4°C. Some sections were counterstained with DAPI and/or NeuroTrace (Life Technologies), a fluorescent Nissl stain, at a concentration of 1:1000 and incubated together with secondary antibodies. Sections were mounted on gelatin-coated slides and coverslipped with Fluoromount-G mounting medium (Southern Biotech) for imaging.

Primary antibodies used include the custom-made polyclonal rabbit anti-FMRP (1:1000, RRID: AB_2861242; Wang et al., 2018; Yu et al., 2020), anti-synaptotagmin 2 (1:1000, DSHB znp-1, RRID: AB_2315626), anti-SNAP25 (1:1000, Abcam 5666, RRID: AB_305033), anti-microtubule associated protein 2 (MAP2; Millipore MAB 3418; RRID: AB_94856), custom-made polyclonal rabbit anti-Lhx2/9 (1:100, I. Sibony and T. Schultheiss, unpublished data; kind gift from T. Schultheiss, Technion-Israel Institute of Technology, Haifa, Israel) and polyclonal rabbit anti VGluT2 (1:150, Synaptic Systems 135402).

Single-cell filling

Following electroporation with *Fmr1*-shRNA plasmids and doxycycline treatment, E15 embryos were used to prepare acute brainstem slices as previously described (Wang et al., 2017). NL cells were individually dye-filled with Alexa Fluor 568 dextran (Invitrogen) following our published protocol (Wang and Rubel, 2012; Wang et al., 2017).

Imaging for illustration

Images for illustration were captured with the Olympus FV1200 at Florida State University and with the E400 microscope (Nikon) with DP70 CCD camera (Olympus) at the Hebrew University of Jerusalem. Image brightness, gamma and contrast adjustments were performed in Adobe Photoshop. All adjustments were applied equally to all images of the same set of staining from the same animal. In some cases, multiple images were taken and stitched by the automatic Image Composite Editor (ICE) software unless otherwise stated.

Data analysis

Quantification of Atoh1-Cre NM ratio

Atoh1-Cre-transfected NM neurons and total NM neurons counterstained by NeuroTrace on each section were counted using Cell Counter of Image J. Sections from the same animal were grouped, and the transfection ratio was calculated as: transfection ratio=number of Atoh1-mGFP$^+$ NM neurons/total NM neurons (*n*=8 embryos).

Quantification of axon terminal morphology

The axon terminal morphology was characterized by numbers of filopodia. Image stacks containing identifiable intact axon terminals were reconstructed using ImageJ, and the numbers of filopodia on each terminal were counted on both ipsilateral and contralateral sides. Number of filopodium per terminal was then calculated and compared between the immature stages (E11-E13) and E15.

Localization analysis of FMRP granules

FMRP granule localization in Atoh1-mGFP-labeled axons were analyzed using ImageJ. Briefly, a straight-line region of interest was drawn across an FMRP granule with Atoh1-mGFP transfection and applied to both channels. The fluorescent intensity profile was then analyzed and plotted using GraphPad Prism 7 software.

Quantification of gRNA-expressing cells

Quantification of expression of gRNA$_{control}$ and gRNA$_{3+4}$ plasmids in dA1 cells was demonstrated by box plot analysis. For each group, two transverse sections obtained from three different embryos at E4.5 were taken. Each data point represents one section. The ratio of cells co-expressing gRNA-GFP$^+$ and the dA1-specific marker Lhx2/9 out of the total gRNA-GFP$^+$-expressing cells is presented.

Quantification of FMRP expression

Quantification of the extent of FMRP expression in gRNA$_{control}$- and gRNA$_{3+4}$-expressing cells is demonstrated by box plot analysis. For each group, electroporated sagittal sections obtained from seven different embryos at E6 were taken. Each data point represents one section for which the ratio of (FMRP$^+$+GFP$^+$)/GFP$^+$ cells was measured.

Quantification of gRNA-expressing cells

Quantification of expression of gRNA$_{control}$ and gRNA$_{3+4}$ plasmids in dA1 cells is demonstrated by box plot analysis. For each group, two transverse sections obtained from three different embryos at E4.5 were taken. Each data point represents one section. The ratio of cells co-expressing gRNA-GFP$^+$ and the dA1-specific marker Lhx2/9 out of the total gRNA-GFP$^+$-expressing cells is presented.

Quantification of axon fascicule width

Axonal width measurement was performed for two different experiments (Fmr1-shRNA and FMRP-CRISPR) at E4.5 and E6.5. Each stage included two groups: (1) gRNA$_{control}$- and gRNA$_{3+4}$-expressing cells and (2) sc-shRNA-GFP- and Fmr1-shRNA-GFP-expressing cells. Box plots are demonstrated for each group, from which cross-sections from seven different embryos (E4.5) or four embryos (E6.5) were taken. Each data point represents one section for which the ratio of the axonal length relative to the mantle-ventricular width was measured using ImageJ software.

Quantification of axonal crossing

Box plot quantification of axonal crossing was performed for two different experiments at E4.5 and E6.5. Each stage contained two groups: (1) gRNA$_{control}$- and gRNA$_{3+4}$-expressing cells and (2) sc-shRNA-GFP- and FMR1-shRNA-GFP-expressing cells. For each group, cross-sections from seven different embryos (E4.5) or four embryos (E6.5) were taken. Each data point represents one section for which the ratio of the signal intensity between commissural axons and non-commissural axons was measured using ImageJ software.

Quantification of neurite length and branch points in cultures

Neurite length (mm/mm^2) and branch point (per mm^2) were calculated in gRNA$_{control}$- and gRNA$_{3+4}$-expressing neurons in each well (*n*=6 wells for each treatment) using the IncuCyte Zoom NeuroTrack software module (Sartorius), as described by Wurster et al. (2019). Microplate graphs were generated using the time plot feature in the graph/export menu of the IncuCyte Zoom software.

Quantification of Atoh1-Cre-expressing cells

The percentage of Atoh1-Cre::nGFP-expressing cells was calculated by counting the number of GFP$^+$ nuclei co-expressing the dA1-specific marker Lhx2/9 out of the total number of GFP-expressing nuclei (*n*=7 embryos).

Quantification of the laminar specificity of axon targeting

Axonal projection was measured from Fmr1-shRNA-transfected embryos at E15 and E19, as well as from scrambled-shRNA-transfected embryos at E15 using ImageJ. For each embryo, transverse sections containing the middle and rostral NL, where NL cell bodies are aligned into a single layer, were used for the analysis. For each section, the dorsal and ventral neuropil regions of the NL on the side contralateral to the transfection were outlined based on NeuroTrace staining. The neuropil area covered by EGFP$^+$ axons was then measured for each neuropil region. The specificity of axon projection was evaluated by calculating the ratio of the dorsal EGFP$^+$ area to the ventral EGFP$^+$ area. The ratios from all sections (usually two or three) of the same embryo were averaged as individual data points and compared between Fmr1-shRNA- and control-shRNA-transfected animals (*n*=5-9 animals for each group).

Statistics

Statistics were performed by Mann–Whitney non-parametric U-test using the GraphPad Prism 7 software package (GraphPad Software). $P<0.05$ was considered statistically significant. Data are displayed as mean±s.d. as indicated in the Results. Each individual data point represents one animal. Two-way ANOVA was used for Tukey multiple comparisons.

Acknowledgements

We thank Edwin W Rubel (University of Washington) for facilitating the collaboration between Y.W. and D.S.-D. We are grateful to David Fradkin for assistance with the IncuCyte system, Itzhak Sibony and Tom Schultheiss for the Lhx2/9 antibody, and Daniel Lazar for help with the tissue culture. We thank Jeniffer Darnell (Rockefeller University) for the hFMRP-EGFP plasmid.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.S.-D., Y.W.; Methodology: A. Klar, D.S.-D., Y.W.; Investigation: X.W., A. Kohl, X.Y., D.A.R.Z.; Writing - original draft: X.W., A. Kohl, D.S.-D., Y.W.; Writing - review & editing: A. Klar, D.S.-D., Y.W.; Supervision: D.S.-D., Y.W.; Funding acquisition: D.S.-D., Y.W.

Funding

This work was supported by the National Institutes of Health (DC13074 and DC17267 to Y.W.), the United States – Israel Binational Science Foundation (2015087 to Y.W. and D.S.-D.), the Israel Science Foundation (1515/16 to D.S.-D.) and the Office of the Chief Scientist, Ministry of Health (3-0000-33793 to D.S.-D.). Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at
https://dev.biologists.org/lookup/doi/10.1242/dev.188797.supplemental

Peer review history

The peer review history is available online at
https://dev.biologists.org/lookup/doi/10.1242/dev.188797.reviewer-comments.pdf

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