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Supplemental Information

Primary Ciliary Deficits in the Dentate Gyrus of Fragile X Syndrome

Bumwhee Lee, Shree Panda, and Hye Young Lee

Supplemental Experimental Procedures

Materials. Paraformaldehyde and D(+)-Sucrose were purchased from Acros Organics; Sodium Citrate Dihydrate, Phosphate Buffered Saline (PBS), and Superfrost Plus Microsope Slides from Fisher Scientific; Tissue-Plus O.C.T. Compound from Fisher Healthcare; Isoflurane from Vetone; ProLong Gold Antifade Reagent with DAPI from Molecular Probes; Embedding Molds from Thermo Scientific; Goat Serum from Gibco; PowerUp SYBR Green Master Mix from Applied Biosystems; and BrdU and SuperScript III First-Strand Synthesis kit from Invitrogen.

Antibodies. The following primary antibodies were used: rabbit anti-AC3 (1:500; Santa Cruz; SC588), rabbit anti-AC3 (1:2000; EnCor Biotechnology Inc.; RPCA-ACIII), rabbit anti-Arl13b (1:750; Proteintech; 17711-1-AP), rabbit anti-HOPX (1:500; Proteintech, 11419-1-AP), rat anti-BrdU (1:150; Novus; NB500-169), mouse anti-BLBP (1:300; Abcam; ab131137), mouse anti-FMRP (1:250; BioLegend; 843701), mouse anti-NeuN (1:500; Chemicon; MAB377), mouse anti-S100β (1:100; Santa Cruz; sc-393919), mouse anti-pericentrin (1:250, BD Biosciences; 611814), and chicken anti-calbindin (1:400; Encor Biotechnology Inc.; CPCA-Calb). For secondary antibodies: donkey anti-rat IgG-Alexa Fluor 488, (1:250), goat anti-rabbit-Cy3 (1:500), donkey anti-mouse Fcγ fragment-Cy5 (1:500), and donkey anti-chicken IgG-Alexa Fluor 647 (1:500) from Jackson ImmunoResearch Laboratories, Inc.

Vaginal Plug Formation. To monitor plug formation, fertile heterozygous female mice were housed with WT male mice. The following morning, females were checked for the presence of vaginal plugs. If a plug was present, the female was removed to a separate cage. If no plug was detected, the female remained in the cage with the male. Plug checks were performed every morning.

RNA Extraction from Mouse Brains and qRT-PCR. The DG was dissected from the hippocampus of postnatal mice in ice-cold PBS. After adding 800 µL of TRIzol, whole embryonic brains or the dissected DG were homogenized, treated with 160 µL of chloroform, and centrifuged for 15 min at 4°C. The aqueous phase of the sample was removed by pipetting and 400 µL of 100% isopropanol was added. After being centrifuged for 10 min, the supernatant was removed from the tube, and then the pellet was washed with 75% ethanol and centrifuged for 5 min. Afterwards, the supernatant was removed and the pellet was dissolved in DNase- and RNase-free water. 1 µg of RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis kit (Invitrogen). qRT-PCR analysis was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) with the primers listed in Table 1. The relative expression from RNA samples was analyzed using the $2^{-\Delta\Delta CT}$ method. Values of *Gli1* mRNA were normalized by the peptidylprolyl isomerase A (*Ppia*) housekeeping gene expression levels.

Table 1. qRT-PCR primers.

Gli1 (Gli1) F	CACTACCTGGCCTCACACCT
Gli1 (Gli1) R	GTACTCGGTTCGGCTTCTCC
Ppia (PPIA) F	GAGCTGTTTGCAGACAAAGTTC
Ppia (PPIA) R	CCCTGGCACATGAATCCTGG

Immunostaining. For immunostaining, all sections were post-fixed by methanol for 10 min at -20°C, followed by antigen retrieval for 15 min. After being washed twice with PBS for 5 min, the sections were incubated overnight with blocking solution (5% goat serum, 0.01% Triton-X 100 in PBS). The following day, the sections were incubated with the appropriate primary antibodies for 4 h at room temperature and then washed 3 times with PBS for 7 min, followed by incubation with secondary antibodies for 2 h at room temperature afterwards. After additionally being washed 3 times with PBS for 7 min and with 70% ethanol for 1 min, the sections were mounted with ProLong Gold Antifade Reagent with DAPI. Immunostained sections were kept in 4°C. For BrdU immunostaining, the brain tissues were additionally pretreated in 2 M HCl for 15-30 min at 37°C as previously reported (Amador-Arjona et al., 2011).

Imaging and Analysis. Images were taken randomly in each brain region of interest by Zeiss LSM710 Confocal microscopy with a 40x oil objective lens and 2.0 zoom at a 10 μm range with 1 μm intervals and a total of 11 slices. To analyze primary cilia or basal bodies of primary cilia, the number of $AC3^+$, $Arl13b^+$ (for primary cilia), or $pericentrin^+$ cells (for basal bodies) were counted and normalized as indicated in the figure legends. To analyze the number of neurons, the number of $NeuN^+$ cells was counted and normalized by the number of $DAPI^+$ cells. Cells were counted by Image J software (NIH). To determine the relative FMRP expression levels in the DNe or the DG during embryonic development, images were taken by Zeiss Apotome fluorescence microscopy with a 10x lens. The fluorescence intensity of FMRP in a defined ROI where HOPX expresses in the DNe or the DG was analyzed using the Image J software (NIH). Mean fluorescence intensities were taken and background fluorescence signals (same region in *Fmr1* KO brain) were subtracted.

Data Availability. All data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplemental References

Amador-Arjona, A., Elliott, J., Miller, A., Ginbey, A., Pazour, G.J., Enikolopov, G., Roberts, A.J., and Terskikh, A.V. (2011). Primary cilia regulate proliferation of amplifying progenitors in adult hippocampus: implications for learning and memory. J Neurosci *31*, 9933-9944.

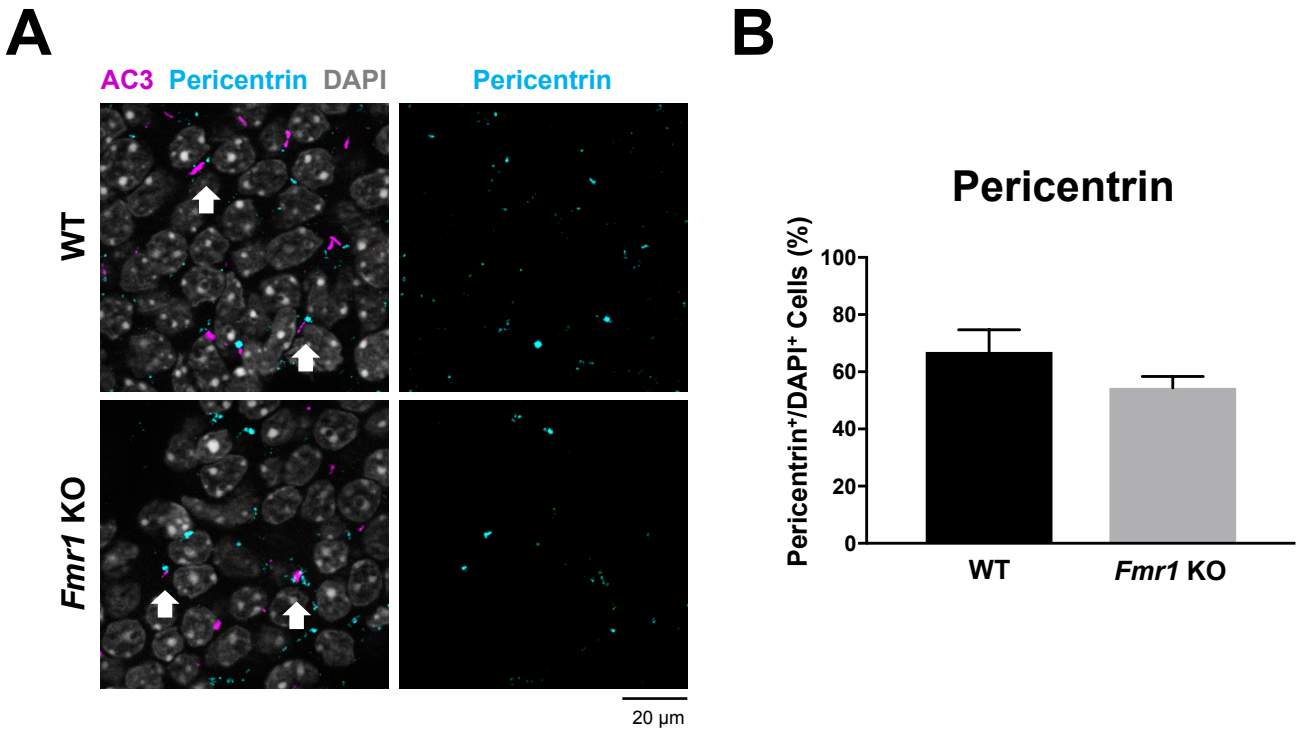


Figure S1. No significant reduction in the number of basal bodies is found in the DG of *Fmr1* KO mice. Related to Figure 1. (A) Immunostaining of AC3 (magenta) and pericentrin (cyan) with DAPI nuclear staining (gray) in the DG of P60 WT or *Fmr1* KO mice. White arrows, $AC3^{+}$;$pericentrin^{+}$ cells. (B) Quantification of the percentage of $pericentrin^{+}$ cells among $DAPI^{+}$ cells. n = 16, mean ± SEM. Student's unpaired t test.

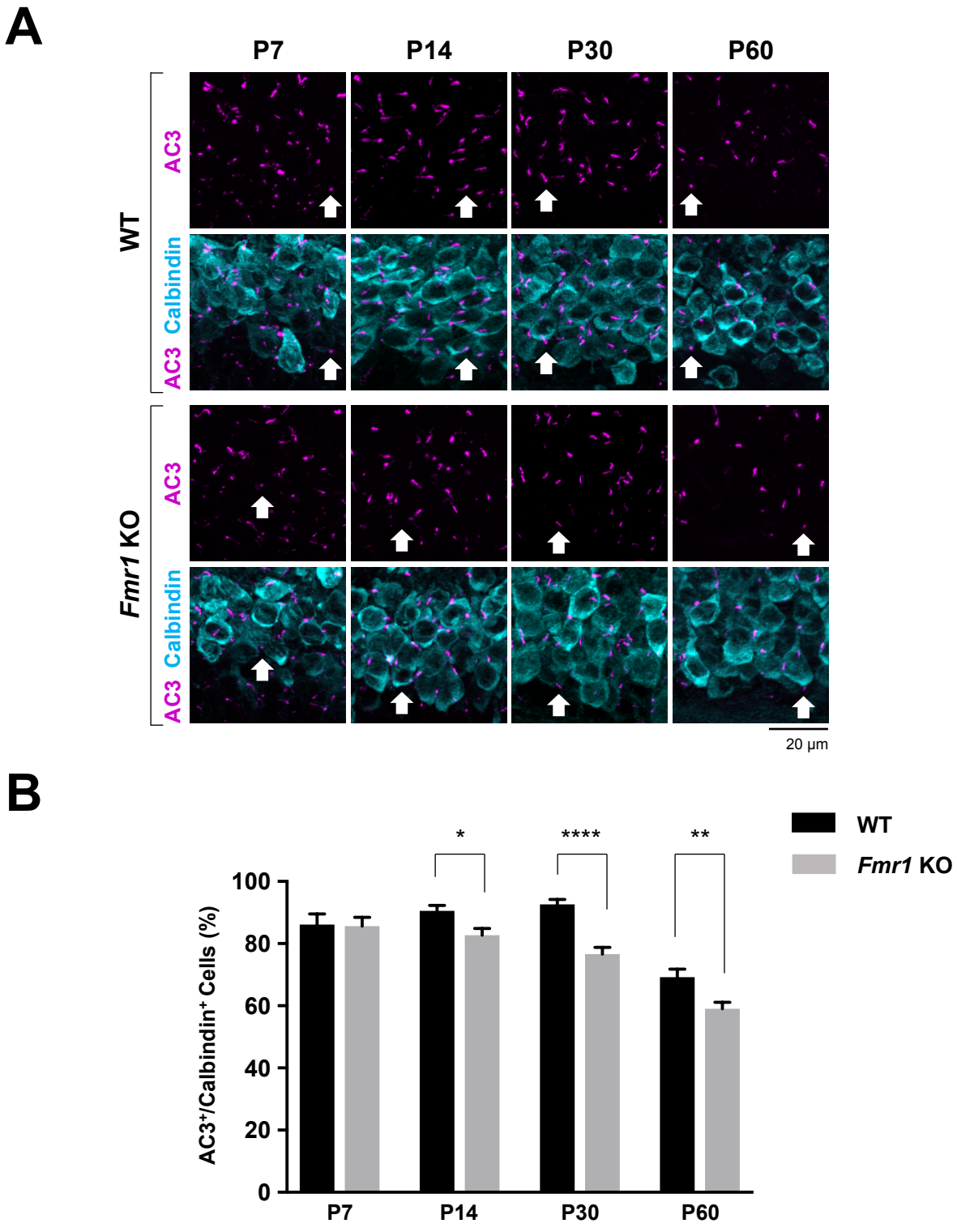
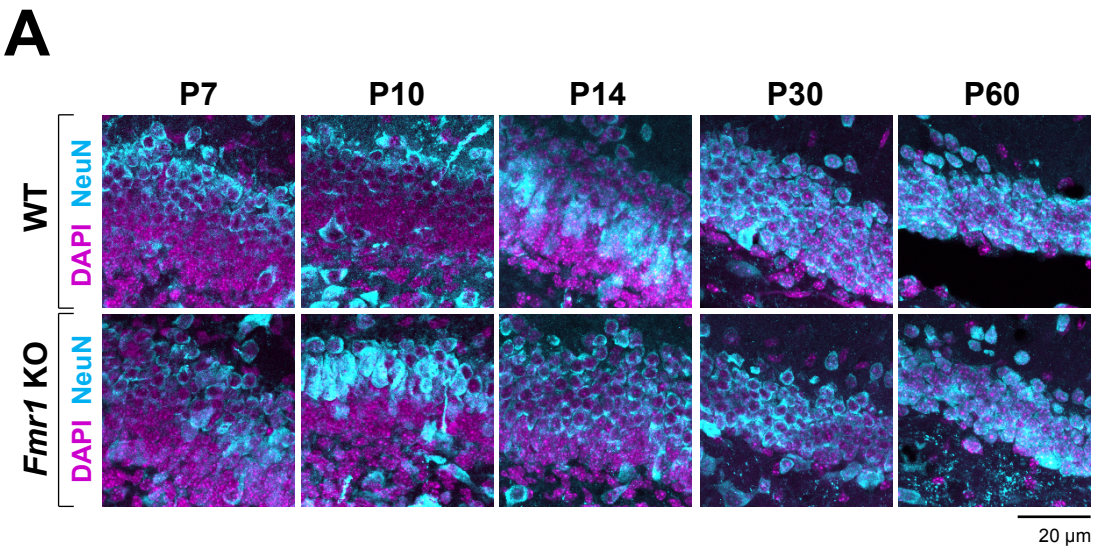


Figure S2. The number of primary cilia is reduced in calbindin$^+$ mature granule neurons in the DG of *Fmr1* KO mice. Related to Figure 4. (A) Immunostaining of AC3 (magenta) and calbindin (cyan) in the DG of P7, P14, P30, and P60 WT or *Fmr1* KO mice. White arrows, AC3$^+$;calbindin$^+$ cells. (B) Quantification of the percentage of AC3$^+$ cells among calbindin$^+$ cells. n = 8, mean ± SEM. Student's unpaired t test. *p < 0.05, **p < 0.01, ****p < 0.0001.



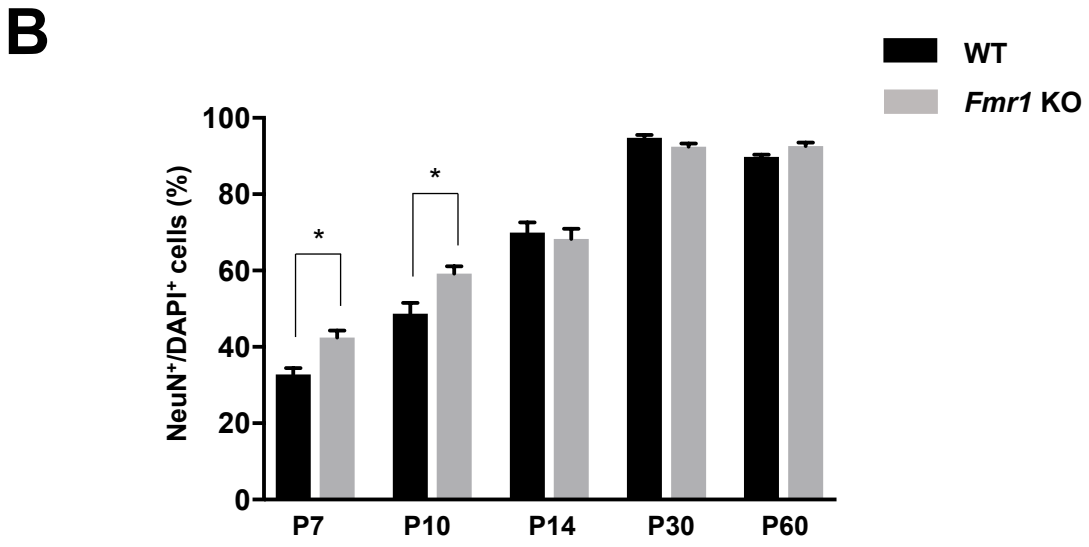


Figure S3. The number of NeuN⁺ mature granule neurons are not reduced during the DG development of postnatal *Fmr1* KO mice. Related to Figure 4. (A) Immunostaining of NeuN (cyan) with DAPI nuclear staining (magenta) in the DG of P7, P10, P14, P30, and P60 WT (top) or *Fmr1* KO (bottom) mice. (B) Quantification of the percentage of NeuN⁺ cells among DAPI⁺ cells. n = 8, mean ± SEM. Student's unpaired t test. *p < 0.05.

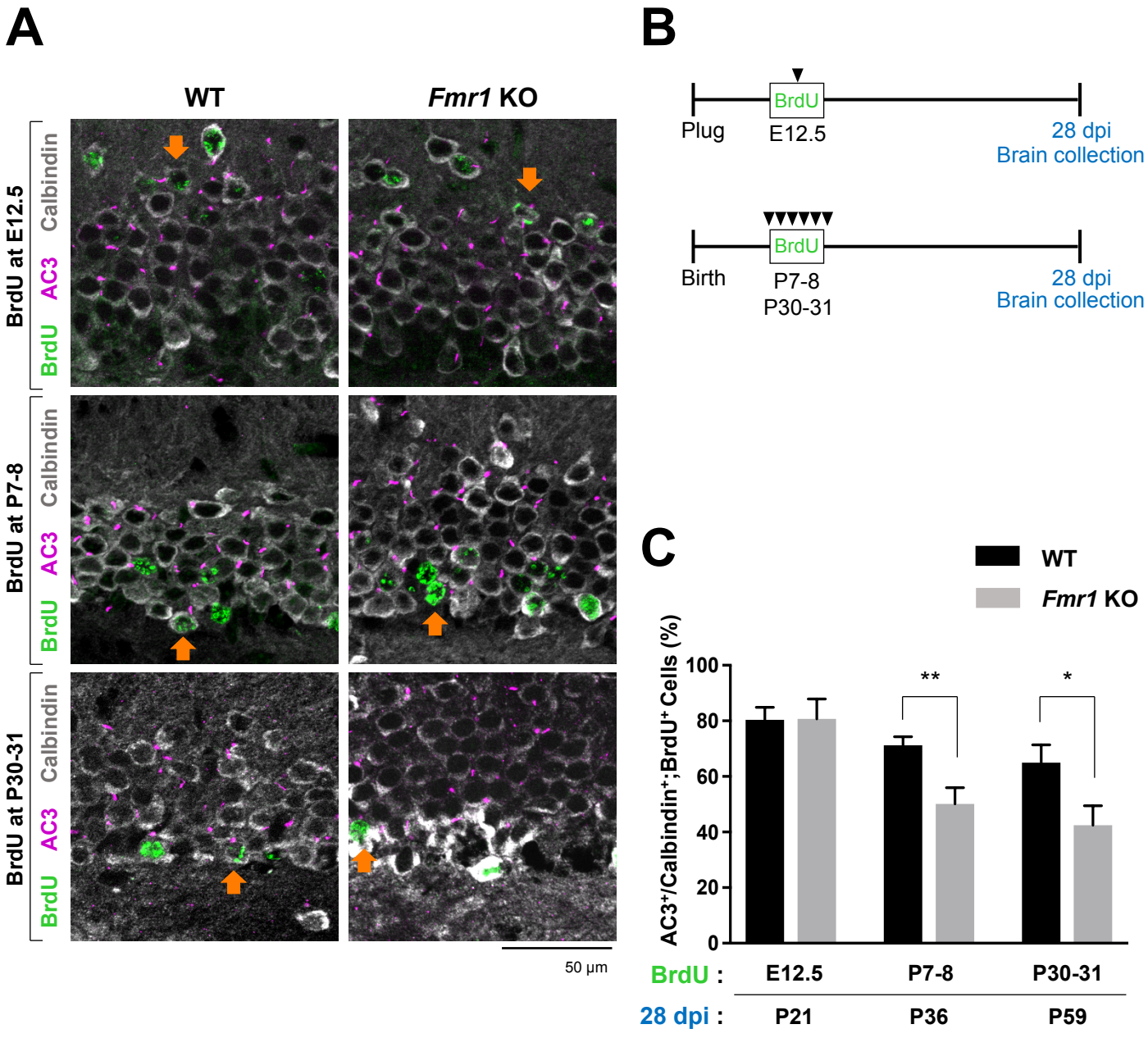


Figure S4. Newborn neurons (calbindin$^+$;BrdU$^+$) originated from the SGZ exhibit primary cilia loss in the DG of *Fmr1* KO mice. Related to Figure 6. BrdU was administrated by intraperitoneal injection into WT or *Fmr1* KO mice at E12.5, P7-8, or P30-31 followed by brain collection at 28 days post injection (dpi). (A) Immunostaining of AC3 (magenta), calbindin (gray), and BrdU (green) in the DG of BrdU-treated WT or *Fmr1* KO mice. Orange arrows, AC3$^+$;calbindin$^+$;BrdU$^+$ cells. Inner layer (toward hilus) of the DG is located on the lower side of images. (B) Schematic view of BrdU treatment and collection of WT or *Fmr1* KO mice. (C) Quantification of the percentage of AC3$^+$ cells among calbindin$^+$;BrdU$^+$ newborn neurons originating at E12.5, P7-8, or P30-31 in WT and *Fmr1* KO mice (28 dpi: P21, P36, or P59). n = 8, mean ± SEM, Student's unpaired t test. *p < 0.05, **p < 0.01.

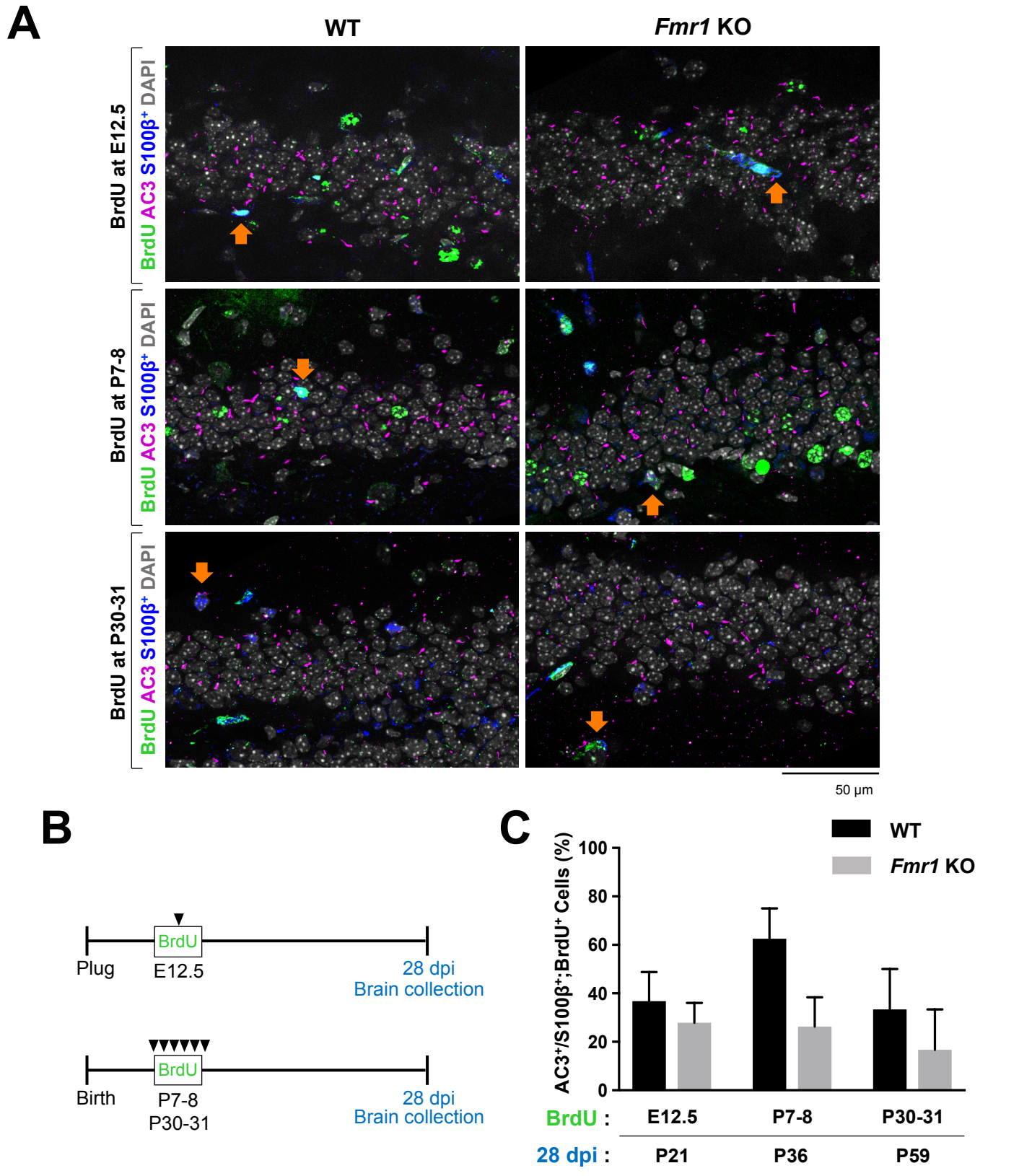
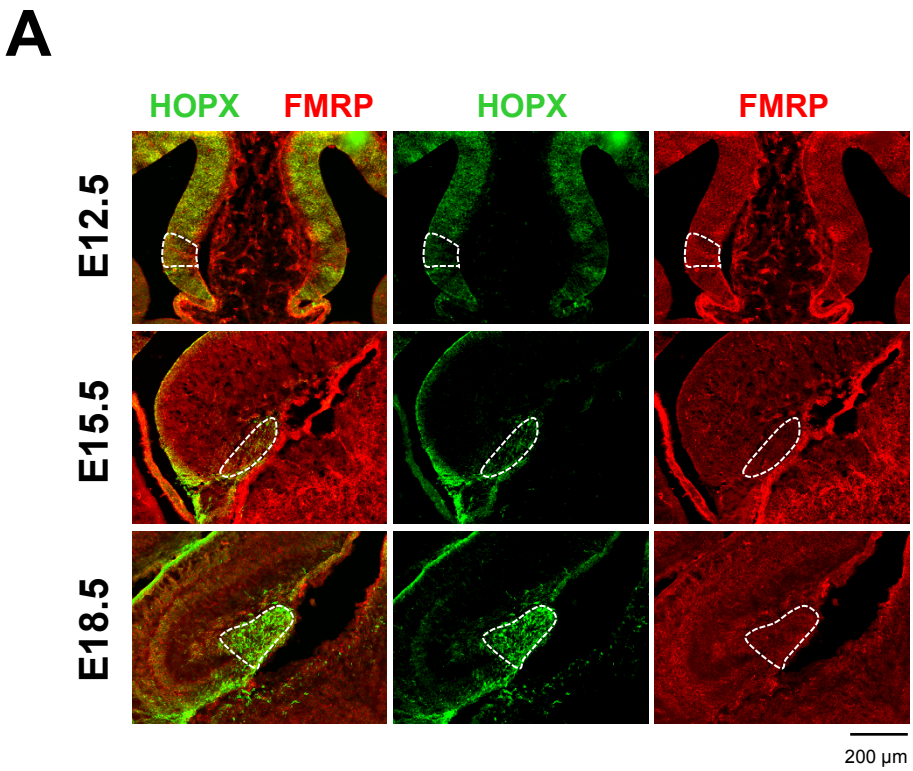


Figure S5. Newly differentiated astrocytes (S100β⁺;BrdU⁺) do not exhibit primary cilia loss in the DG of *Fmr1* KO mice. Related to Figure 6. BrdU was administrated by intraperitoneal injection into WT or *Fmr1* KO mice at E12.5, P7-8, or P30-31 followed by brain collection at 28 days post injection (dpi). (A) Immunostaining of AC3 (magenta), S100β (blue), and BrdU (green) with DAPI nuclear staining (gray) in the DG of BrdU-treated WT or *Fmr1* KO mice. Orange arrows, AC3⁺;S100β⁺;BrdU⁺ cells. Inner layer (toward hilus) of the DG is located on the lower side of images. (B) Schematic view of BrdU treatment and collection of WT or *Fmr1* KO mice. (C) Quantification of the percentage of AC3⁺ cells among S100β⁺;BrdU⁺ newly differentiated astrocytes originating at E12.5, P7-8, or P30-31 in WT and *Fmr1* KO mice (28 dpi: P21, P36, or P59). n = 8, mean ± SEM, Student's unpaired t test.



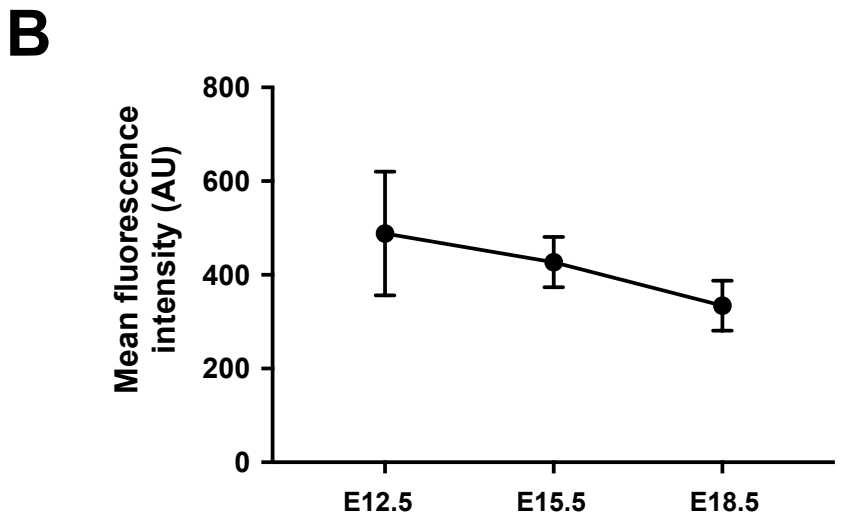


Figure S6. FMRP expression levels are not significantly changed during embryonic development between E12.5 and E18.5. Related to Figure 6. (A) Immunostaining of HOPX (green) and FMRP (red) in the DNe or DG of E12.5, E15.5, and E18.5 WT embryos. FMRP expression levels were analyzed in the DNe or DG (white dashed line) where HOPX expresses. (B) Mean intensity of FMRP was analyzed in E12.5, E15.5, and E18.5 from the DNe or DG of WT embryos and was subtracted by the mean intensity of FMRP in *Fmr1* KO mice. n = 4. mean ± SEM. One-way ANOVA.