FMRP REGULATES NEURONAL MIGRATION VIA MAP1B

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ABSTRACT

The Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability, and the first monogenic cause of Autism Spectrum Disorder. FXS is caused by the absence of the RNA-binding protein FMRP (Fragile X Messenger Ribonucleoprotein).

Neuronal migration is an essential step of brain development allowing displacement of neurons from their germinal niches to their final integration site. The precise role of FMRP in neuronal migration remains mostly unexplored. We studied the consequences of FMRP absence on migration, by live-imaging neurons of the postnatal Rostral Migratory Stream (RMS).

In *Fmr1-null* RMS, neurons exhibit a slowed-down migration and an impaired trajectory, associated with defects of their centrosomal movement. RNA-interference-induced knockdown of *Fmr1* shows that these migratory defects are cell-autonomous. Finally, the FMRP mRNA target involved in these defects is MAP1B (Microtubule-Associated Protein 1B), since its knockdown rescues most migratory defects.

Our results thus unveil a new neurodevelopmental role of FMRP, as a crucial actor of neuronal migration linked to MAP1B, potentially important for the understanding of FXS pathophysiology.

INTRODUCTION

The Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability and a leading cause of autism spectrum disorder. FXS is due to the silencing of the gene *FMR1* and loss of the encoded protein, FMRP (Fragile X Messenger Ribonucleoprotein) (Davis & Broadie, 2017). FMRP is an ubiquitous RNA-binding protein, with high level of expression in the central nervous system (Gholizadeh *et al*, 2015). It is a general regulator of RNA metabolism and especially of mRNA local translation in neurons (Banerjee *et al*, 2018). Its cognate mRNA targets are numerous and diverse, including mRNAs encoding cytoskeletal proteins like MAP1B (Microtubule-Associated Protein 1B) (Ascano *et al*, 2012)(Brown *et al*, 2001)(Darnell *et al*, 2001)(Maurin *et al*, 2018). *Fmr1*-null mice are the murine model of FXS and have

allowed characterization of neurodevelopmental and plasticity defects consecutive to the absence of FMRP.

Neuronal migration is a crucial step for the establishment of neuronal circuitry, allowing the displacement of neurons from their site of birth to their final destination of differentiation. Migration defects lead to severe brain pathologies like lissencephaly and cortical heterotopia and might be involved in psychiatric disorders (Romero *et al*, 2018). Interestingly, migration in the human infant brain appears to be even more extended than anticipated from the rodent data (Sanai *et al*, 2011)(Paredes *et al*, 2016). In addition, periventricular heterotopia was described in two FXS patients, suggesting a possible role for FMRP in migration (Moro *et al*, 2006). However, the question of potential migration defects in FXS remains mostly unexplored. In *Fmr1*-null mice, radially migrating embryonic glutamatergic cortical neurons display a defect in the multipolar to bipolar transition (La Fata *et al*, 2014). Additionally, FMRP overexpression of knockdown leads to misplacement of cortical glutamatergic neurons, also suggesting its role in radial embryonic migration (Wu *et al*, 2019). However, to our knowledge, tangential neuronal migration in the absence of FMRP has not been studied so far and, more generally, the dynamics of mutated *Fmr1* neurons have never been analyzed in detail.

Here, using the postnatal rostral migratory stream (RMS) as a migration model as in (Stoufflet *et al*, 2020), we show entirely novel migratory defects induced by the absence of FMRP and the causal role of its mRNA target MAP1B.

RESULTS

FMRP is expressed in migrating neurons of the postnatal RMS

A massive number of neuroblasts are constantly produced in the ventricular/subventricular zone (V/SVZ) and migrate over a long distance along the RMS to the olfactory bulb (OB) (Lim & Alvarez-Buylla, 2016)(Fig. 1A). They display a cyclic saltatory mode of migration, in which the nucleus and centrosome move forward in a “two-stroke” cycle (Bellion *et al*, 2005). The centrosome moves first within a swelling in the leading process, which we call centrokinesis (CK) and the nucleus follows subsequently (nucleokinesis, NK)(Fig. 1B). The neurons then pause and the cycle can reinitiate.

After an *in vivo* intraventricular electroporation of a GFP-expressing plasmid in neonate mice, a cohort of GFP-positive neurons can be visualized in the RMS a few days later (Fig. 1.C).

FMRP is expressed in most neurons of the brain (Gholizadeh *et al*, 2015). Accordingly, immunostaining for FMRP reveals that FMRP is strongly expressed in the RMS, where most neurons appear labeled. In individual GFP-positive neurons, FMRP labeling appears as a discrete and punctate staining visible mainly in the cytoplasm both at the rear of the neuron and in the leading process and growth cone (Fig. 1.D,E).

FMRP cell-autonomously regulates neuronal migration

To investigate the involvement of FMRP in RMS migration, we used the *Fmr1-null* mouse line (Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium, 1994). Time-lapse imaging of GFP positive neurons was performed in the control and *Fmr1-null* RMS (movies S.1 and S.2). *Fmr1-null* neurons display a slowed-down migration, an increased pausing time, a more sinuous trajectory, and a defective directionality (Fig. 2.A-D). Additionally, the NK is less frequent and the mean distance per NK is reduced (Fig. 2.E,F).

Given the crucial role of the centrosome in neuronal migration (Higginbotham & Gleeson, 2007), we analyzed its dynamics by performing co-electroporation of GFP and Centrine-RFP in *Fmr1-null* and control neonate mice to co-label migrating neurons and their centrosome (Fig. 3.A). The CK is slowed-down and less frequent in *Fmr1-null* neurons, as compared to controls (Fig. 3.B,C). A CK leading to a subsequent NK was defined as an efficient CK, as opposed to a CK not leading to an NK. CK efficiency is reduced in *Fmr1-null* neurons as compared to controls (Fig. 3.D).

Taken together, these results show that FMRP plays a key role in neuronal migration.

To assess whether these migration defects are cell autonomous, we designed an interfering RNA coupled to GFP to cell-autonomously knock-down *Fmr1* mRNA in RMS neurons, similar to (Scotto-Lomassese *et al*, 2011). The interfering miR*Fmr1*-GFP was co-electroporated with centrin-RFP to perform live-imaging (movie S. 3). Analysis of migration and centrosome dynamics (Fig. S1 and S2) showed that *Fmr1* KD is sufficient to recapitulate the entire migratory phenotype described in *Fmr1-null* mutants, demonstrating that FMRP cell-autonomously regulates neuronal migration.

Together, these data show that FMRP is cell-autonomously necessary for proper neuronal migration of RMS neurons, by regulating their speed and directionality as well as centrosome/nucleus coupling.

FMRP regulates neuronal migration through MAP1B

MAP1B is a neuron-specific microtubule-associated protein widely expressed in the developing CNS with crucial roles in diverse steps of neural development including neuronal migration (Yang *et al*, 2012). MAP1B is also a well-know FMRP mRNA target (Zhang *et al*, 2001)(Darnell *et al*, 2001)(Brown *et al*, 2001). It thus appeared as an interesting FMRP target to investigate. As FMRP is a repressor of MAP1B mRNA translation (Brown *et al*, 2001)(Darnell *et al*, 2001)(Lu *et al*, 2004), the overall level of MAP1B usually appears increased in an *Fmr1-null* context (Lu *et al*, 2004)(Hou *et al*, 2006). Accordingly, MAP1B expression is increased in the RMS, as assessed by immunostaining and Western Blot (Fig. S3 A,B)

To assess whether the upregulation of MAP1B is responsible for the migratory phenotype observed in *Fmr1-null* neurons, we cell-autonomously knocked-down *Map1b* in RMS neurons with an interfering RNA. The miR*Map1b*-GFP was electroporated in *Fmr1-null* neonate mice and time-lapse imaging was performed in acute sections of the RMS (movie S. 4). *Fmr1-null* neurons expressing the miR*Map1b*-GFP exhibit a completely restored migration speed, pausing time, NK distance and frequency, which become comparable to miRNeg-GFP control neurons (Fig.6.A,B,D,E). Of note, the sinuosity of *Fmr1-null* neurons expressing miR*Map1b*-GFP was not rescued (Fig.6.C), suggesting that this parameter is MAP1B independent. Overall, our results demonstrate that MAP1B is the main FMRP mRNA target involved in the regulation of neuronal migration.

DISCUSSION

FMRP is commonly described as a critical regulator of neuronal plasticity and neural development (Richter & Zhao, 2021). However, its role in neuronal migration remains poorly understood.

A role for FMRP in radial embryonic migration was evidenced by (La Fata *et al*, 2014) and (Wu *et al*, 2019). However, to our knowledge, tangential migration in the absence of FMRP has never been analyzed so far, and neither has been the effect of its absence on the dynamics of saltatory migration.

We report strong defects in postnatal migration with a slowed-down and erratic migration. Of importance, even though the mutated neurons are delayed in their movement and lose time finding the proper direction, they ultimately properly arrive in the OB, as we previously showed in the adult (Scotto-Lomassese *et al*, 2011). Live imaging allowed us to perform detailed analysis of both nucleokinesis and centrosome dynamics, which proved both to be deeply perturbed. As microtubules are essential regulators of both processes (Kuijpers & Hoogenraad, 2011) (Tsai & Gleeson, 2005), we suspected MAP1B to be the key FMRP mRNA target involved in the migratory phenotypes. MAP1B is one of the historic targets of FMRP (Brown *et al*, 2001)(Darnell *et al*, 2001)(Zhang *et al*, 2001). It is expressed early in the embryonic brain (Tucker *et al*, 1989) and is essential to different steps of neural development (Gonzalez-Billault *et al*, 2004) and in particular the regulation of embryonic radial migration (González-Billault *et al*, 2005).

We show MAP1B overexpression in *Fmr1*-mutated neurons and rescue the migratory defects through its RNA-interference-induced KD. This identifies MAP1B as the critical FMRP mRNA target involved in the regulation of cyclic saltatory migration.

The importance of FMRP-regulated MAP1B translation was initially evidenced in *drosophila*, where it appeared essential for proper synaptogenesis (Zhang *et al*, 2001). This was later confirmed in the mouse hippocampus, where increased MAP1B levels in the *Fmr1*-null context also induced defective synaptogenesis (Lu *et al*, 2004). To our knowledge, the importance of the FMRP-MAP1B duo for neuronal migration described here is the only other described neurodevelopmental function of FMRP-regulated MAP1B translation.

In the context of FXS, analyzing migration in the FXS human organoids or assembloïds will be of course of major interest (Levy & Paşca, 2023). It is to be noted that postnatal tangential migration in the infant human brain was recently described to be even more extensive than in mice (Paredes *et al*, 2016)(Sanai *et al*, 2011) so that, if conserved in humans, the defects that we observe might be critical for proper postnatal corticogenesis.

As a conclusion, we report here an entirely new function of FMRP as a regulator of migration linked to microtubules *via* MAP1B. This participates in the fundamental understanding of this multifaceted protein and might also be important for the pathophysiological understanding of FXS.

MATERIAL AND METHODS

Mouse lines

Mice were housed in a 12 hours light/dark cycle, in cages containing one or two females and one male. The postnatal mice were housed in their parents' cage. Animal care was conducted in accordance with standard ethical guidelines [National Institutes of Health (NIH) publication no. 85-23, revised 1985 and European Committee Guidelines on the Care and Use of Laboratory Animals 86/609/EEC]. The experiments were approved by the local ethics committee (Comité d'Ethique en Expérimentation Animale Charles Darwin C2EA-05 and the French Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche APAFIS#13624-2018021915046521_v5). We strictly performed this approved procedure. The mice used were in a C57BL6-J background. *Fmr1-null* mice were genotyped according to the original protocol (Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium, 1994).

MiRNA production

Silencing of *Fmr1* and *Map1b* has been performed using BLOCK-iT Pol II miR RNAi Expression Vector Kits (Invitrogen) and the RNAi Designer (Invitrogen). The sequences of the single-stranded oligos are:
Fmr1 Top:
TGCTGTACAAATGCCTTGTAGAAAGCGTTTTGGCCAACTGACTGACGCTTTCTAAGGCATTTGTA,
Fmr1 Bottom:
CCTGTACAAATGCCTTAGAAAGCGTCAGTCAGTGGCCAAAACGCTTTCTACAAGGCATTTGTAC,
Map1b Top:
TGCTGTGTTGATGAAGTCTTGGAGATGTTTTGGCCACTGACTGACATCTCCAACTTCATCAACA,
Map1b Bottom:
CCTGTGTTGATGAAGTTGGAGATGTCAGTCAGTGGCCAAAACATCTCCAAGACTTCATCAACAC.
The double-stranded oligos were inserted in a pcDNA 6.2-GW/EmGFP-miR. The resulting constructions were sequenced before use.

Postnatal electroporation

Postnatal injection and electroporation were performed at postnatal day 2 (P2). Postnatal mice were anesthetized by hypothermia. Pseudo-stereotaxic injection [from lambda medial-lateral (M/L): 0,9; anterior-posterior (A/P): 1,1; dorsal-ventral (D/V): 2] using a glass micropipette (Drummond Scientific Company, Wiretrol I, 5-000-1050) was performed, and 2ul of plasmid (between 5 and 8 μg/ml) was injected. Animals were subjected to 5 pulses of 99.9V during 50ms separated by 950ms using the CUY21 SC Electroporator and 10-mm tweezer electrodes (Harvard Apparatus, Tweezertrode, 10mm, 45-0119). The animals were placed on 37°C plates to restore their body temperature before returning in their parents' cage. Animals were considered as fully restored when moving naturally and their skin color returned to pink.

Postnatal acute brain slices

Brain slices of mice aged from P6 to P10 were prepared as previously described in (Stoufflet *et al*, 2020). Pups were sacrificed by decapitation and the brain was removed

from the skull. Sagittal brain sections (250 μm) were cut with a VT1200S microtome (Leica). Slices were prepared in an ice-cold cutting solution of the following composition: 125 mM NaCl, 0.4 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5 mM sodium pyruvate, 20 mM glucose and 1 mM kynurenic acid, saturated with 5% CO_2 and 95% O_2. Slices were incubated in this solution for 30 minutes at room temperature and then placed in recording solution (identical to the cutting solution, except that the $CaCl_2$ concentration is 2 mM and kynurenic acid is absent) for at least 30 minutes at 32°C before image acquisition.

Time-lapse video microscopy of postnatal slices

To analyze neuronal migration and centrosome dynamics, images were obtained with an inverted SP5D confocal microscope (Leica) using a 40x/1.25-numerical aperture (N.A.) objective with 1.5 optical zoom or an upright SP5 MPII two-photon microscope (Leica) using a 25x/0.95-N.A. objective with 1.86 optical zoom. Images were acquired every 3 minutes for 2 to 3 hours. The temperature in the microscope chamber was maintained at 32°C during imaging and brain slices were continuously perfused with heated recording solution (see above) saturated with 5% CO_2 and 95% O_2.

Analyses of neuronal migration and centrosome movement

Analyses were performed using ImageJ (NIH Image; National Institutes of Health, Bethesda, MD) software and MTrackJ plugin (Meijering, Dzyubachyk, et Smal 2012). The nucleus and the centrosome (when centrin-RFP was co-injected) were tracked manually on each time frame during the whole movie. We considered a NK as a movement superior to 6 μm between two consecutive time points (3 minutes-interval). For cell migration, calculation of speed, percentage of pausing time, sinuosity, directionality, NK distance and frequency was performed using the x,y,t coordinates of the nucleus of each cell. Cells tracked for less than 30 minutes and cells that did not perform any NK during the whole tracking were excluded. A CK was defined as a forward movement superior to 2 μm followed by a backward movement superior to 2 μm. For centrosome movement, calculation of CK speed, frequency and efficiency was performed using the x,y,t coordinates of the centrosome of each cell and the x,y,t coordinates of each corresponding nucleus.

Immunohistochemistry

P7 to P10 mice were lethally anesthetized using Euthasol. Intracardiac perfusion with 4% paraformaldehyde was performed. The brain was post-fixed overnight in 4% paraformaldehyde and then rinsed three times with phosphate-buffered saline (PBS) 1x (Gibco, 1400-067). 50 μm sagittal sections were made with VT1200S microtome (Leica). Slices were placed 1 hour in a saturation solution (10% fetal bovine serum; 0.5% Triton X-100 in PBS). Primary antibodies used in this study are: GFP (Aves; GFP-1020; 1/1000), FMRP (Developmental Studies Hybridoma Bank; 2F5-1; 1/200), MAP1B (Santa Cruz Biotechnology; sc-365668; 1/300). The antibodies were diluted in saturation solution. Slices were incubated for 48 to 72 hours at 4°C under agitation with the antibodies and then rinsed three times with PBS 1x. Secondary antibodies used are: anti-chicken immunoglobulin Y (IgY) Alexa Fluor 488 (1/1000; Jackson ImmunoResearch; 703-545-155) against anti-GFP, anti-mouse immunoglobulin G2b (IgG2b) Alexa Fluor 555 (1/2000; Jackson ImmunoResearch; 703-545-155) against anti-FMRP and anti-MAP1B. The antibodies were diluted in saturation solution. Slices were incubated with secondary antibodies for 1 hour at room temperature under

agitation, protected from light. After rinsing three times with PBS 1x, slices were counter-colored with Hoechst and mounted in Mowiol.

Tissue collection and Western blotting

RMS were manually micro-dissected from 5 to 6 postnatal mouse brains and pooled in a PBS 1x (0.5% glucose) solution. After centrifugation, protein extraction was performed on the tissue pellet. Samples were homogenized in a lysis buffer with the following composition: 25 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% NaDeoxycholate, 1 mM EDTA, 5% glycerol, 1X EDTA-free protease inhibitor cocktail (Sigma, 4693132001). After centrifugation, samples were loaded and run in NuPAGE 3-8% Tris-Acetate Gel (Invitrogen, EA0378BOX) at 120V for 15 minutes then 180V for 40 minutes. Transfer to nitrocellulose Immobilon-PVDF-P membrane (Millipore, IPVH00010) was performed at 40V overnight at 4°C. The membrane was then saturated for 1 hour in TBSt containing 5% powder milk. Primary antibodies used are: MAP1B (Santa Cruz Biotechnology, sc-365668, 1/100), Vinculin (Cell Signaling Technology, 13901S, 1/1000). The antibodies were diluted in TBSt containing 5% powder milk. Secondary antibodies used are: ECL anti-mouse immunoglobulin G (IgG) horseradish peroxidase linked whole antibody (1/10 000; Fisher Scientific; NXA931V) for anti-MAP1B, Peroxidase-conjuguated AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit IgG (H+L) (1/5 000; Jackson ImmunoResearch; 711-036-152) for anti-Vinculin. The antibodies were diluted in TBSt containing 5% powder milk. Labeling was visualized using Pierce ECL Western Blotting Substrate (Thermo Scientific; 32209) and luminescent image analyzer LAS-3000.

Statistics

All manipulations and statistical analyses were implemented with R (4.2.1, R Foundation for Statistical Computing, Vienna, Austria). Normality in the variable distributions was assessed by the Shapiro-Wilk test. Furthermore, the Levene test was performed to probe homogeneity of variances across groups. Variables that failed the Shapiro-Wilk or the Levene test were analyzed with non-parametric statistics using the one-way Kruskal-Wallis analysis of variance on ranks followed by Nemenyi test post hoc and Mann-Whitney rank sum tests for pairwise multiple comparisons. Variables that passed the normality test were analyzed by means of one-way ANOVA followed by Tukey post hoc test for multiple comparisons or by Student's *t* test for comparing two groups. Categorical variables were compared using Pearson's c^2 test or Fisher's exact test. A *p*- value of <0.05 was used as a cutoff for statistical significance. Results are presented as the means ± SEM. The statistical tests are described in each figure legend.

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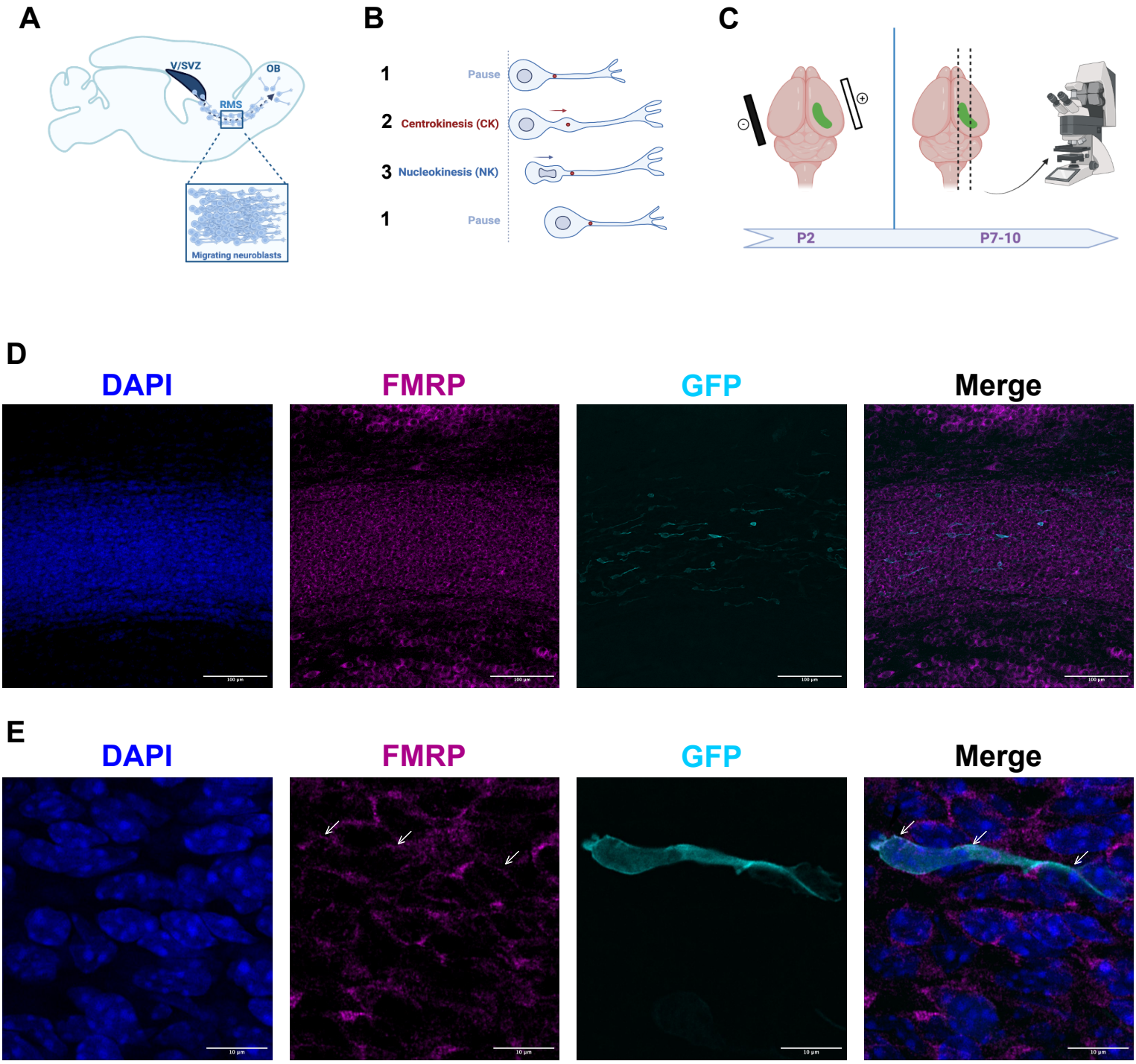

FIGURE 1. ***FMRP is expressed in migrating neurons of the murine postnatal RMS.*** *(A) Scheme of a sagittal section of the postnatal RMS connecting the V/SVZ to the OB. V/SVZ, ventricular/sub-ventricular zone; OB, olfactory bulb; RMS, rostral migratory stream. The inset shows the high density of homotypically migrating neurons in the RMS. (B) Representation of neuroblasts' cyclic saltatory migration. 1. The neuron is in pause. 2. The leading process (LP) extends, and the centrosome moves within a swelling in the LP. 3. The nucleus moves forward. CK, centrokinesis; NK, nucleokinesis. (C) Scheme of the experimental procedure. 2-day old neonates are intraventricularly electroporated with a GFP-expressing plasmid to label a cohort of migrating neurons that can be subsequently visualized in fixed or acute sections of the RMS. (D) Immunohistochemistry of the RMS showing FMRP expression (magenta) along the stream, in which a cohort of GFP-positive neurons (cyan) are visible. Scale bar: 100 µm. (E) Immunohistochemistry of a GFP-positive RMS neuron (cyan) showing FMRP subcellular expression (magenta). The GFP-positive neuron displays a cytoplasmic expression of FMRP around the nucleus (indicated by white arrows), along the leading process and in the growth cone. The surrounding GFP-negative neurons express FMRP as well, according to the same pattern. Scale bar: 10 µm.*

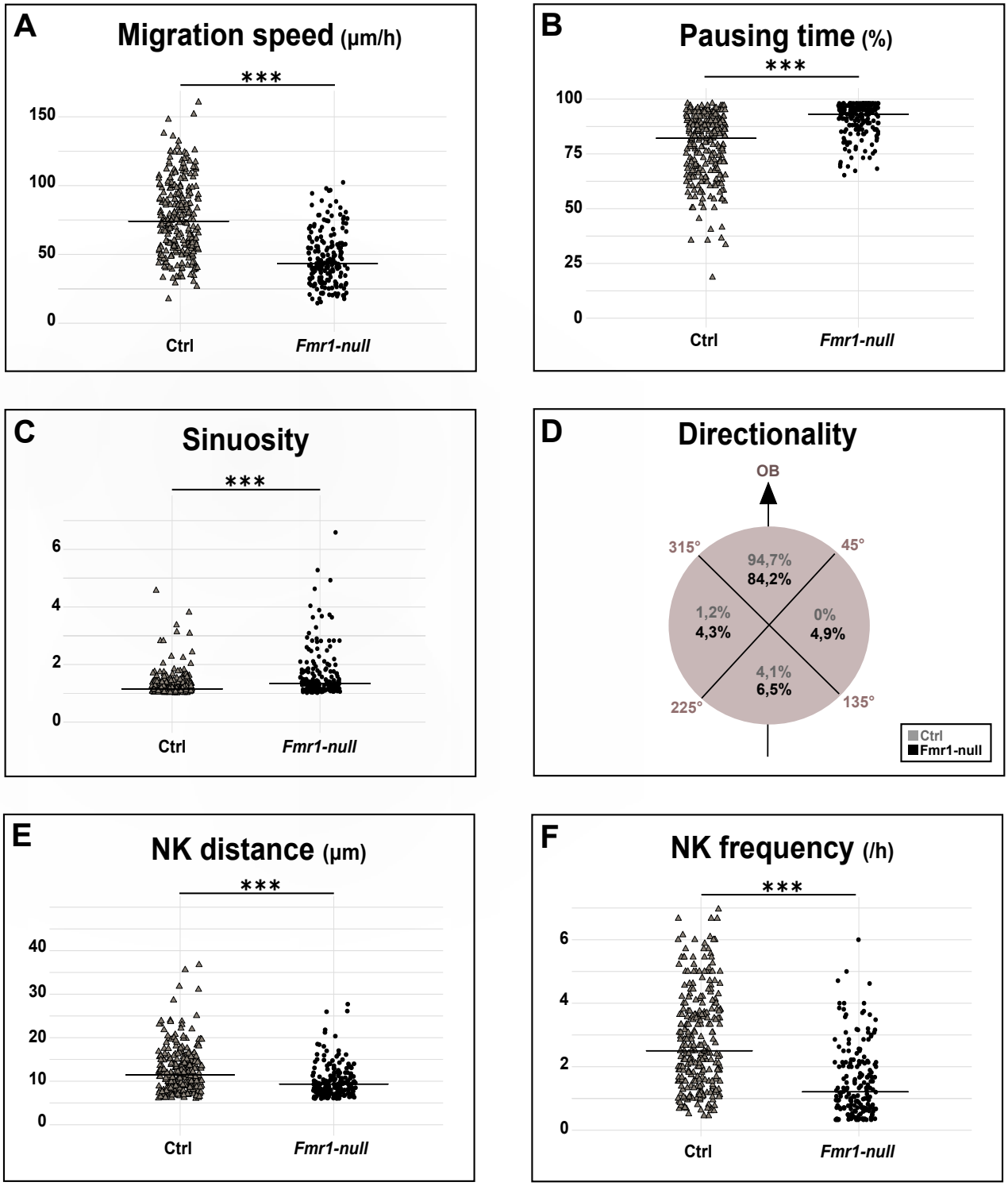

FIGURE 2. ***Migration defects in Fmr1-null neurons.*** *(A) Migration speed of control (Ctrl) and Fmr1-null neurons. Ctrl: 76 ± 2 µm/h; Fmr1-null: 47 ± 1 µm/h (Mann-Whitney test, p-value < 0.001). (B) Percentage of pausing time of control and Fmr1-null neurons. Ctrl: 78 %; Fmr1-null: 91 % (Mann-Whitney test, p-value < 0.001). (C) Sinuosity index of control and Fmr1-null neurons. Ctrl: 1.32 ± 0.04; Fmr1-null: 1.93 ± 0.16 (Mann-Whitney test, p-value < 0.001). (D) Migration directionality radar represented in four spatial dials. Percentage of cells migrating in each spatial direction in control and Fmr1-null neurons, relatively to the vector going straight from the SVZ to the OB. (Fisher's Exact test, p-value < 0.001). (E) NK mean distance of control and Fmr1-null neurons. Ctrl: 12.5 ± 0.3 µm; Fmr1-null: 10.9 ± 0.8 µm (Mann-Whitney test, p-value < 0.001). (F) NK frequency of control and Fmr1-null neurons. Ctrl: 2.9 ± 0.1 NK/h; Fmr1-null: 1.5 ± 0.1 NK/h (Mann-Whitney test, p-value < 0.001). The black line represents the median. Ctrl: N = 3, n = 275; Fmr1-null: N = 3, n = 184. ***p-value < 0.001.*

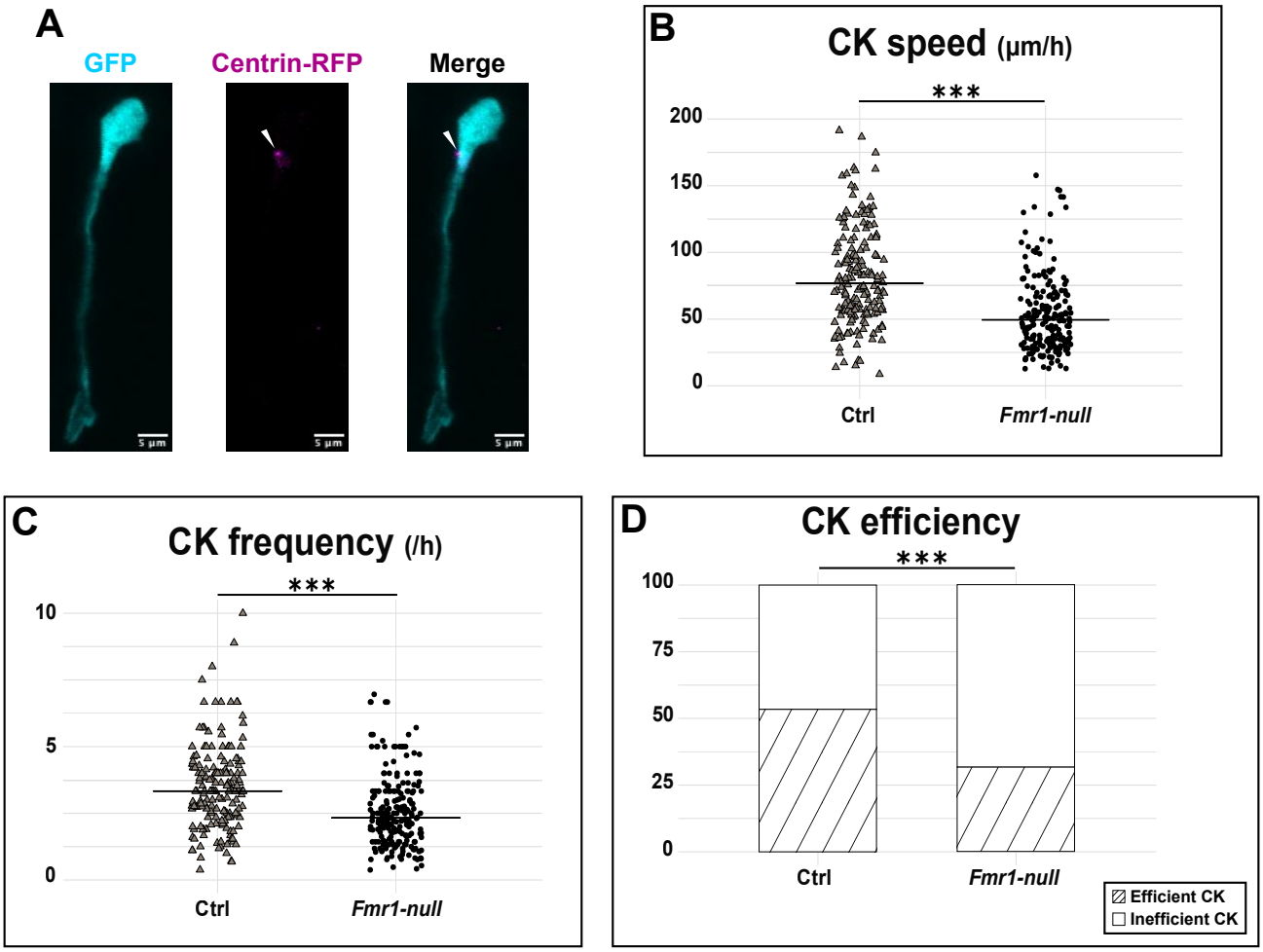

FIGURE 3. *CK defects in Fmr1-null neurons.* *(A) Illustration of an RMS-migrating neuron (cyan) co-injected with centrin-RFP (magenta). Scale bar: 5 µm. (B) CK speed of control and Fmr1-null neurons. Ctrl: 82 ± 3 µm/h; Fmr1-null: 54 ± 2 µm/h (Mann-Whitney test, p-value < 0.001). (C) CK frequency of control and Fmr1-null neurons. Ctrl: 3.5 ± 0.1 CK/h; Fmr1-null: 2.6 ± 0.1 CK/h (Mann-Whitney test, p-value < 0.001). (D) Percentage of efficient CKs in control and Fmr1-null neurons. Ctrl: 54 %; Fmr1-null: 33 % (Chi2 = 57.611, p-value < 0.001). The black line represents the median. Ctrl: N = 3, n = 178; Fmr1-null: N = 3, n = 216. *** p-value < 0.001.*

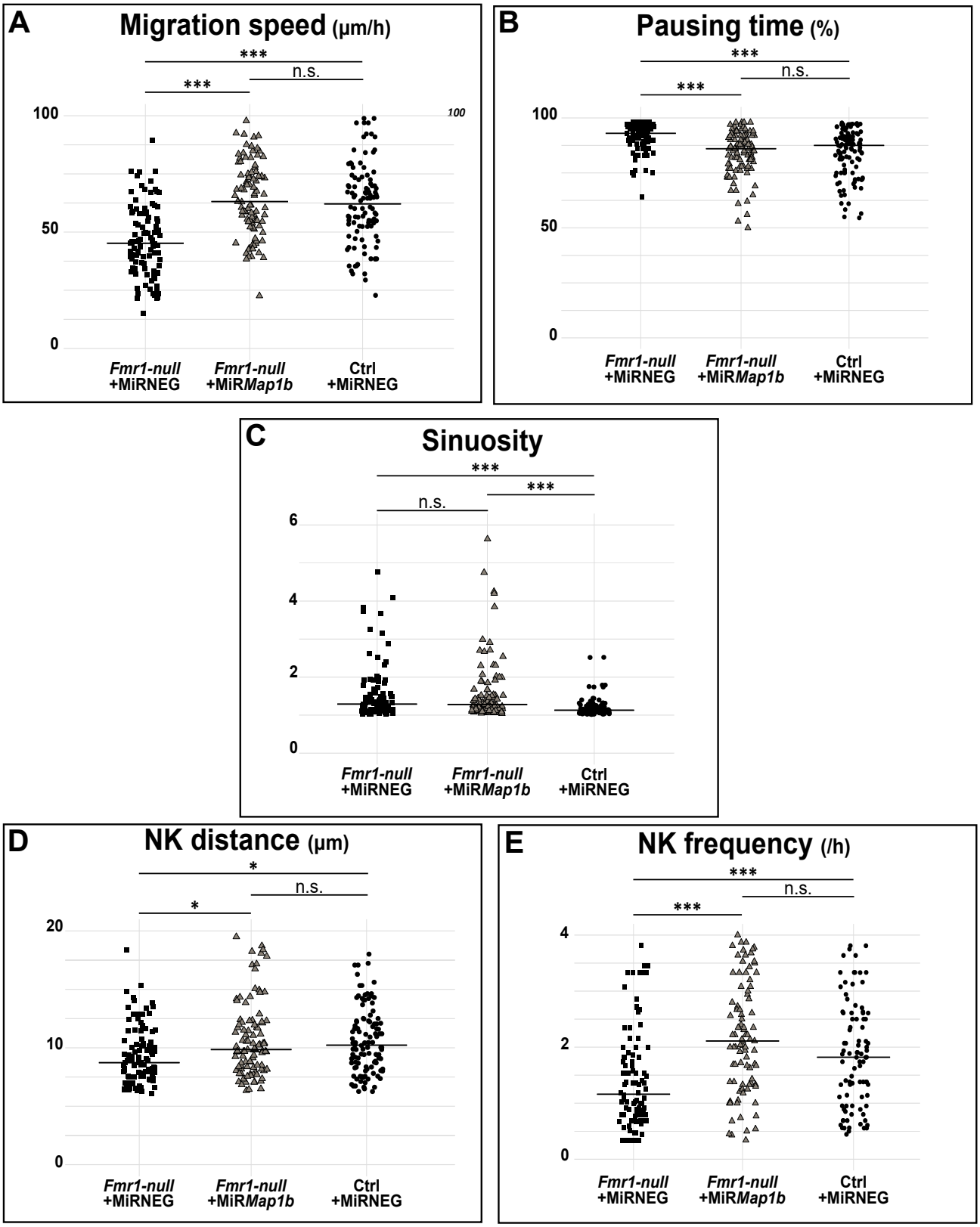

FIGURE 4. *Map1b KD rescues Fmr1-null neurons migration defects.* *(A) Migration speed of Fmr1-null neurons expressing MiRNEG and MiRMap1b and control neurons expressing MiRNEG. Fmr1-null neurons + MiRNEG: 47 ± 2 µm/h; Fmr1-null neurons + MiRMap1b: 67 ± 2 µm/h; control neurons + MiRNEG: 64 ± 2 µm/h (Kruskall-Wallis Test: Chi2 = 61.168, p-value < 0.001, df = 2; followed by Dunn's posthoc test). (B) Percentage of pausing time of Fmr1-null neurons expressing MiRNEG and MiRMap1b and control neurons expressing MiRNEG. Fmr1-null neurons + MiRNEG: 92 %; Fmr1-null neurons + MiRMap1b: 84 %; control neurons + MiRNEG: 84 % (Kruskall-Wallis Test: Chi2 = 45.716, p-value < 0.001, df = 2; followed by Dunn's posthoc test). (C) Sinuosity index of Fmr1-null neurons expressing MiRNEG and MiRMap1b and control neurons expressing MiRNEG. Fmr1-null neurons + MiRNEG: 1.77 ± 0.18; Fmr1-null neurons + MiRMap1b: 1.90 ± 0.20; control neurons + MiRNEG: 1.18 ± 0.02 (Kruskall-Wallis Test: Chi2 = 39.807, p-value < 0.001, df = 2; followed by Dunn's posthoc test). (D) NK mean distance of Fmr1-null neurons expressing MiRNEG and MiRMap1b and control neurons expressing MiRNEG. Fmr1-null neurons + MiRNEG: 9.7 ± 0.3 µm; Fmr1-null neurons + MiRMap1b: 11.1 ± 0.4 µm; control neurons + MiRNEG: 10.6 ± 0.3 µm (Kruskall-Wallis Test: Chi2 = 11.573, p-value = 0.003, df = 2; followed by Dunn's posthoc test). (E) NK frequency of Fmr1-null neurons expressing MiRNEG and MiRMap1b and control neurons expressing MiRNEG. Fmr1-null neurons + MiRNEG: 1.5 ± 0.1 NK/h; Fmr1-null neurons + MiRMap1b: 2.4 ± 0.1 NK/h; control neurons + MiRNEG: 2.5 ± 0.2 NK/h (Kruskall-Wallis Test: Chi2 = 39.272, p-value < 0.001, df = 2; followed by Dunn's posthoc test). The black line represents the median. Fmr1-null neurons + MiRNEG: N = 6, n = 102; Fmr1-null neurons + MiRMap1b: N = 3, n = 101; control neurons + MiRNEG: N = 3, n = 78. * p-value < 0.05; *** p-value < 0.001; n.s. (not significant), p-value > 0.05.*