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Supplementary Information for

Astroglial FMRP Deficiency Cell-autonomously Upregulates miR-128 and Disrupts Developmental Astroglial mGluR5 Signaling

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Includes:

Figs. S1 to S7

Legend for Figs. S1 to S7

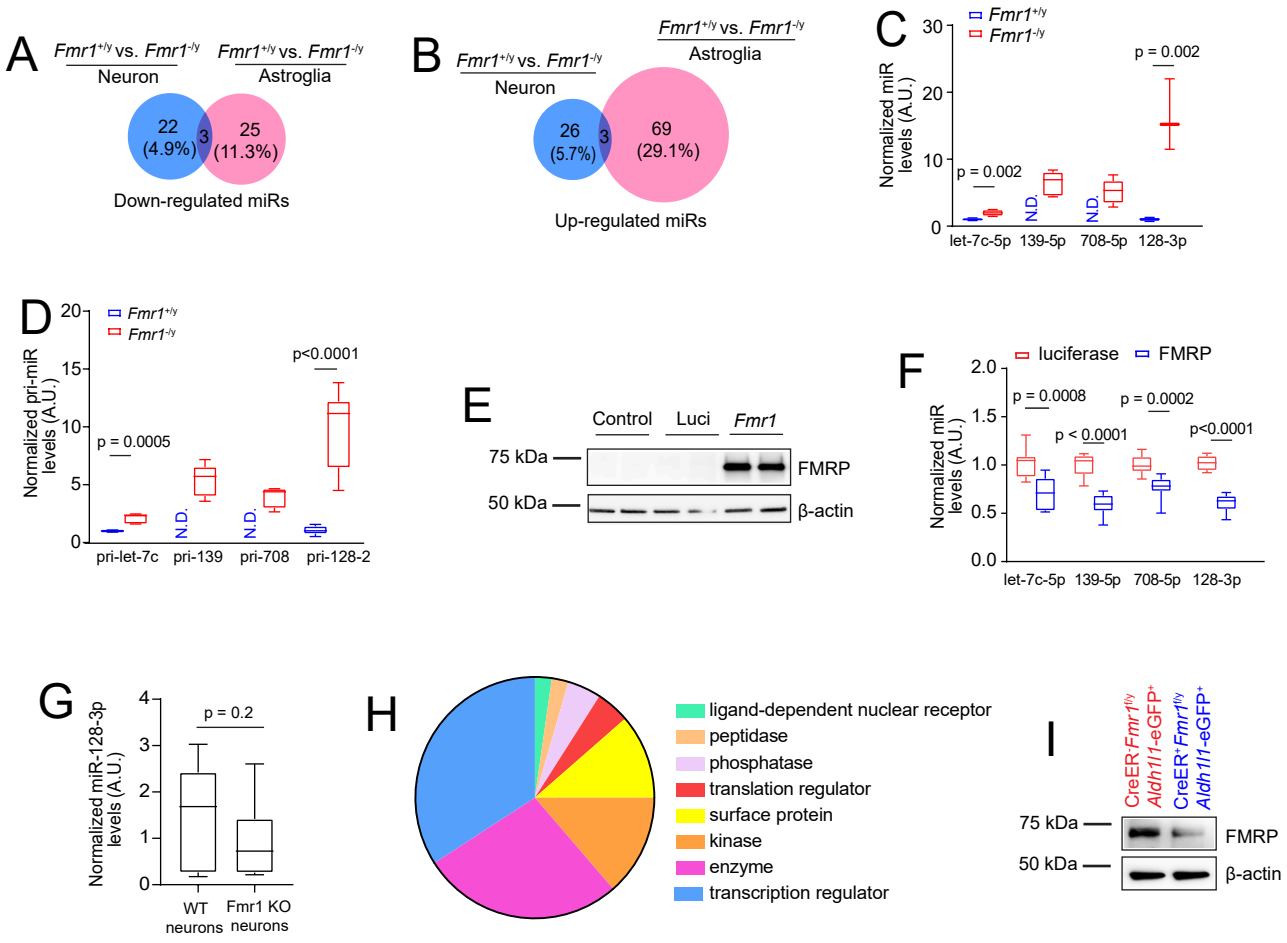

A
Fmr1+/y vs. Fmr1-/y
Neuron
Fmr1+/y vs. Fmr1-/y
Astroglia
22 (4.9%)
3
25 (11.3%)
Down-regulated miRs
B
Fmr1+/y vs. Fmr1-/y
Neuron
Fmr1+/y vs. Fmr1-/y
Astroglia
26 (5.7%)
3
69 (29.1%)
Up-regulated miRs
C
Fmr1+/y
Fmr1-/y
Normalized miR levels (A.U.)
p = 0.002
p = 0.002
N.D.
N.D.
let-7c-5p
139-5p
708-5p
128-3p
D
Fmr1+/y
Fmr1-/y
Normalized pri-miR levels (A.U.)
p = 0.0005
p<0.0001
N.D.
N.D.
pri-let-7c
pri-139
pri-708
pri-128-2
E
Control
Luci
Fmr1
75 kDa
50 kDa
FMRP
β-actin
F
luciferase
FMRP
Normalized miR levels (A.U.)
p = 0.0008
p = 0.0002
p < 0.0001
p<0.0001
let-7c-5p
139-5p
708-5p
128-3p
G
Normalized miR-128-3p levels (A.U.)
p = 0.2
WT neurons
Fmr1 KO neurons
H
ligand-dependent nuclear receptor
peptidase
phosphatase
translation regulator
surface protein
kinase
enzyme
transcription regulator
I
CreER+Fmr1f/y Aldh1l1-eGFP+
CreER+Fmr1f/y Aldh1l1-eGFP+
75 kDa
50 kDa
FMRP
β-actin

Figure. S1

Fig. S1 FMRP preferentially suppress miR biogenesis in astroglia

Venn diagram of the down- (**A**) and up-regulated (**B**) miRs in cultured *Fmr1*$^{+/y}$ and *Fmr1*$^{-/y}$ astroglia and neurons. Differentially expressed miRs were identified by miR microarray hybridization. n=3 biologically independent samples per condition. miRs with expression values < 2^3 arbitrary unit (A.U.) from the microarray hybridization were deemed not expressed. Percentage in the Venn diagram was calculated by up- or down-regulated miRs divided by total number of detectable miRs in astroglia or neurons; **C,** Relative expression levels of representative miRs in *Fmr1*$^{+/y}$ and *Fmr1*$^{-/y}$ astroglia by qPCR. All original Ct values are within 26-33 range; N.D.; not detected (Ct ≥ 35). U6 small nuclear (sn) RNA was used as the endogenous control; n = 5 biologically independent samples per condition. **D,** Relative expression levels of primary miR transcript (pri-miR) of representative miRs in *Fmr1*$^{+/y}$ and *Fmr1*$^{-/y}$ astroglia by qPCR. All original Ct values are within 28-34 range; N.D.: not detected (Ct ≥ 35). β-actin mRNA was used as the endogenous control; n= 5 biologically independent samples per condition. **E,** Representative immunoblot of FMRP re-expression following *Fmr1* cDNA transfection in cultured *Fmr1*$^{-/y}$ astroglia. **F,** Relative expression levels of representative miRs in FMRP re-expressed *Fmr1*$^{-/y}$ astroglia by qPCR. All original Ct values are within 22-32 range. U6 RNA was used as the endogenous control. n= 6 biologically independent samples per condition. **G,** Expression levels of miR-128-3p in *Fmr1*$^{+/y}$ and *Fmr1*$^{-/y}$ astroglia primary neurons by qPCR. n = 9 independent neuronal cultures. p values were determined using two-tailed unpaired t-test. **H,** Pie chart showing functional categories of genes that are expressed in human and mouse astroglia and are also predicted miR-128-3p binding targets. TargetScan was used to identify putative miR-128-3p target mRNAs. Ingenuity pathway analysis was used for characterizing functional categories. Only genes that are expressed in both human and mouse astroglia (FPKM > 10 and the miR-128-3p-binding

site was conserved in both mouse and human) were included in generating the pie chart. **I,** Representative immunoblot of FMRP in acutely sorted cortical astroglia from 4-OHT-injected astroglial cKO (*Slc1a3*-CreER^{+}*Fmr1*$^{f/y}$*Aldh1l1*-eGFP^{+}) mice at P14. p values were determined using two-tailed unpaired t-test. For **C**, **D**, and **F**, the data was presented in the box and whisker plot with defined elements, median (center line), upper and lower quartiles (bounds of box), and highest and lowest values (whiskers).

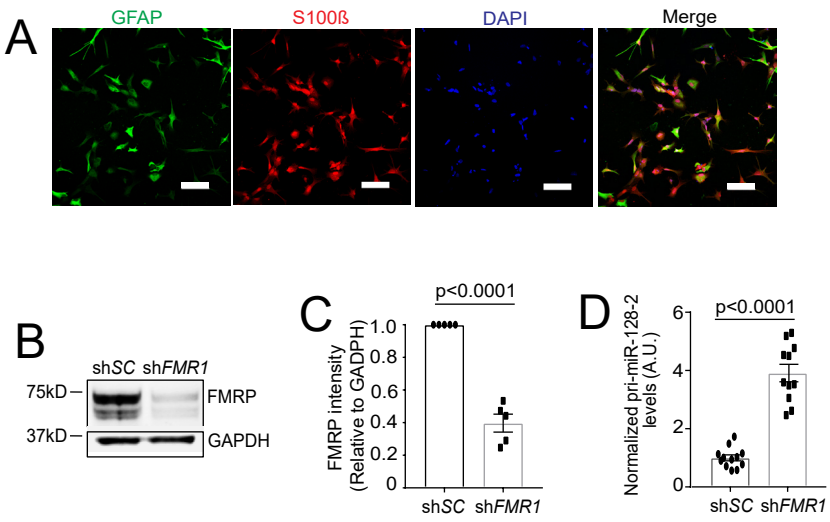
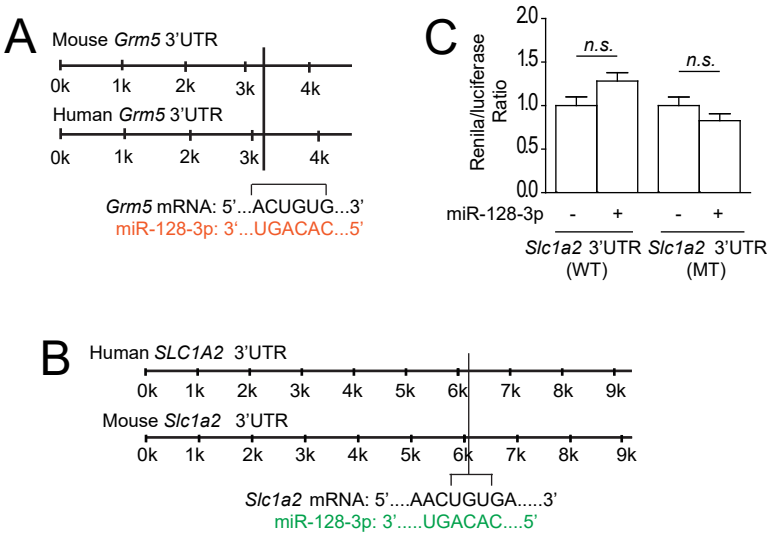

A
GFAP
S100ß
DAPI
Merge
B
shSC shFMR1
75kD
FMRP
37kD
GAPDH
C
p<0.0001
FMRP intensity
(Relative to GADPH)
1.0
0.8
0.6
0.4
0.2
0
shSC shFMR1
D
p<0.0001
Normalized pri-miR-128-2
levels (A.U.)
6
4
2
0
shSC shFMR1

Figure. S2

Fig. S2 FMRP knockdown promotes miR-128 transcription in human astroglia

A, Representative immunostaining images of glial markers GFAP and S100 β in H1 astroglia (7-days post-differentiation) following 7-month astroglial progenitor cell (APC) culture. Scale bar: 100 μm. Representative immunoblot (**B**) and quantification (**C**) of FMRP following lentiviral shRNA-mediated knockdown of FMRP in H1 astroglia. Data are mean ± SEM of n = 5 biologically independent samples/group with Welch's t-test. **D,** Quantification of pri-miR-128-2 levels by qPCR in lentiviral sh-s*crambled control* (sh*SC*)and sh*FMR1* infected H1 astroglia. Data are mean ± SEM of n = 11-12 wells/group with Mann-Whitney U test.


A
Mouse Grm5 3'UTR
0k 1k 2k 3k 4k
Human Grm5 3'UTR
0k 1k 2k 3k 4k
Grm5 mRNA: 5'...ACUGUG...3'
miR-128-3p: 3'...UGACAC...5'
C
Renila/luciferase Ratio
2.0
1.5
1.0
0.5
0.0
n.s.
n.s.
miR-128-3p - + - +
Slc1a2 3'UTR (WT)
Slc1a2 3'UTR (MT)

B
Human SLC1A2 3'UTR
0k 1k 2k 3k 4k 5k 6k 7k 8k 9k
Mouse Slc1a2 3'UTR
0k 1k 2k 3k 4k 5k 6k 7k 8k 9k
Slc1a2 mRNA: 5'....AACUGUGA.....3'
miR-128-3p: 3'.....UGACAC....5'

Figure. S3

Fig. S3 Bioinformatic analysis of predicted miR-128-3p target mRNAs that are expressed in astroglia

Bioinformatic analysis of the conserved miR-128-3p binding site on human and mouse *Grm5* (**A**) or *Slc1a2* (**B**) mRNA 3'-UTR sequence. **C,** Wild-type (WT) and miR-128-3p mutant (MT) *Slc1a2* 3′ UTR luciferase activity in HEK 293 cells following miR-128-3p transfection. n = 3 independent experiments with six replicates per experiment per condition; p value was determined using two-tailed unpaired t-test.

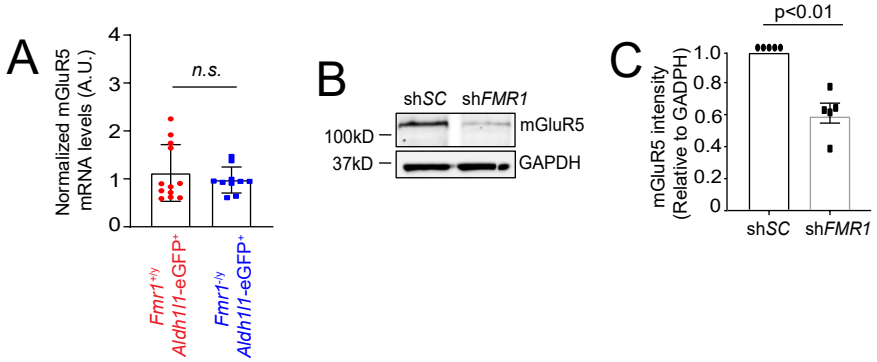

A
Normalized mGluR5 mRNA levels (A.U.)
4
3
2
1
0
n.s.
Fmr1+/y Aldh1l1-eGFP+
Fmr1-/y Aldh1l1-eGFP+
B
shSC
shFMR1
100kD
37kD
mGluR5
GAPDH
C
p<0.01
mGluR5 intensity (Relative to GADPH)
1.0
0.8
0.6
0.4
0.2
0
shSC
shFMR1

Figure. S4

Fig. S4 Loss of astroglial *Fmr1* down-regulates mGluR5 protein but not mRNA expression

A, Expression levels of mGluR5 mRNA in FAC sorted cortical astroglia from *Fmr1*$^{+/y}$*Aldh1l1*-eGFP^{+} and *Fmr1*$^{-/y}$*Aldh1l1*-eGFP^{+} mice at P14; n=5-6 biologically independent samples per condition. p value was determined using two-tailed unpaired t-test. Representative immunoblot (**B**) and quantification (**C**) of mGluR5 protein following lentiviral shRNA-mediated knockdown of FMRP in H1 astroglia. Data are mean ± SEM of n = 5 biologically independent samples/group with Welch's t-test test.

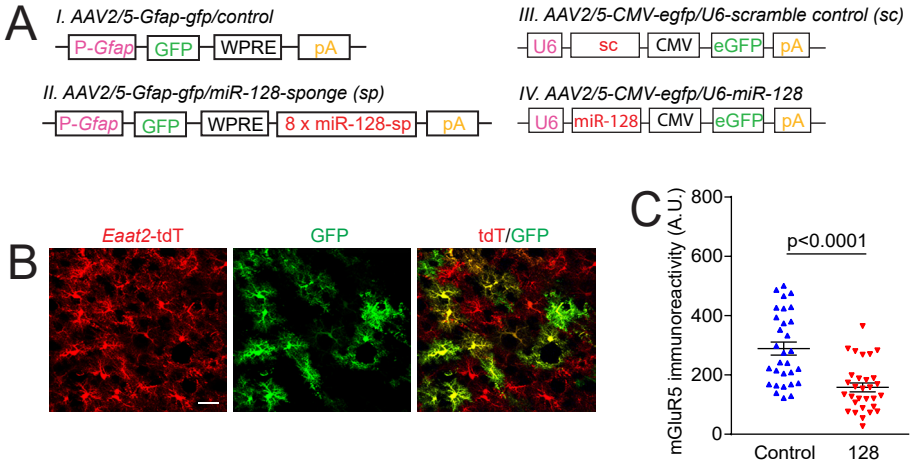

A
I. AAV2/5-Gfap-gfp/control
P-Gfap
GFP
WPRE
pA
II. AAV2/5-Gfap-gfp/miR-128-sponge (sp)
P-Gfap
GFP
WPRE
8 x miR-128-sp
pA
III. AAV2/5-CMV-egfp/U6-scramble control (sc)
U6
sc
CMV
eGFP
pA
IV. AAV2/5-CMV-egfp/U6-miR-128
U6
miR-128
CMV
eGFP
pA
B
Eaat2-tdT
GFP
tdT/GFP
C
mGluR5 immunoreactivity (A.U.)
800
600
400
200
0
p<0.0001
Control
128

Figure. S5

Fig. S5 Developmental expression of astroglial mGluR5 is cell-autonomously regulated by miR-128-3p *in vivo*

A, Diagram of AAV constructs for gain- and loss-of-function analysis of miR-128-3p *in vivo*. Subpanels: AAV2/5-*Gfap*-GFP/miR-128-control (I), AAV5-*Gfap*-GFP/miR-128-sponge (II), AAV2/5-*CMV*-GFP/U6-scramble control (III), and AAV2/5-*CMV*-GFP/U6-miR-128 (IV). **B,** Representative images of GFP-miR-128-sp expression in cortex of *Fmr1*$^{-/y}$*Eaat2*-tdT^{+} mice. Scale bar: 30 μm. **C,** Quantification of mGluR5 immunoreactivity within individual astroglial domains after miR-128-3p overexpression in *Fmr1*$^{+/y}$*Eaat2*-tdT^{+} mice n = 30–45 cells/4 mice per group; p values were determined using two-tailed unpaired t-test.

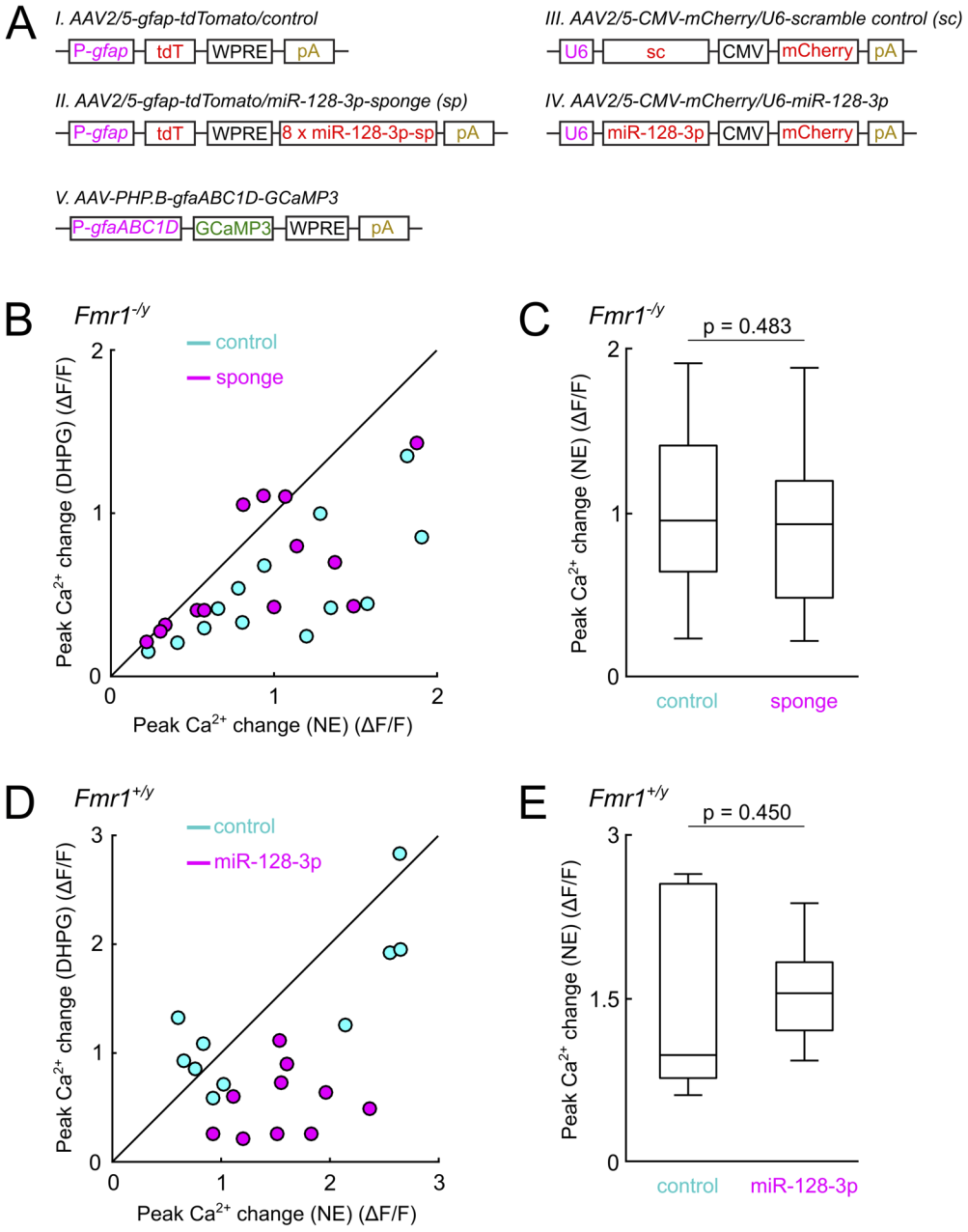

A
I. AAV2/5-gfap-tdTomato/control
P-gfap
tdT
WPRE
pA
II. AAV2/5-gfap-tdTomato/miR-128-3p-sponge (sp)
P-gfap
tdT
WPRE
8 x miR-128-3p-sp
pA
III. AAV2/5-CMV-mCherry/U6-scramble control (sc)
U6
sc
CMV
mCherry
pA
IV. AAV2/5-CMV-mCherry/U6-miR-128-3p
U6
miR-128-3p
CMV
mCherry
pA
V. AAV-PHP.B-gfaABC1D-GCaMP3
P-gfaABC1D
GCaMP3
WPRE
pA
B
Fmr1-/y
control
sponge
Peak Ca2+ change (DHPG) (ΔF/F)
Peak Ca2+ change (NE) (ΔF/F)
C
Fmr1-/y
p = 0.483
Peak Ca2+ change (NE) (ΔF/F)
control
sponge
D
Fmr1+/y
control
miR-128-3p
Peak Ca2+ change (DHPG) (ΔF/F)
Peak Ca2+ change (NE) (ΔF/F)
E
Fmr1+/y
p = 0.450
Peak Ca2+ change (NE) (ΔF/F)
control
miR-128-3p

Figure. S6

Fig. S6 *In vivo* expression of miR-128-3p cell-autonomously regulate developmental astroglial mGluR5 signaling

A, Diagram of viral constructs used in two-photon Ca^{2+} imaging. Subpanels: I: AAV2/5-*Gfap*-tdTomato/control; II. AAV2/5-*Gfap*-tdTomato/miR-128-sponge; III. AAV2/5-*CMV*-mCherry/U6-scramble control; IV. AAV2/5-*CMV*-mCherry/U6-miR-128; V. AAV-PHP.B-gfaABC1D-GCaMP3; **B,** Scatter plot comparing averaged Ca^{2+} changes in astroglia from individual slice in response to either DHPG or NE in control or sponge AAV injected *Fmr1*$^{-/y}$ mice, respectively. Cyan color filled circle: scrambled control; Magenta color filled circle: miR-128-3p sponge. Diagonal line: identical responses to DHPG and NE; **C,** Boxplot of astroglial peak Ca^{2+} response in *Fmr1*$^{-/y}$ slice induced by NE following control (median: 0.942; minimum to maximum: 0.230 to 1.906) or sponge (median: 0.936; minimum to maximum: 0.221 to 1.875) AAV injections; p values were calculated from the Student's t-test. **D,** Scatter plot comparing averaged Ca^{2+} changes in astroglia from individual slice in response to either DHPG or NE in control of miR-128 AAV injected *Fmr1*$^{+/y}$ mice, respectively. Cyan color filled circle: scrambled control; Magenta color filled circle: miR-128. **E,** Boxplot of astroglial peak Ca^{2+} response in *Fmr1*$^{+/y}$ slice induced by NE following control (median: 0.975; minimum to maximum: 0.608 to 2.646) or miR-128 (median: 1.545; minimum to maximum: 0.929 to 2.368) AAV injection; p values were calculated from the Student's t-test. For **C** and **E**, the data was presented in the box and whisker plot with defined elements, median (center line), upper and lower quartiles (bounds of box), and highest and lowest values (whiskers).

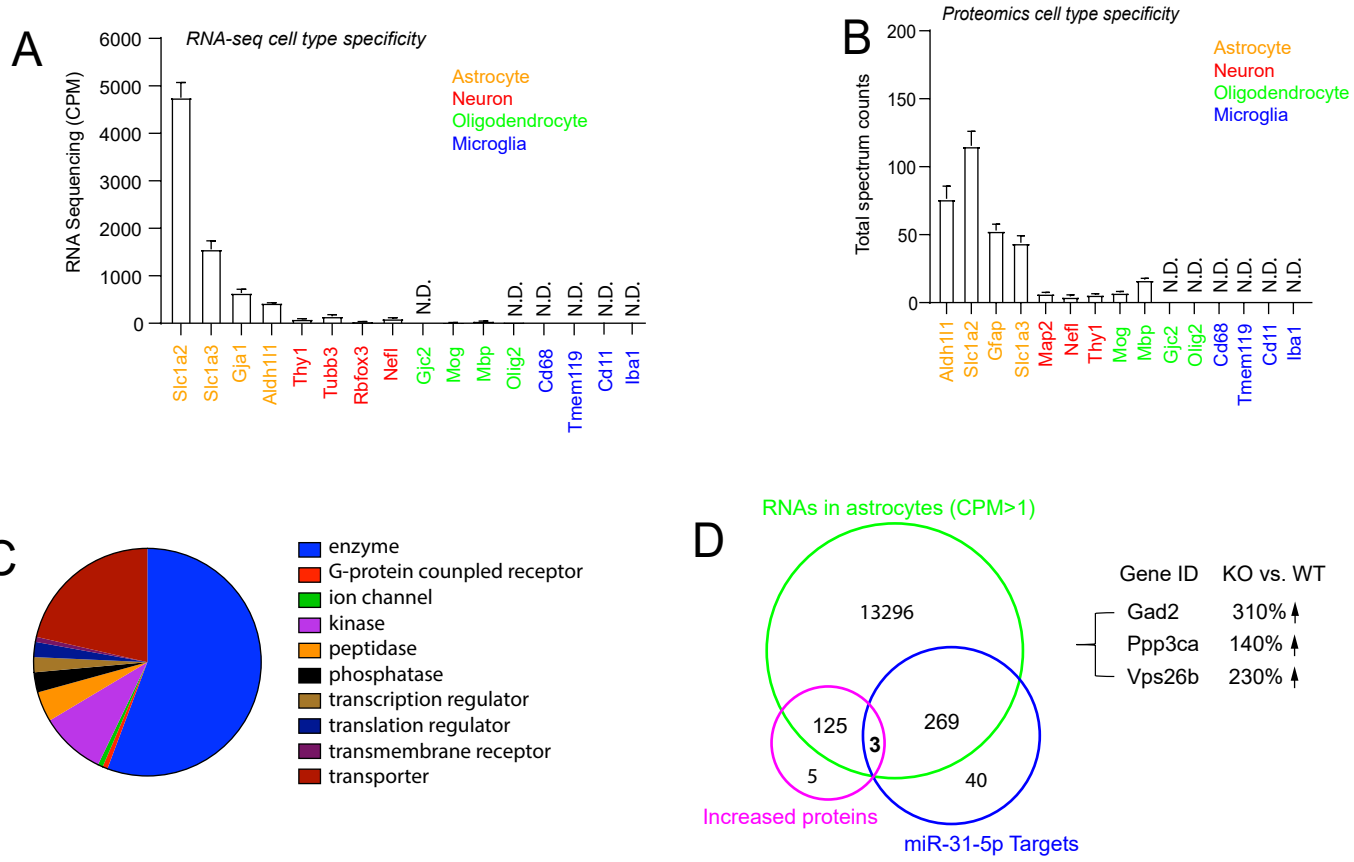

A
RNA-seq cell type specificity
RNA Sequencing (CPM)
6000
5000
4000
3000
2000
1000
0
Astrocyte
Neuron
Oligodendrocyte
Microglia
N.D.
Slc1a2
Slc1a3
Gja1
Aldh1l1
Thy1
Tubb3
Rbfox3
Nefl
Gjc2
Mog
Mbp
Olig2
Cd68
Tmem119
Cd11
Iba1
B
Proteomics cell type specificity
Total spectrum counts
200
150
100
50
0
Astrocyte
Neuron
Oligodendrocyte
Microglia
N.D.
Aldh1l1
Slc1a2
Gfap
Slc1a3
Map2
Nefl
Thy1
Mog
Mbp
Gjc2
Olig2
Cd68
Tmem119
Cd11
Iba1
C
enzyme
G-protein counpled receptor
ion channel
kinase
peptidase
phosphatase
transcription regulator
translation regulator
transmembrane receptor
transporter
D
RNAs in astrocytes (CPM>1)
13296
125
3
269
5
40
Increased proteins
miR-31-5p Targets
Gene ID
KO vs. WT
Gad2
310%
Ppp3ca
140%
Vps26b
230%

Figure. S7

Fig. S7 Genome-wide transcriptome and proteomics analysis of *Fmr1*$^{+/y}$ and *Fmr1*$^{-/y}$ astroglia

A, Expression levels (CPM) of representative cell type specific gene markers for astroglia, neurons, oligodendrocytes, and microglia in TRAP/RNA-seq data from *Fmr1*$^{+/y}$*Aldh1l1*-TRAP and *Fmr1*$^{-/y}$*Aldh1l1*-TRAP mice. n=3 biologically independent samples per condition. N.D.: not detectable; **B**, Expression levels (total spectral counts) of representative cell type specific protein markers for astroglia, neurons, oligodendrocytes, and microglia in LC-MS/MS proteomics data. n=3 biologically independent samples per condition. **C,** Pie chart showing functional categories of DEPs between *Fmr1*$^{+/y}$ and *Fmr1*$^{-/y}$ astroglia using Ingenuity pathway analysis. **D,** Identification of up-regulated proteins in *Fmr1*$^{-/y}$ astroglia whose mRNAs have predicted miR-31-5p binding sites. Venn diagram showing the number of expressed mRNAs (CPM>1) and up-regulated proteins, as well as predicted miR-31-5p mRNA targets, respectively. There are 3 mRNAs having miR-31-5p binding targets and also encoding for proteins that are up-regulated in *Fmr1*$^{-/y}$ astroglia compared to in *Fmr1*$^{+/y}$ astroglia. These 3 genes and their protein expression changes in *Fmr1*$^{-/y}$ vs. *Fmr1*$^{+/y}$ astroglia were shown.