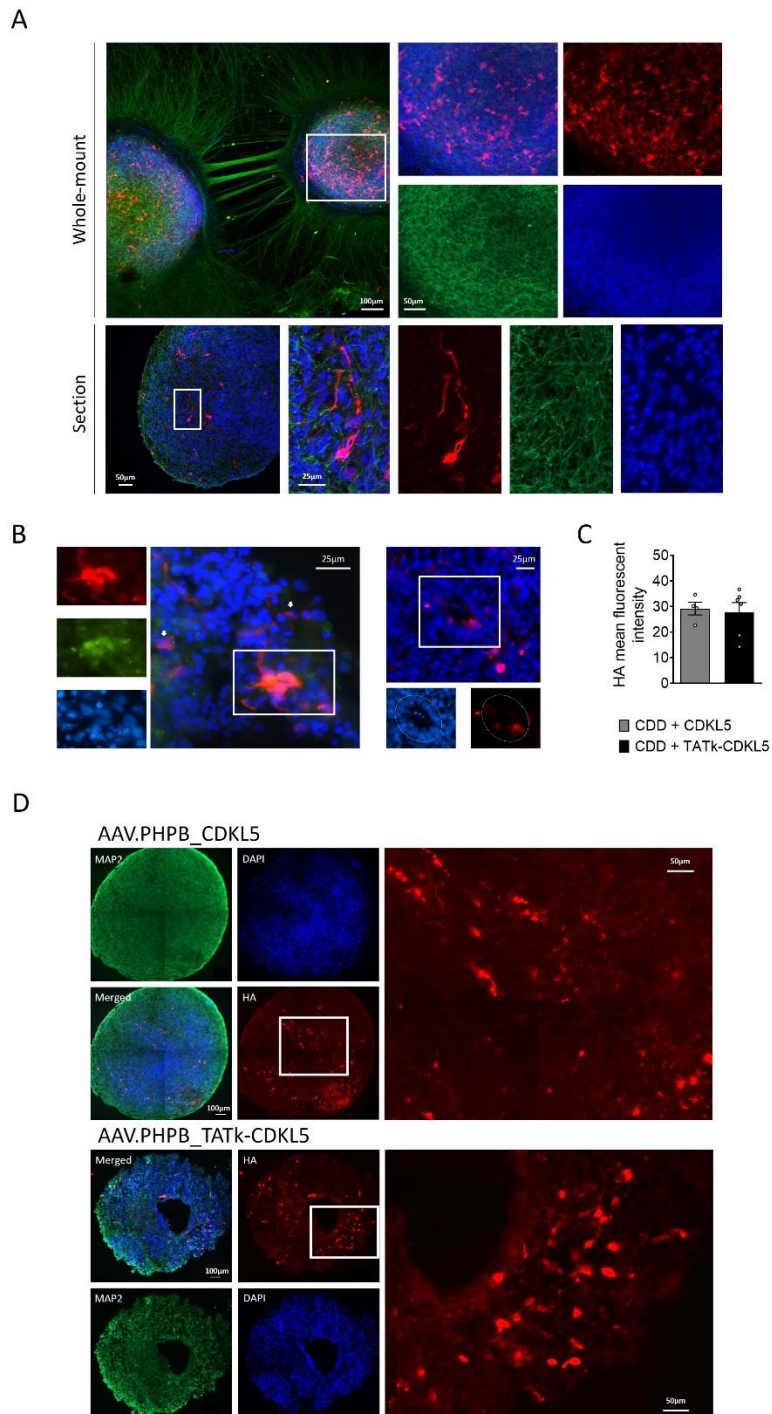


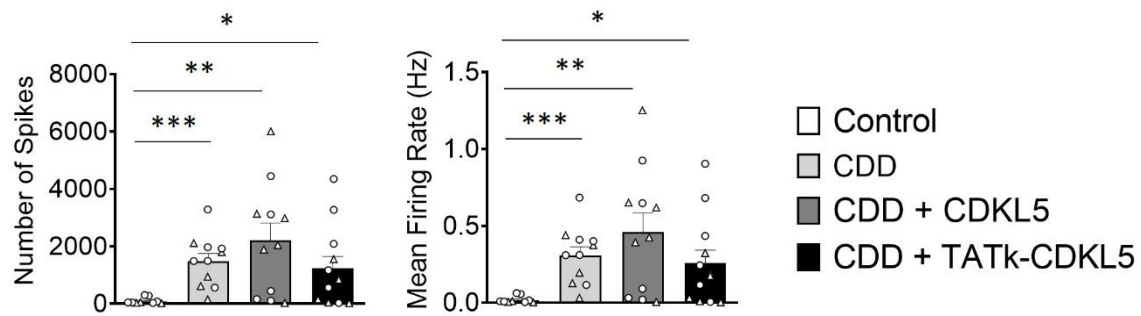
Supplementary Material

1 Supplementary Figures and Tables

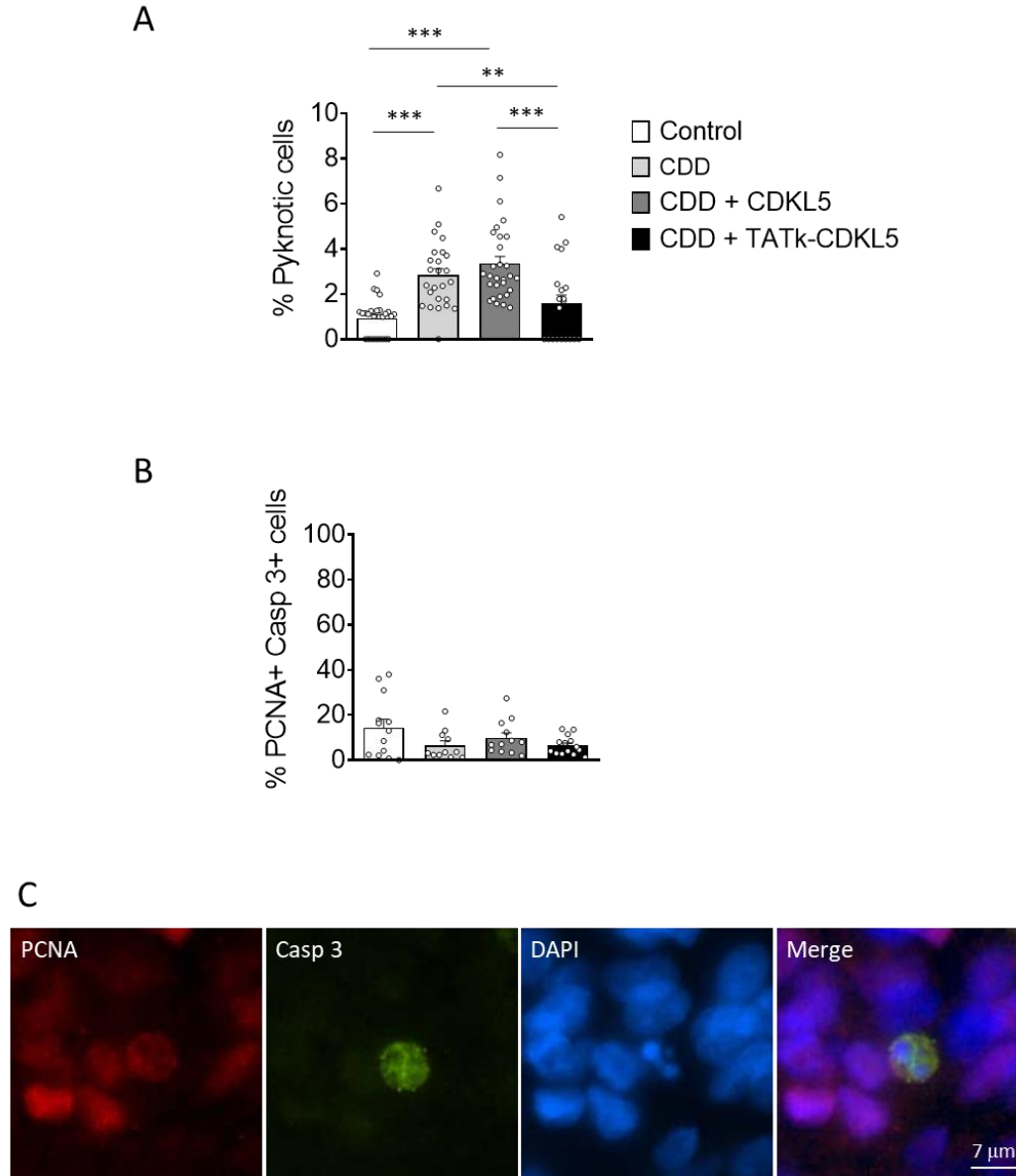
1.1 Supplementary Figures



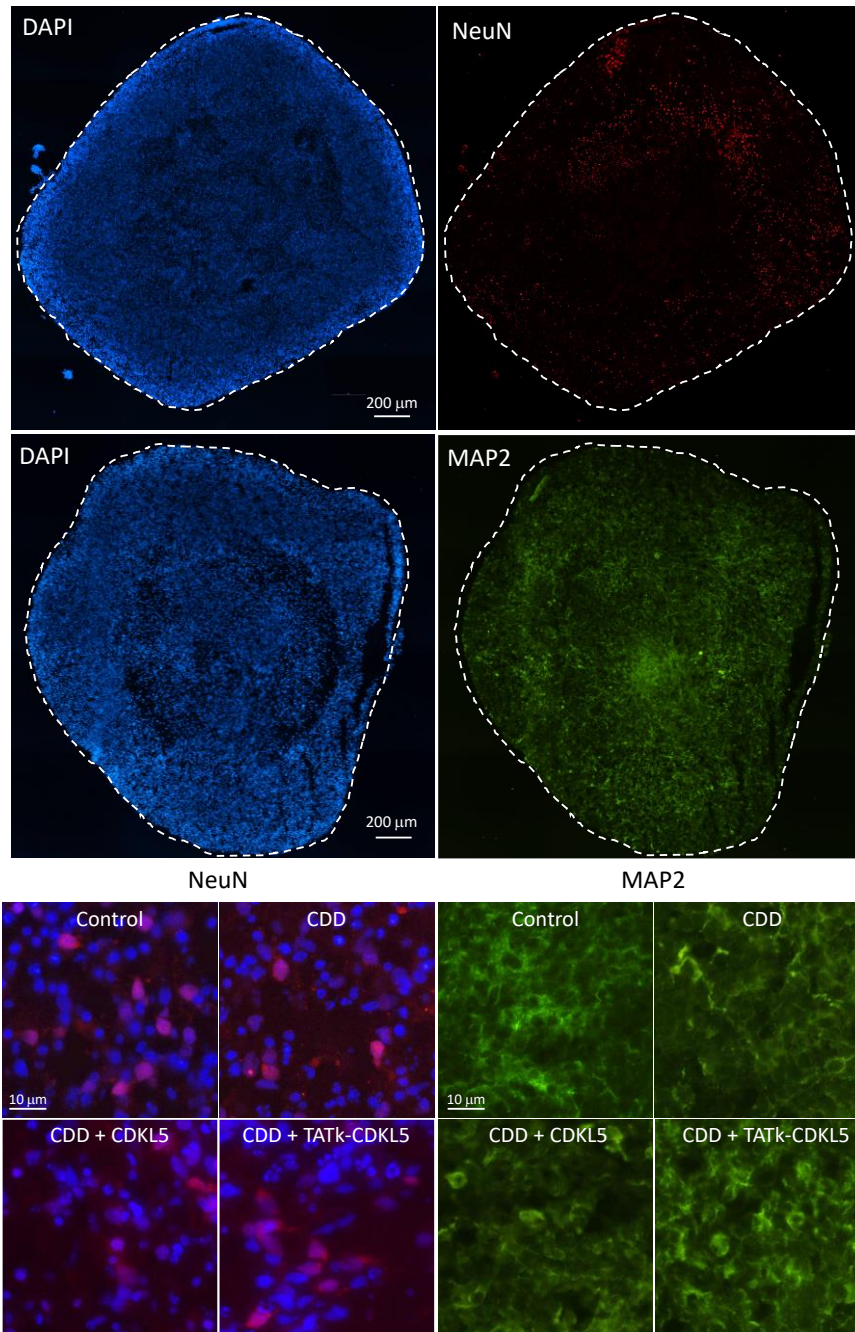
Supplementary Figure S1. CDKL5 protein expression in CDD cortical organoids following gene therapy. (A) Representative fluorescence images of whole-mount immunostaining (upper panels) and a cortical organoid slice (lower panels) showing CDKL5 expression (HA, in red) in 11-week-old control cortical organoids infected with TATk-CDKL5 gene therapy vector at the dose of 7.2×10^{10} vg/org and collected 1 week after infection; MAP2 (in green) and nuclei counterstained with DAPI (in blue). White rectangles indicate the region shown in the high-magnification images on the right. Scale bar = 100 μm and 50 μm (low magnification); 50 μm and 25 μm (high magnification). (B) Representative fluorescence images of cortical organoid slice showing different cell subtypes expressing CDKL5 protein (HA, in red) in control cortical organoids infected at 11 weeks old with TATk-CDKL5 gene therapy vector at the dose of 3.6×10^{10} vg/org and collected 3 weeks after infection for immunostaining. Left panels show CDKL5 expression in differentiated neurons (NeuN-positive cells, in green); white arrows indicate HA-positive non-neuronal cells (NeuN-negative). White rectangles indicate the region shown in the magnification images. Right panels show a rosette with progenitor cells expressing CDKL5 protein. The dotted outline delineates rosette structure. Nuclei counterstained with DAPI (in blue). Scale bar = 25 μm . (C) Quantification of HA mean intensity in organoid slices; organoids were infected as described in (D). Values are represented as means \pm SEM. (Unpaired t-test). (D) Representative fluorescence images of CDD cortical organoid slices showing distribution of TATk-CDKL5 or CDKL5 expression (HA, in red) in CDD cortical organoids infected at 11 weeks old with TATk-CDKL5 or CDKL5 gene therapy vector at a dose of 3.6×10^{10} vg/org and collected 3 weeks after infection for immunostaining; MAP2 (in green) and nuclei counterstained with DAPI (in blue). The boxed areas indicate representative HA-positive cells; higher-magnification images show a punctate intracellular HA signal corresponding to CDKL5 protein within infected cells. Scale bar = 100 μm (low magnification); 50 μm (high magnification).



Supplementary Figure S2. Electrophysiological recordings in CDD cortical organoids following gene therapy. Electrophysiological recording of CDD and control cortical organoids grown on MEA plates, treated at 11 weeks old with TATk-CDKL5 or CDKL5 gene therapy vectors at a dose of 3.6×10^{10} vg/org, or vehicle as a control. Electrical activity was recorded 2 weeks after treatment. Graphs show the result of several electrical parameters as indicated during 3 minutes of recording (Control and CDD conditions: $n = 9-12$ wells, 3 org per well; 2 cell line, 1 differentiation batch). Dots and triangles in the scatter plots identify the two independent CDD iPSC lines. Values are represented as means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Unpaired t-test after Welch's ANOVA).



Supplementary Figure S3. Cell death in CDD cortical organoids following gene therapy. (A) Graph shows pyknotic nuclei quantification in cortical organoids following gene therapy (Control and CDD conditions: $n = 15-30$ images, from 6 organoids, 1 cell line). (B) Graph shows the percentage of PCNA-positive cells that are also positive for cleaved caspase-3 (Control and CDD conditions: $n = 12-15$ images, from 6 organoids; 1 cell line, 2 differentiation batches). (C) Representative fluorescence images of a cortical organoid slice immunolabeled for PCNA (in red) and cleaved Caspase-3 (in green), with cell nuclei counterstained using DAPI (in blue). Scale bar = $7 \mu\text{m}$. Data in (A) and (B) are expressed as mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$ (Dunn's test after Kruskal-Wallis).



Supplementary Figure S4. Neuronal complexity and maturation of CDD cortical organoids following gene therapy. Representative fluorescence images of CDD and control cortical organoid slices showing the expression of the neuronal markers NeuN or MAP2, as indicated, in cortical organoids treated at 11 weeks old with TATk-CDKL5 or CDKL5 gene therapy vectors at a dose of 3.6×10^{10} vg/org, or vehicle as a control, and collected 3 weeks after treatment for immunostaining; nuclei were counterstained with DAPI (in blue). Upper panels show lower magnification images with dotted lines indicating the manually traced area of organoids in which signal intensity was quantified, while lower panels show representative high-magnification images. Scale bar = 200 μm (low-magnification); 10 μm (high-magnification).

1.2 Supplementary Tables

Supplementary Table S1. Primary and secondary antibodies used for Western blot and Immunostaining.

Antibody	Company	Dilution	Code
<i>Immunohistochemistry</i>			
HA	Cell Signaling Technologies	1:500	#3724
Ki-67	Invitrogen	1:200	#MA5-14520
PCNA	Sigma	1:500	#P8825
NeuN	Millipore	1:250	#MAB377
MAP2	Millipore	1:500	#AB5622
Cleaved Caspase 3	Cell Signaling Technologies	1:200	#9661S
Goat anti-Rabbit IgG- Alexa Fluor 555	Invitrogen	1:500	#A-21428
Goat anti-Mouse IgG- Alexa Fluor 555	Invitrogen	1:500	#A-21422
Donkey anti-Rabbit IgG- Alexa Fluor 488	Invitrogen	1:500	#A-21206
<i>Western blot</i>			
hCDKL5	Santa Cruz	1:500	#sc-376314
EB2	Abcam	1:1000	#ab234843
P(Ser222)-EB2	Covalab	1:1000	#pab01032-P
B-Actin	Abcam	1:1000	#8226
IRDye 680RD Goat anti-Mouse IgG	LICORbio	1:5000	#926-68070
IRDye® 800CW Goat Anti-Rabbit IgG	LICORbio	1:5000	#926-32211

Supplementary Table S2. Descriptive statistics.

<i>Figure</i>	<i>Test</i>	<i>Format</i>	<i>Values</i>	<i>Significance</i>
Fig. 1C	Two-Way ANOVA	F (DFn, DFd)	35,74 (1, 39)	$p < 0.0001$
Fig. 1D	Unpaired t test	t (df)	0.2703 (8)	$p = 0.7938$
Fig. 2C	Welch's ANOVA	W (DFn, DFd)	33.55 (2, 5.088)	$p = 0.0012$
Fig. 2E	Welch's ANOVA	W (DFn, DFd)	9.584 (3, 5.712)	$p = 0.0118$
Fig. 3B (Burst)	Welch's ANOVA	W (DFn, DFd)	5.804 (3, 21.57)	$p = 0.0046$
Fig. 3B (Network burst)	Kruskal-Wallis test	H (DF)	6.491 (3)	$p = 0.0900$
Fig. 3B (Synchrony)	Welch's ANOVA	W (DFn, DFd)	2.561 (3, 22.68)	$p = 0.0801$
Fig. 4B	Kruskal-Wallis test	H (DF)	24.16 (3)	$p < 0.0001$
Fig. 4D	One-Way ANOVA	F (DFn, DFd)	5.040 (3, 28)	$p = 0.0064$
Fig. 4F	One-Way ANOVA	F (DFn, DFd)	3.516 (3, 10)	$p = 0.0569$
Fig. 5A	Welch's ANOVA	W (DFn, DFd)	2.347 (3, 21.44)	$p = 0.1012$
Fig. 5C	Kruskal-Wallis test	H (DF)	18.51 (3)	$p = 0.0003$
Fig. 5E	Welch's ANOVA	W (DFn, DFd)	23.03 (3, 33.68)	$p < 0.0001$
Fig. 5F	One-Way ANOVA	F (DFn, DFd)	14.23 (3, 66)	$p < 0.0001$
Sup. Fig. S1D	Unpaired t test	t (df)	0.7684 (29)	$p = 0.4485$
Sup. Fig. S2 (Spikes)	Welch's ANOVA	W (DFn, DFd)	14.48 (3, 17.54)	$p < 0.0001$
Sup. Fig. S2 (Firing rate)	Welch's ANOVA	W (DFn, DFd)	14.48 (3, 17.54)	$p < 0.0001$
Sup. Fig. S3A	Kruskal-Wallis test	H (DF)	40.45 (3)	$p < 0.0001$
Sup. Fig. S3B	Kruskal-Wallis test	H (DF)	3.239 (3)	$p = 0.3562$