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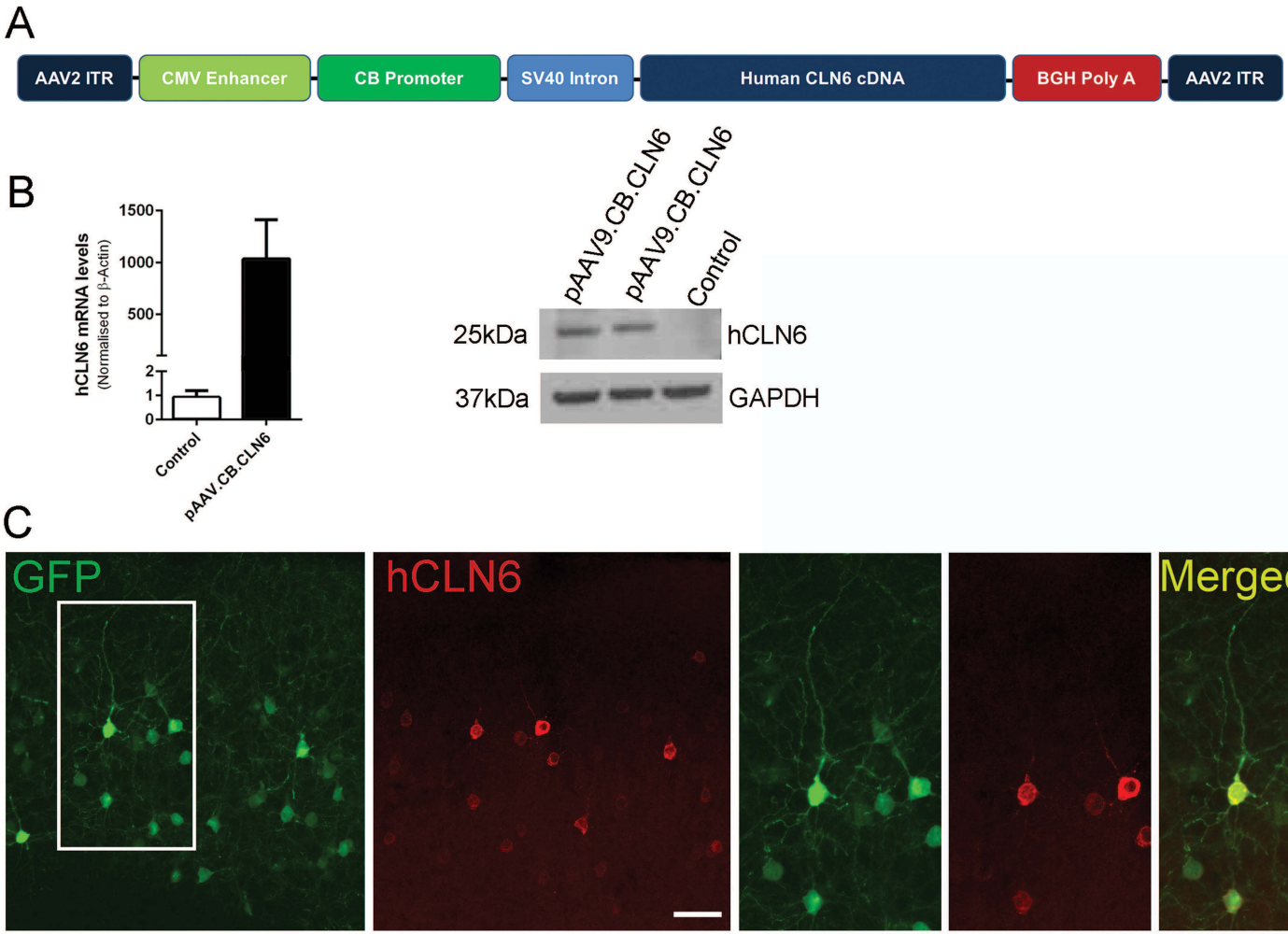
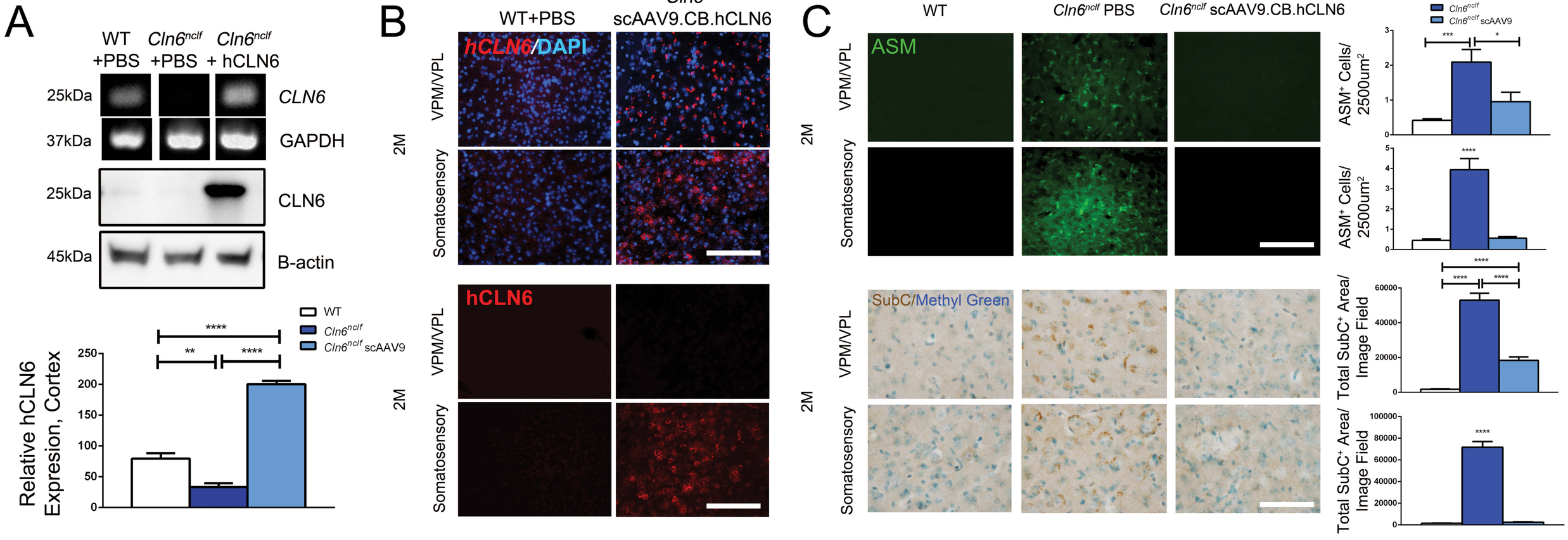
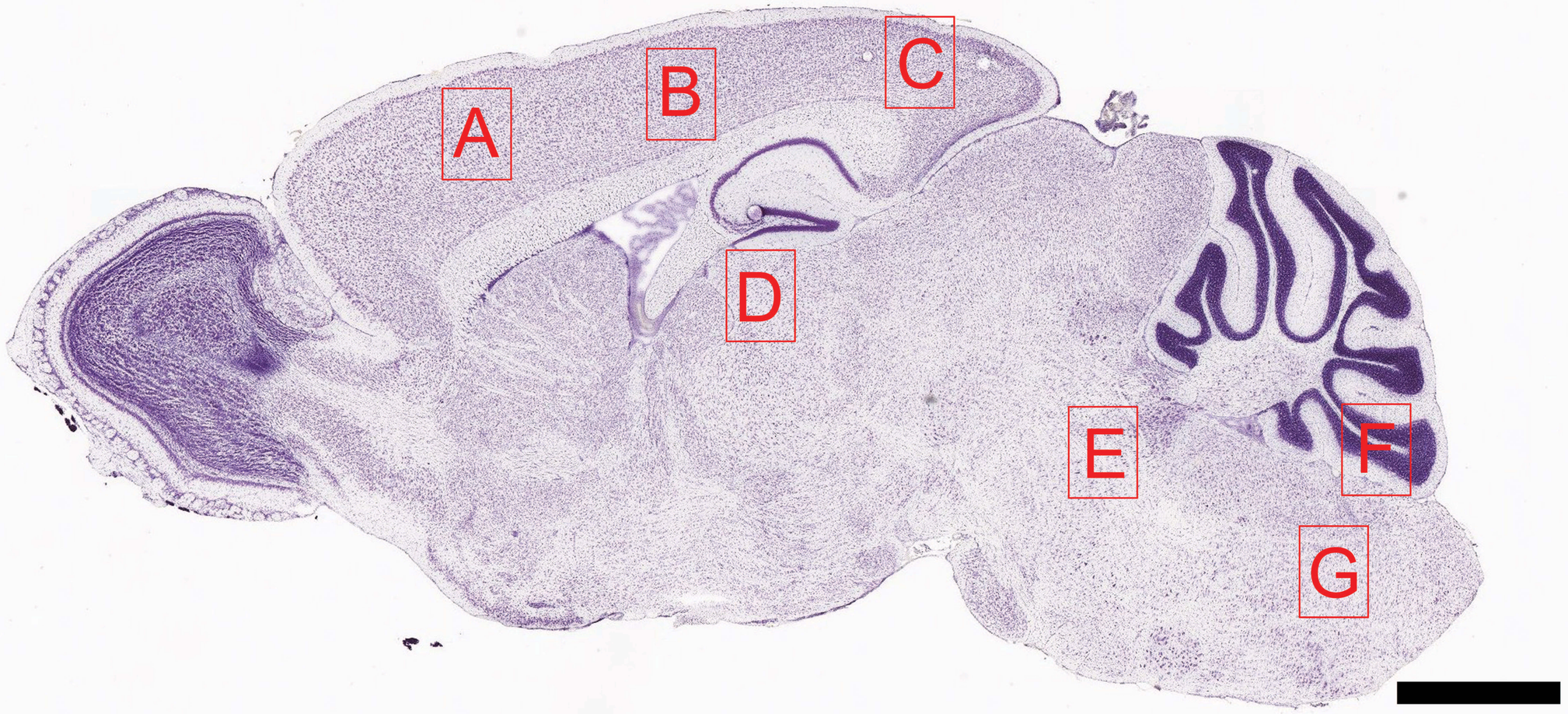
Supplemental Information

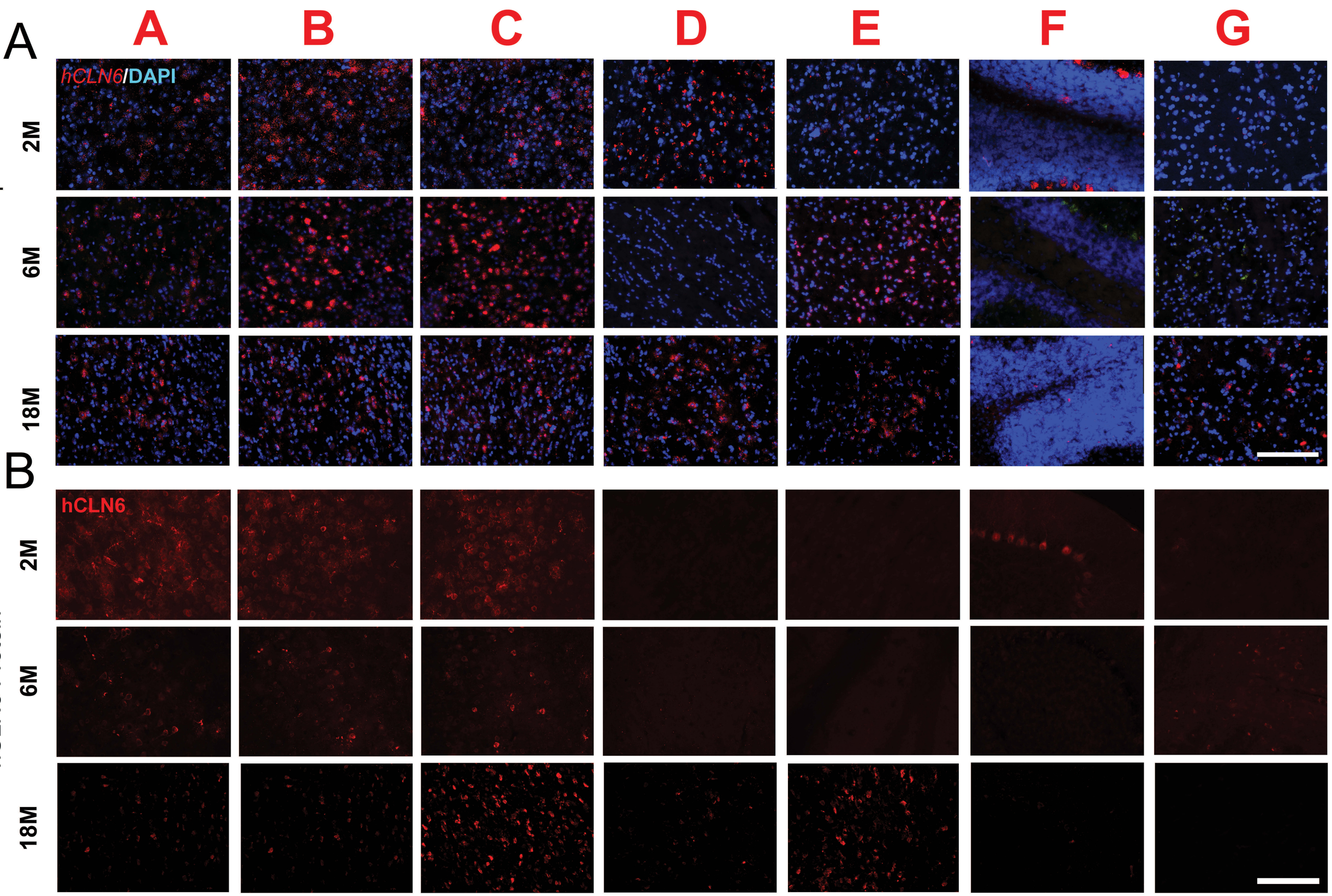
Gene Therapy Corrects Brain and Behavioral Pathologies in CLN6-Batten Disease

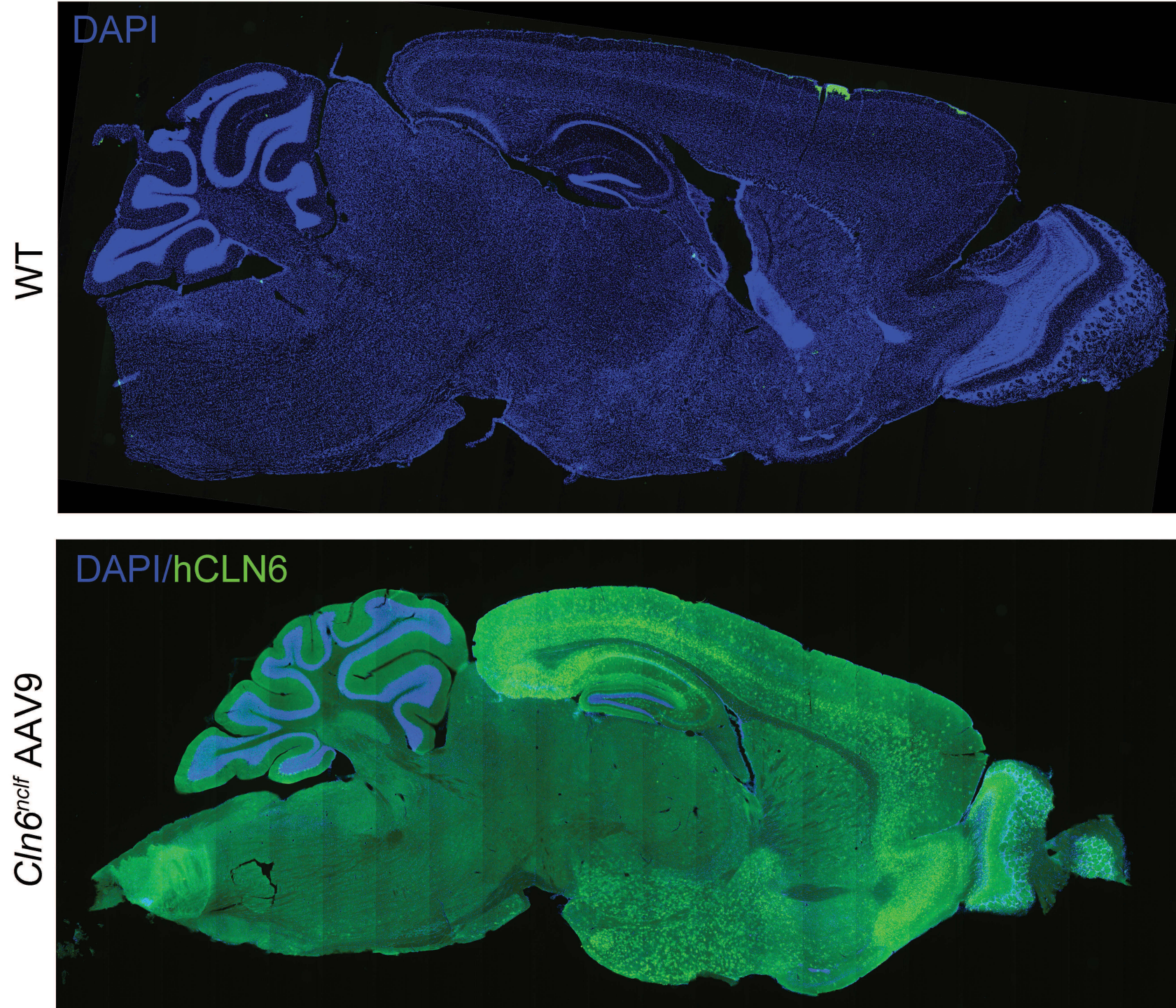
Jacob T. Cain, Shibi Likhite, Katherine A. White, Derek J. Timm, Samantha S. Davis, Tyler B. Johnson, Cassandra N. Dennys-Rivers, Federica Rinaldi, Dario Motti, Sarah Corcoran, Pablo Morales, Christopher Pierson, Stephanie M. Hughes, Stella Y. Lee, Brian K. Kaspar, Kathrin Meyer, and Jill M. Weimer


A
AAV2 ITR
CMV Enhancer
CB Promoter
SV40 Intron
Human CLN6 cDNA
BGH Poly A
AAV2 ITR
B
hCLN6 mRNA levels
(Normalised to β-Actin)
1500
1000
500
2
1
0
Control
pAAV.CB.CLN6
pAAV9.CB.CLN6
pAAV9.CB.CLN6
Control
25kDa
hCLN6
37kDa
GAPDH
C
GFP
hCLN6
Merged


A
WT +PBS
Cln6nclf +PBS
Cln6nclf + hCLN6
25kDa
CLN6
37kDa
GAPDH
25kDa
CLN6
45kDa
B-actin
WT
Cln6nclf
Cln6nclf scAAV9
Relative hCLN6 Expresion, Cortex
****
**
****
250
200
150
100
50
0
B
WT+PBS
Cln6nclf scAAV9.CB.hCLN6
hCLN6/DAPI
hCLN6
VPM/VPL
Somatosensory
2M
C
WT
Cln6nclf PBS
Cln6nclf scAAV9.CB.hCLN6
ASM
SubC/Methyl Green
ASM+ Cells/ 2500um2
Total SubC+ Area/ Image Field
***
*
****
60000
40000
20000
100000
80000
60000


A
B
C
D
E
F
G


A
A
B
C
D
E
F
G
hCLN6 RNAscope
hCLN6/DAPI
2M
6M
18M
B
hCLN6 Protein
hCLN6
2M
6M
18M


DAPI
WT
DAPI/hCLN6
Cln6nclf AAV9

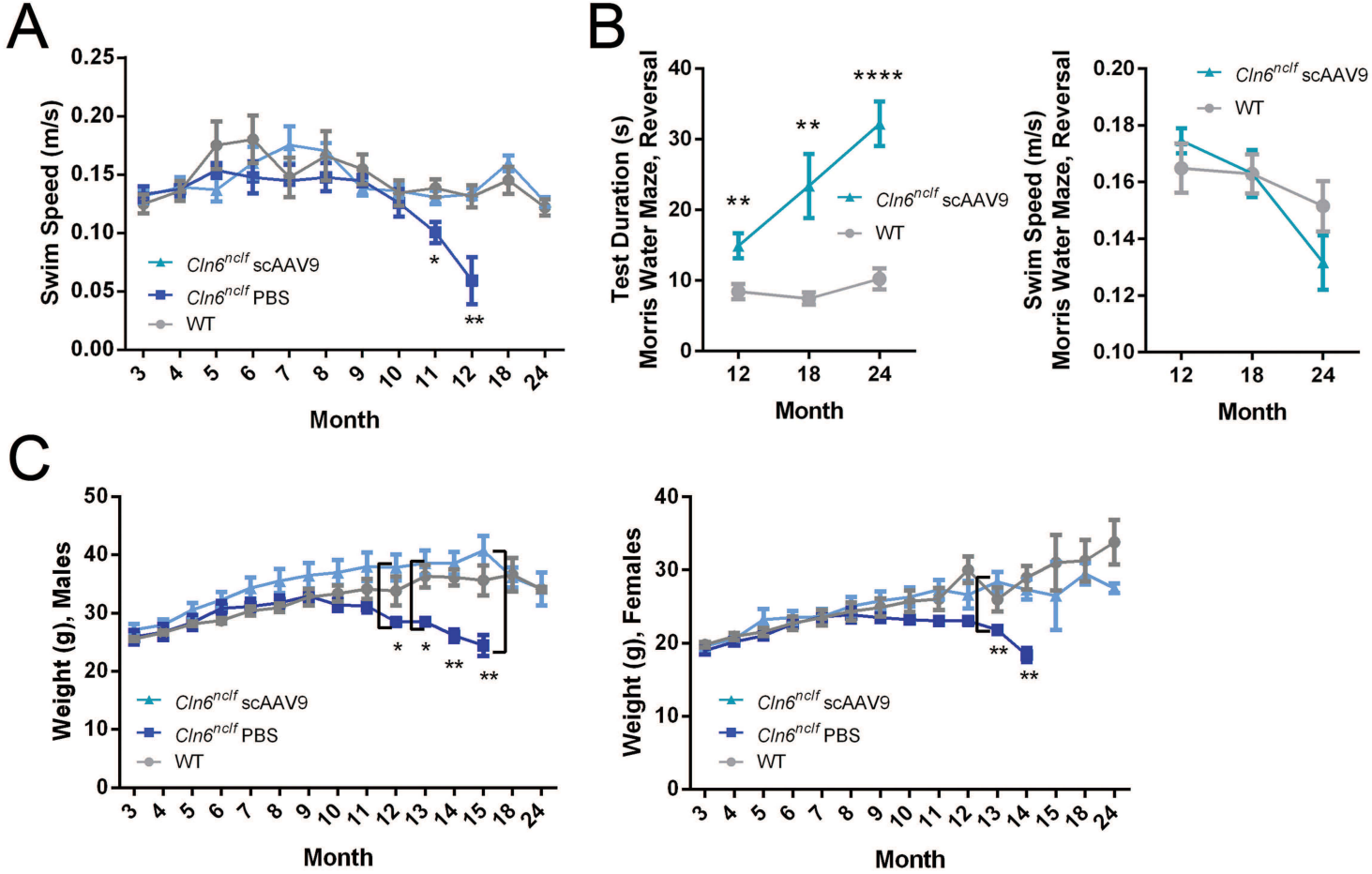

A
Swim Speed (m/s)
0.25
0.20
0.15
0.10
0.05
0.00
Cln6nclf scAAV9
Cln6nclf PBS
WT
*
**
3 4 5 6 7 8 9 10 11 12 18 24
Month
B
Test Duration (s)
Morris Water Maze, Reversal
40
30
20
10
0
**
**
****
Cln6nclf scAAV9
WT
12 18 24
Month
Swim Speed (m/s)
Morris Water Maze, Reversal
0.20
0.18
0.16
0.14
0.12
0.10
Cln6nclf scAAV9
WT
12 18 24
Month
C
Weight (g), Males
50
40
30
20
10
0
* * ** **
Cln6nclf scAAV9
Cln6nclf PBS
WT
3 4 5 6 7 8 9 10 11 12 13 14 15 18 24
Month
Weight (g), Females
40
30
20
10
0
** **
Cln6nclf scAAV9
Cln6nclf PBS
WT
3 4 5 6 7 8 9 10 11 12 13 14 15 18 24
Month

Figure Legends:

Figure S1: scAAV9.CB.CLN6 construct induces robust expression of *hCLN6* in vitro and targets the CNS in vivo. (A) Design of the pscAAV9.CB.CLN6 plasmid construct, with CMV enhancer and chicken-β-actin promoter. Self-complementary AAV9.CB.CLN6 was produced by transient transfection procedures using a double-stranded AAV2-ITR–based CB-CLN6 vector, with a plasmid encoding Rep2Cap9 sequence. (B) Transient transfection of HEK293 cells confirms *hCLN6* RNA and protein expression (25kDa). (C) *In utero* electroporation of pscAAV9.CB.CLN6 and pscAAV9.CB.GFP plasmids followed by immunohistochemical staining confirms proper neuronal trafficking and expression of hCLN6 protein on a cellular basis. Electroporation utilized embryonic day 15.5 animals to target excitatory cortical neurons specifically.

Figure S2: Effect of a single scAAV9.CB.CLN6 injection in 2 month old *Cln6* mutant animals.
(A): Representative RT-PCR gels and densitometry (normalized to a *GAPDH*) shows increased *hCLN6* gene expression (25kDa) in 2 month old animals following scAAV9.CB.CLN6 delivery (ICV, postnatal day 1 (P1), $5x10^{10}$ vg/animal) compared to PBS-injected *Cln6*^{nclf} mice. Probed by western blotting, ICV delivery of the scAAV9.CB.CLN6 vector (P1, $5x10^{10}$ vg/animal) shows a marked increase in hCLN6 protein expression (25 kDa) in 2 month *Cln6*^{nclf} mice. . Gene expression represented in the graph. Mean ± SEM. N=3-9 animals/treatment groups. One-Way ANOVA, Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (B) *Top:* Representative images of RNAscope analysis confirms widespread transduction of *hCLN6* mRNA in the brain of 2 month scAAV9.CB.CLN6-injected *Cln6*^{nclf} mice vector (ICV, P1, $5x10^{10}$ vg/animal). *Bottom:* Representative images of immunohistochemistry using anti-hCLN6 antibodies show protein expression in various brain regions of 2 month old scAAV9.CB.CLN6-injected *Cln6*^{nclf} mice (ICV, P1, $5x10^{10}$ vg/animal). Scale bar 50μm (C) *Top*: A single ICV injection of scAAV9.CB.CLN6 at P1 ($5x10^{10}$ vg/animal) reduces accumulation of autofluorescent storage material (ASM) in the VPM/VPL and somatosensory cortex of 2 month *Cln6*^{nclf} mice. Mean ± SEM, N=3-10 animals/treatment gruop. One-Way ANOVA, Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar 50μm. *Bottom*: scAAV9.CB.CLN6 injection (ICV, P1, $5x10^{10}$ vg/animal) prevents the accumulation of mitochondrial ATP synthase subunit C (SubC) in the VPM/VPL and somatosensory cortex of 2 month *Cln6*^{nclf} mice. Brown immunolabeling represents SubC, while blue stain represents methyl green (nuclei). Mean ± SEM, N=21-72 images/treatment group, biological N=3-10 animals/treatment group. One-Way ANOVA, Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar 50μm.

Figure S3: RNAscope and immunohistochemistry using species-specific hCLN6 RNA probes and antibodies show widespread expression throughout the brain. (A) *hCLN6* expression throughout the brain, at various time points in scAAV9.CB.CLN6 treated *Cln6*^{nclf} mice (ICV, P1, $5x10^{10}$ vg/animal), most notably in the cortex. (B) hCLN6 expression throughout the brain, at various time points in scAAV9.CB.CLN6 treated *Cln6*^{nclf} mice (ICV, P1, $5x10^{10}$ vg/animal), most notably in the cortex. (A: motor cortex, B: somatosensory cortex; C: visual cortex; D: thalamus; E: pons; F: cerebellum; G: brainstem) Scale bar 50 μm. Sagittal mouse Nissl slice image courtesy of Allen Mouse Brain Reference Atlas [1, 2].

Figure S4: Overall expression of hCLN6 in treated 2 month old *Cln6* mutant animals. Widespread immunolabeling of hCLN6 (green) can be visualized throughout the brain, most notably in the cortex, of 2 month old scAAV9.CB.CLN6 treated *Cln6*^{nclf} animals (ICV, P1, $5x10^{10}$ vg/animal). DAPI (blue) indicates nuclei dye.

Figure S5: Additional behavior data in 12-24 month old animals.
(A) Untreated *Cln6*^{nclf} animals have significantly slower swim speeds at 11 and 12 month of age in the Morris water maze test than either wildtype or treated *Cln6*^{nclf} counterparts (ICV, P1, $5x10^{10}$ vg scAAV9.CB.CLN6/animal). (B) scAAV9.CB.CLN6 (ICV, P1, $5x10^{10}$ vg/animal) does not significantly improve memory and learning deficits of *Cln6*^{nclf} mice in the Morris water maze reversal task at 12, 18 and 24 months of age when compared to wildtype counterparts. Swim speeds are shown as a control. (C) Male and Female scAAV9.CB.CLN6 treated animals have similar body weights to WT animals. N=5-15 animals/treatment group for water maze assays (panels A-B), N=3-13 animals/treatment group for weight (panel C). One-Way ANOVA or unpaired t-test where appropriate, Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Group	Sex	Age (months)	Reason for Death
WT PBS	F	11	Found dead
	F	17	Moribund, Ulcerative Dermatitis
	F	25	Moribund, Fighting wounds, hunched, broken teeth, BCS 2, rectal bleeding
	F	25	NA, censored at end of study
	F	25	NA, censored at end of study
	F	25	NA, censored at end of study
	F	29	NA, censored at end of study
	M	10	Moribund, Ulcerative Dermatitis
	M	18	Found dead
	M	19	Found dead
	M	23	Moribund
	M	25	NA, censored at end of study
	M	28	Moribund
	M	28	Moribund
	M	28	Moribund
	M	28	Moribund
***Cln6^{nclf}* PBS**	F	10	Moribund, Ulcerative Dermatitis
	F	13	Found dead
	F	14	Moribund, Neurologic
	F	14	Moribund, abdominal bleeding and reduced spleen size
	F	14	Found dead
	F	14	Moribund, Neurologic
	F	14	Moribund, Neurologic
	F	14	Moribund, Neurologic
	F	14	Found dead
	M	14	Moribund, Neurologic
	M	14	Found dead
	M	15	Moribund, Neurologic
	M	15	Moribund, Neurologic
	M	15	Moribund, Neurologic
	M	15	Moribund, abdominal bleeding and reduced spleen size
	M	15	Moribund, Neurologic
	M	15	Moribund, Neurologic

***Cln6^{nclf}* scAAV9.CB.CLN6**	F	15	Moribund; Rectal bleeding, anal prolapse; no signs of neurological disorder; spleen enlarged; CBC suggests anemia
	F	17	Found dead
	F	25	Moribund, Fighting wounds, hunched, broken teeth, BCS 2, rectal bleeding
	F	25	Moribund, Fighting wounds, hunched, broken teeth, BCS 2, rectal bleeding
	M	15	Found dead
	M	18	Moribund, Ulcerative Dermatitis
	M	20	Moribund, Ulcerative Dermatitis
	M	23	Moribund; Orbital Abscess
	M	24	Found dead
	M	24	Moribund; Labored Breathing

Table S1: Reason for death in mice monitored for survival curve, supplemental for Figure 5. If animals were deemed moribund per animal staff, the reason for euthanasia is listed (ie, "Moribund, Ulcerative Dermatitis" indicates that the animal was euthanized due to excessive, untreatable ulcerative dermatitis).

Supplemental References:

1. Lein, ES, Hawrylycz, MJ, Ao, N, Ayres, M, Bensinger, A, Bernard, A, *et al.* (2007). Genome-wide atlas of gene expression in the adult mouse brain. *Nature* **445**: 168-176.
2. Institute, A (2019). Allen Mouse Brain Atlas, P56, Sagittal, Nissl http://atlas.brain-map.org/atlas?atlas=2&plate=100883888#atlas=2&plate=100883867&resolution=17.08&x=7687.92687240912&y=3903.8151332310267&zoom=-4.