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Supplemental information

Correcting tau isoform ratios with a long-acting antisense oligonucleotide alleviates 4R-tauopathy phenotypes

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Supplemental Figure Legends

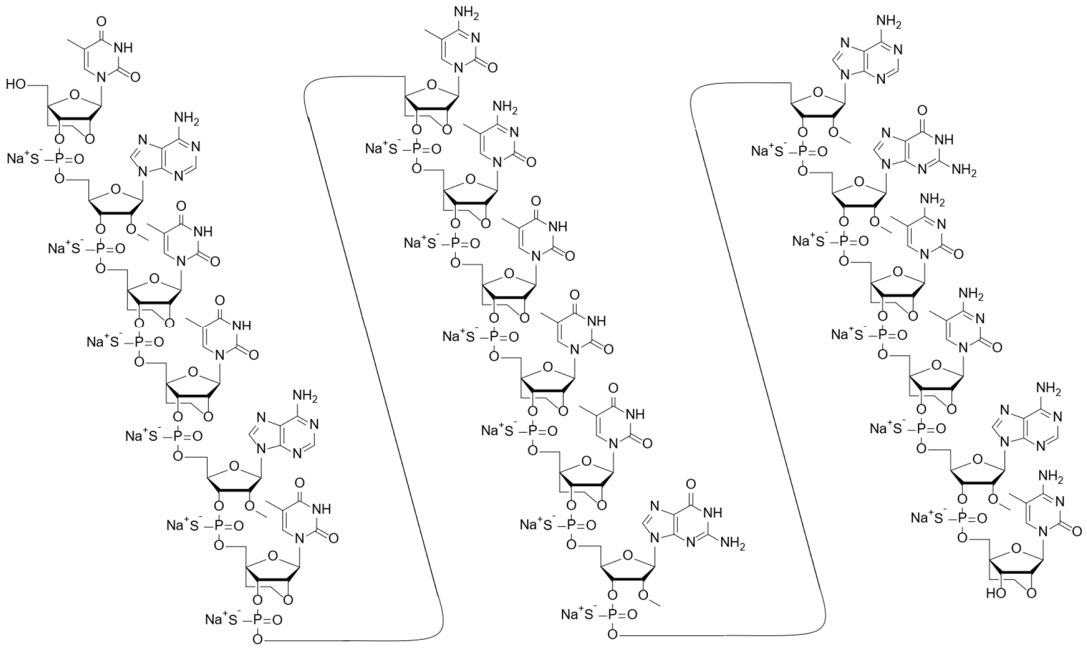


Figure S1. The structural formula of EN-06.

The structural formula of EN-06 sodium salt is shown.

Molecular Formula: $C_{210}H_{253}N_{59}O_{110}P_{17}S_{17}Na_{17}$

Molecular Weight: 6826.0370

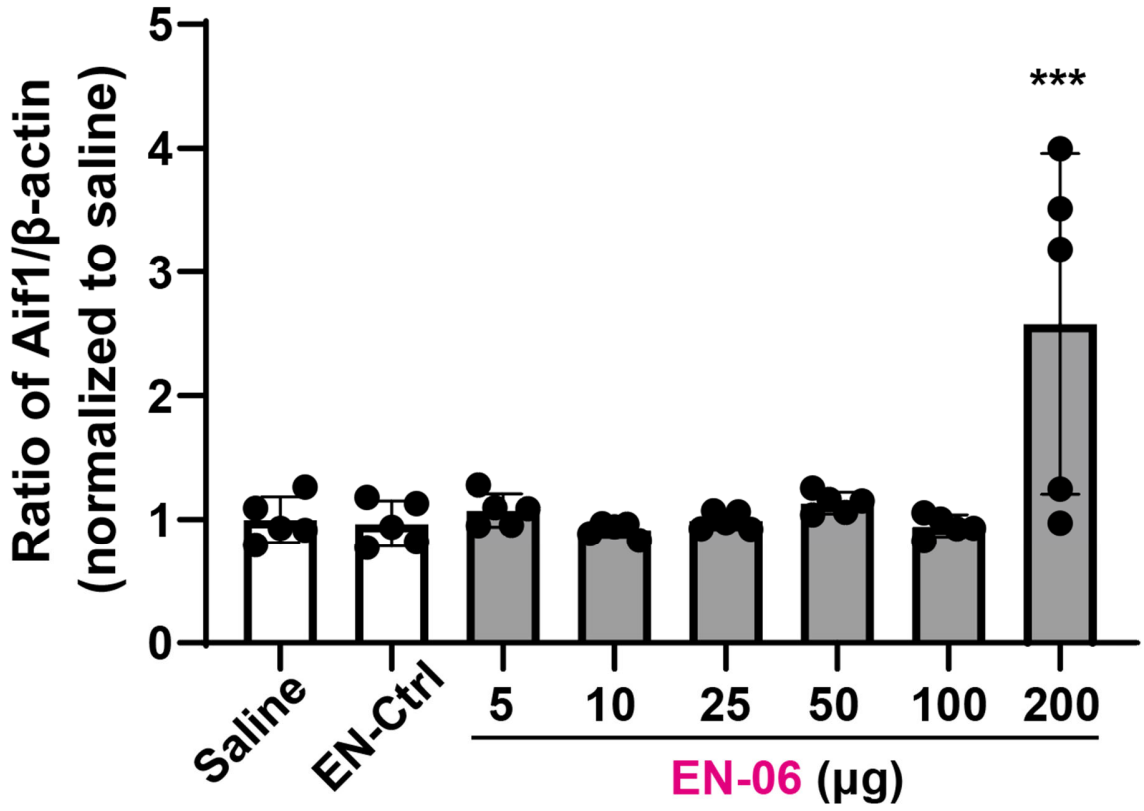
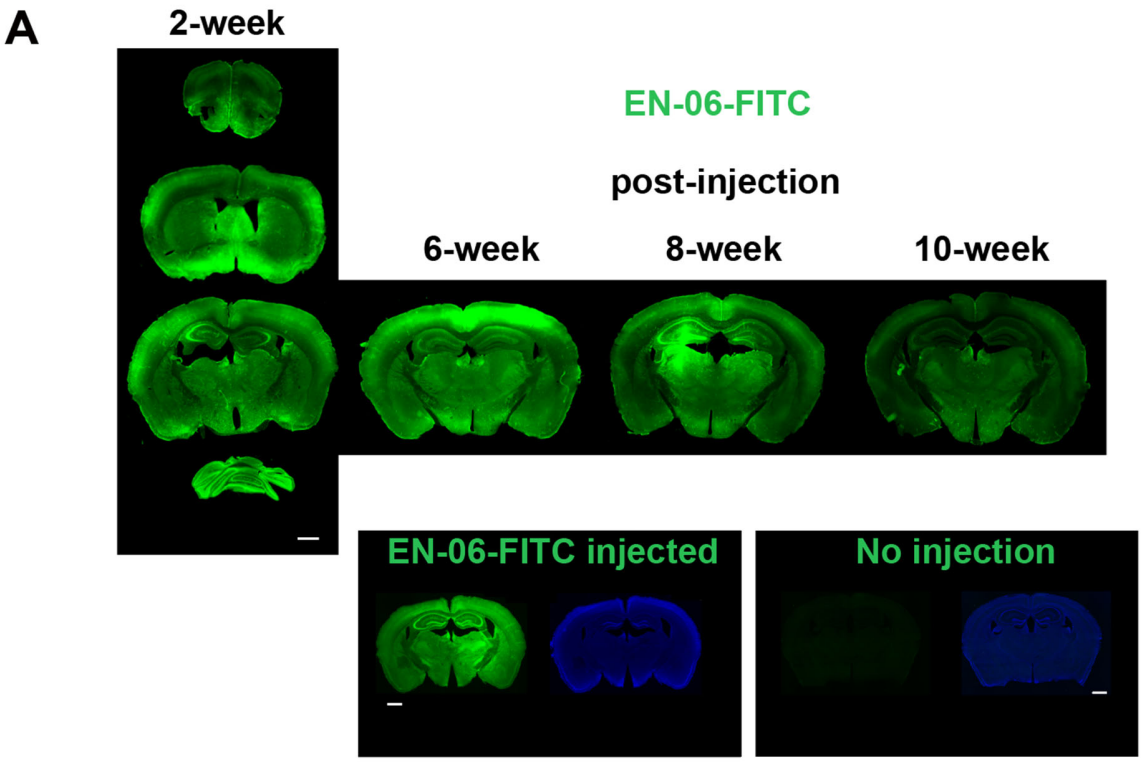
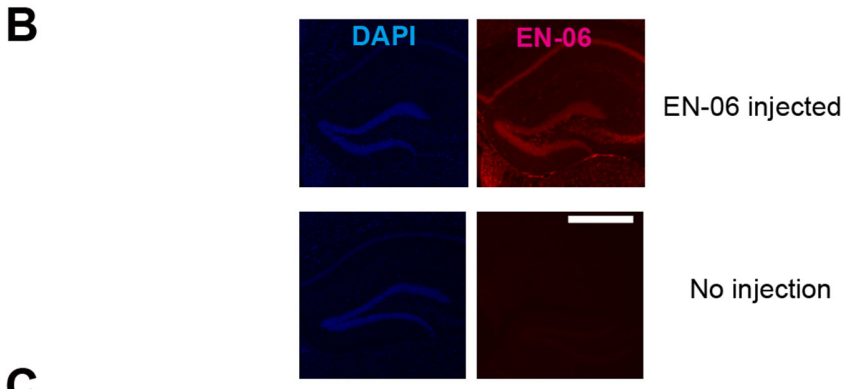


Figure S2. Inflammatory gene expression in the brain after a single ICV administration of EN-06.

The expression level of *Aif1*, which encodes the Iba1 protein, in brain homogenates from mice administered increasing doses (5, 10, 25, 50,100, and 200 μg) of EN-06 were assessed. The data shown represent the mean ± SD and are shown as *Aif1* per *β-actin* (n = 5 for each; one-way ANOVA; ***P < 0.001).


A
2-week
EN-06-FITC
post-injection
6-week
8-week
10-week
EN-06-FITC injected
No injection


B
DAPI
EN-06
EN-06 injected
No injection

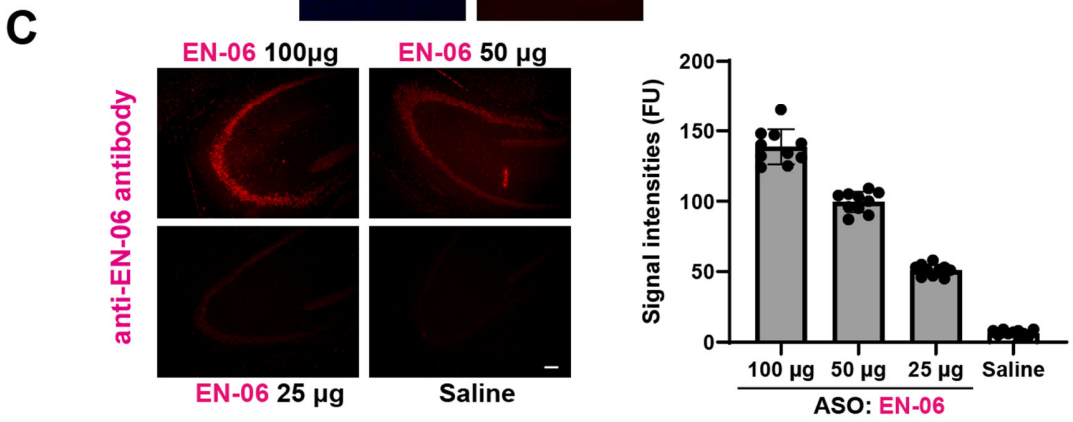

C
EN-06 100µg
EN-06 50 µg
anti-EN-06 antibody
EN-06 25 µg
Saline
Signal intensities (FU)
200
150
100
50
0
100 µg
50 µg
25 µg
Saline
ASO: EN-06

Figure S3. Brain tissue distribution of ENA-ASOs targeting human *MAPT* exon 10.

(A) The brain distribution of FITC-labelled EN-06 at 2-, 6-, 8-, and 10 weeks post-ICV injection of 50 μg. A negative control section without injecting FITC-labelled EN-06 is shown with the section of FITC-labelled EN-06 at 6 weeks below. Scale bar = 1.0 mm. (B) Immunofluorescent study using a custom antibody against EN-06 in the brain. Mouse brain sections at 6 weeks post-ICV injection of 50 μg EN-06 were stained with an antibody against EN-06 (top). The negative control brain sections without EN-06 injection were stained with the same antibody (bottom). Scale bar = 0.5 mm. (C) Immunofluorescence imaging of hippocampal sections was done with a custom antibody against EN-06 after ICV injection of three different EN-06 doses (left panel). Scale bar = 100 μm. Signal intensities were quantified and plotted (right panel). The data shown represent the mean ± SD.

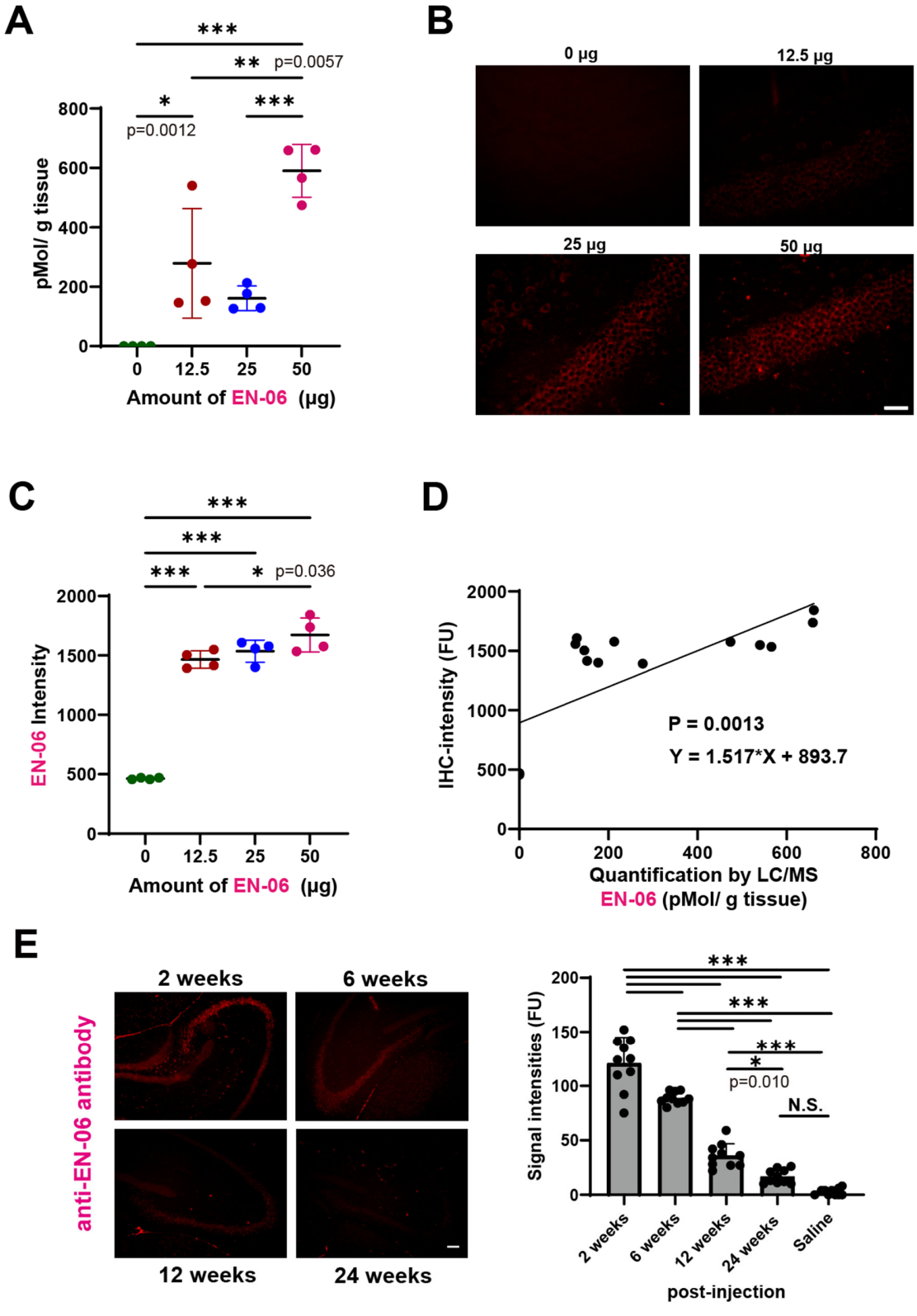

A
***
** p=0.0057
*
p=0.0012
***
pMol/ g tissue
800
600
400
200
0
0
12.5
25
50
Amount of EN-06 (µg)
B
0 µg
12.5 µg
25 µg
50 µg
C
***
***
***
* p=0.036
EN-06 Intensity
2000
1500
1000
500
0
0
12.5
25
50
Amount of EN-06 (µg)
D
IHC-intensity (FU)
2000
1500
1000
500
0
P = 0.0013
Y = 1.517*X + 893.7
0
200
400
600
800
Quantification by LC/MS
EN-06 (pMol/ g tissue)
E
2 weeks
6 weeks
anti-EN-06 antibody
12 weeks
24 weeks
Signal intensities (FU)
200
150
100
50
0
***
***
***
*
p=0.010
N.S.
2 weeks
6 weeks
12 weeks
24 weeks
Saline
post-injection

Figure S4. LC/MS-based and immunofluorescence-based quantification of EN-06 in the brain.

(A) The absolute tissue concentration of EN-06 was determined in the hippocampus of mice administered 4 doses (0, 12.5, 25, and 50 μg) of EN-06 by combining solid-phase extraction and LC/MS. The concentrations of EN-06 in the hippocampus of mice at 6 weeks post-injection were plotted and are shown as pMol/ g tissue (n = 4 for each dose; one-way ANOVA). (B) Immunofluorescent imaging was performed with a custom antibody against EN-06 using hippocampal sections of the mice shown in (A). Scale bar = 40 μm. (C) Signal intensities of the images in (B) were quantified and plotted. (D) Correlation of the absolute tissue concentrations of EN-06 as determined by LC/MS and immunofluorescence imaging ($P = 0.0013$). (E) Immunofluorescence imaging of hippocampal sections at various time points post-ICV injection of a 50-μg dose of EN-06 into 6-week-old hTau mice. Hippocampal sections were stained with a custom antibody against EN-06 (left panel). Scale bar = 100 μm. Signal intensities of the immunofluorescent images were quantified and plotted (right panel). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

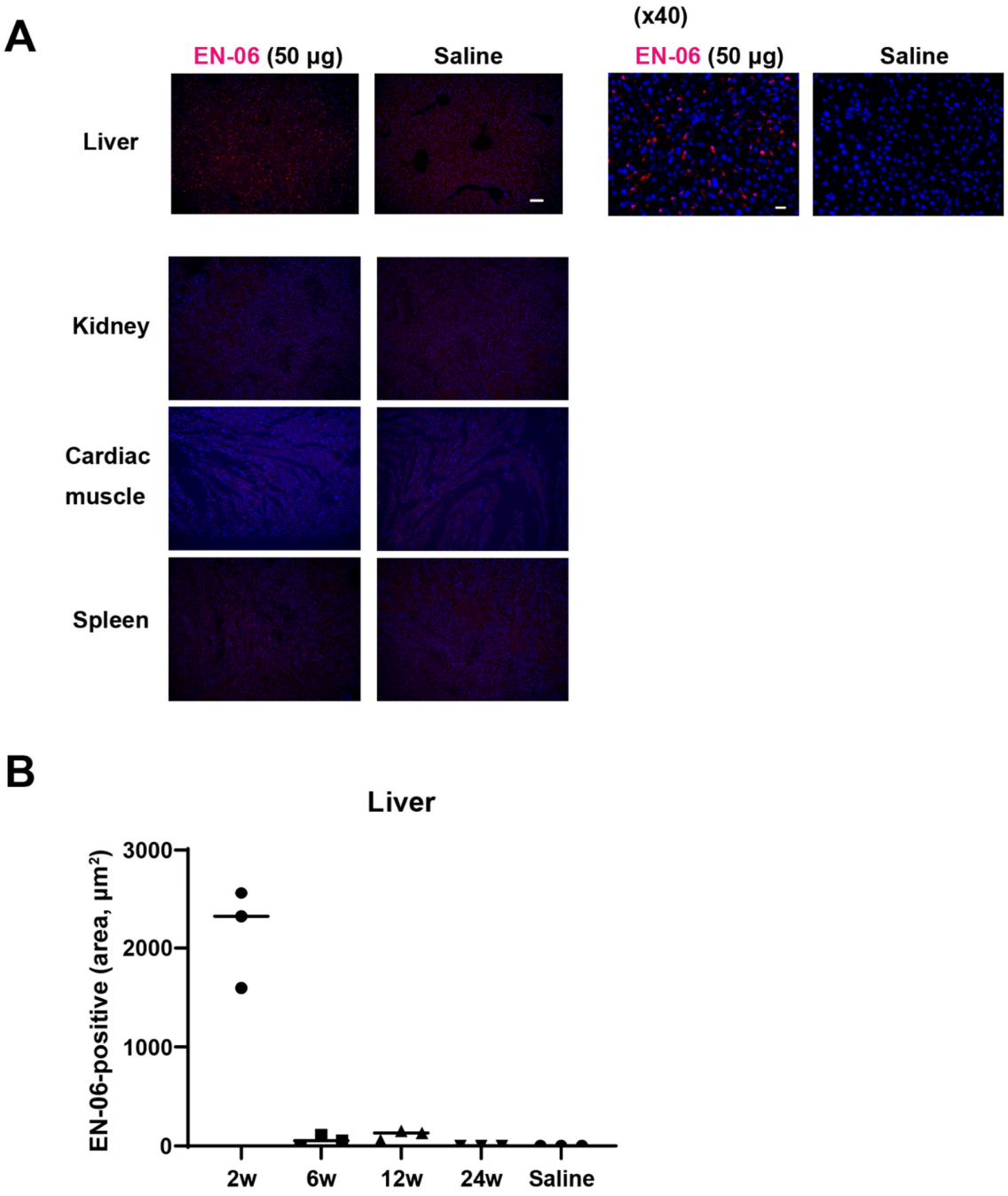


Figure S5. General tissue distribution of EN-06. (A) Immunofluorescent images of liver, kidney, cardiac muscle, and spleen sections of mice ICV injected 50 μg of EN-06 or saline. Sections were stained with a custom antibody against EN-06. Scale bar = 100 μm for left

images, 20 μm for right images. (B) The signal intensities in the liver sections were quantified and plotted (right panel). The data shown represent the mean ± SD (n = 3 for each).

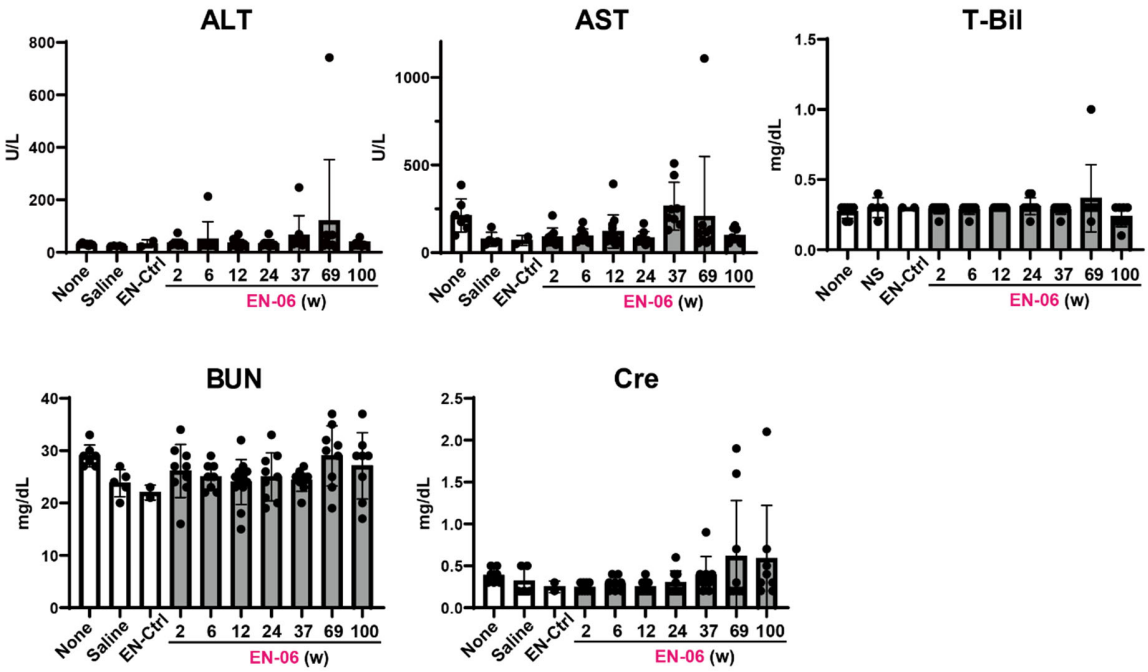


Figure S6. EN-06 has no significant negative effects on liver and renal function.

A panel of blood biochemical analytes associated with liver and renal function was assessed at various time points (2, 6, 12, 24, 37, 69, and 100 weeks) post-EN-06 administration. Mice administered the EN-Ctrl were assessed at 6 weeks, those administered the saline control at 2 weeks, and the non-injected control group was assessed immediately. Analytes measured included alanine aminotransferase (ALT), aspartate transaminase (AST), total bilirubin (T-Bil), blood urea nitrogen (BUN), and creatinine (Cre). There were no significant differences between the control group and any of the experimental groups (one-way ANOVA). The data shown represent the mean ± SD.

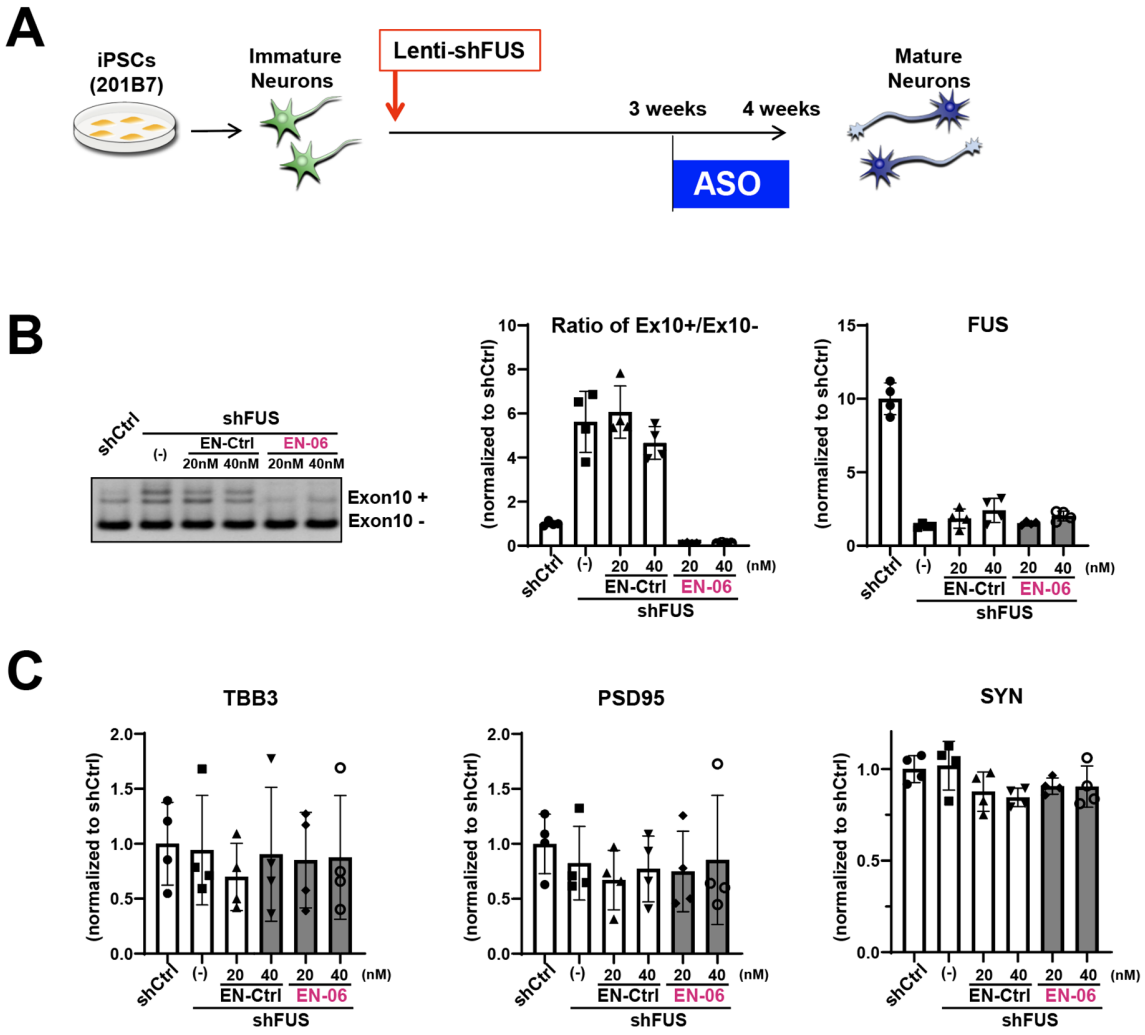


Figure S7. EN-06 restores the FUS-silencing induced increase in 4R-tau/3R-tau ratios in human-induced pluripotent stem cell (iPSC)-derived neurons. (A) Experimental scheme. Briefly, neurons infected with a lentivirus encoding shRNA were treated with 20 or 40 nM of each of the ASOs at 7 days post-differentiation. (B) Lentivirus-mediated shRNA targeting human FUS and a scramble control shRNA (shCtrl) were introduced into human iPSC-derived neurons. Neurons were then treated with 20 or 40 nM EN-06 or EN-Ctrl.

Alternative splicing of *MAPT* exon 10 was assessed by RT-PCR (left panel). The signals were quantified, and the isoform ratio was determined (middle graph). *FUS* expression was assessed by qRT-PCR (right graph). (C) Neuronal transcript levels of *βIII-tubulin* (TBB3), *PSD95*, and *synaptophysin* (SYN) were determined by qRT-PCR. The relative amount for each transcript was normalized to the housekeeping β-actin. The data shown represent the mean ± SD (n = 4 for each).

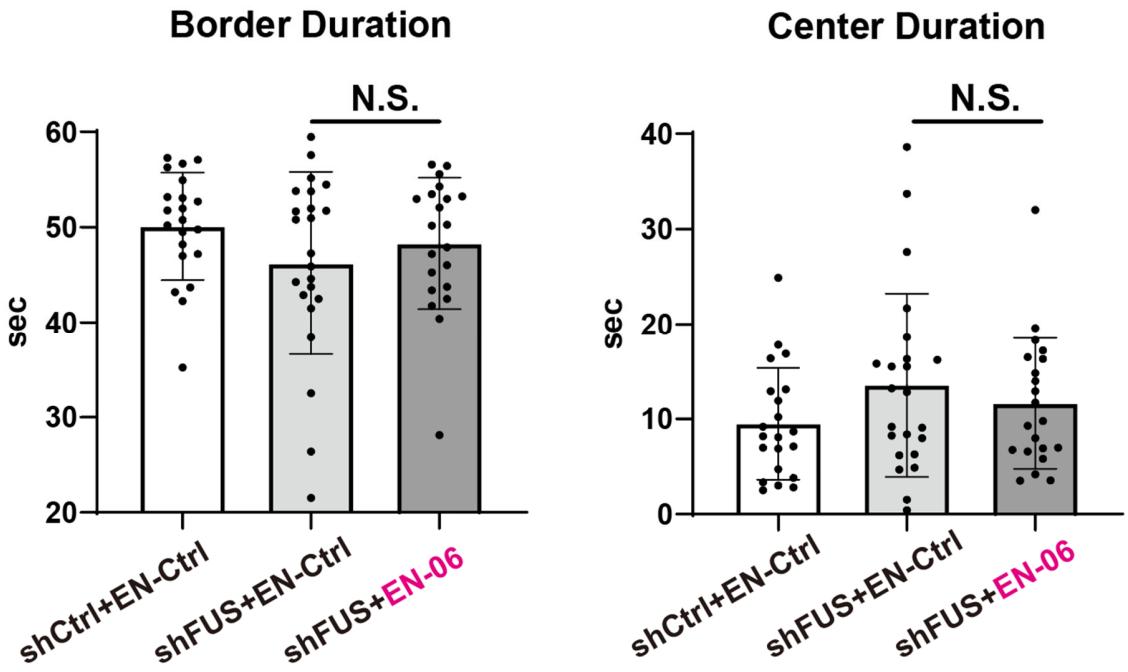


Figure S8. Open-field test behavioral assessment of the FUS knockdown hTau mouse model administered EN-06. Mice were treated with shCtrl + EN-Ctrl, shFUS + EN-Ctrl, or shFUS + EN-06. Time spent in the border (left graphs) and the center (middle graphs) area of an open field was determined (n = 21 for shCtrl + EN-Ctrl, n = 23 for shFUS +EN-Ctrl, and n = 21 for shFUS + EN-06; one-way ANOVA). N.S. denotes not significant. The data shown represent the mean ± SD.

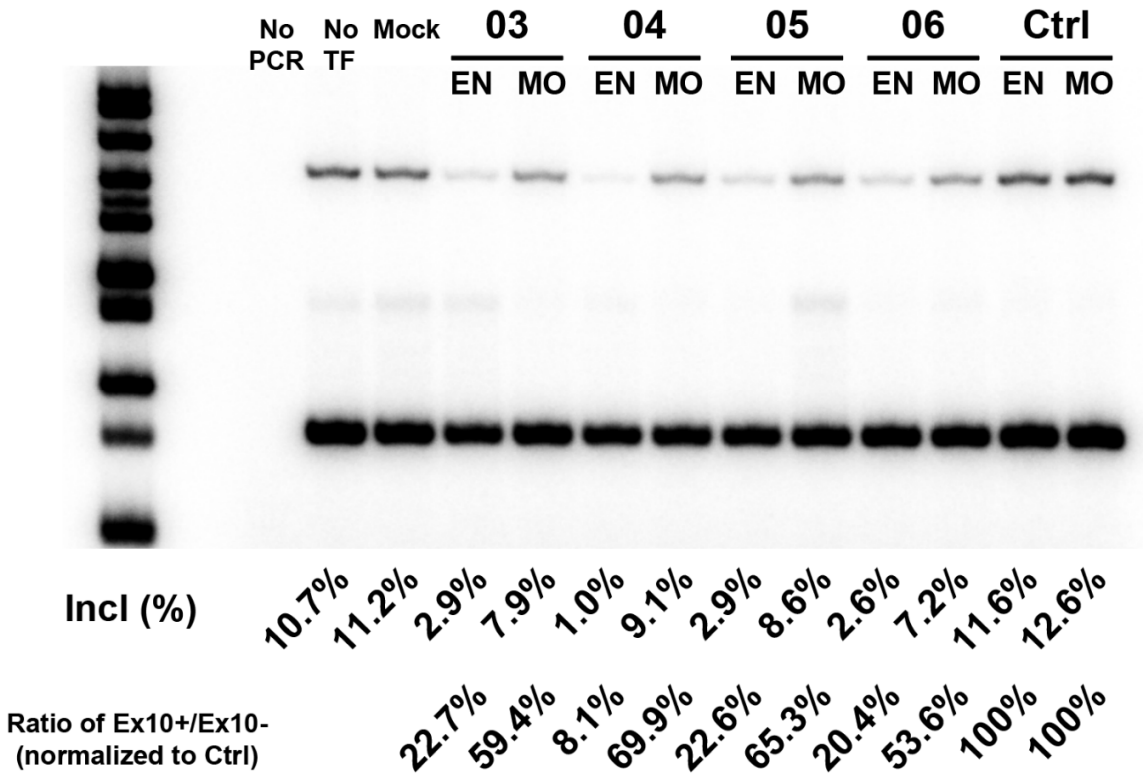


Figure S9. Efficiency of ENA- and MOE-modified ASOs targeting the same sequence to induce skipping of *MAPT* exon 10. RT-PCR was utilized to assess the effects of ENA- and MOE-modified ASOs (EN-03, MO-03, EN-04, MO-04, EN-05, MO-05, EN-06, and MO-06) and control ASOs (EN-Ctrl and MO-Ctrl) on the splicing of *MAPT* exon 10 in HEK293 cells. The percentage of exon 10-skipped transcripts was calculated relative to the total transcripts (sum of exon 10-skipped and exon 10-included transcripts) for each ASO. The ratio of exon 10-included to exon 10-skipped transcripts is shown, normalized to the ratio observed in the control, which was set to 100%.

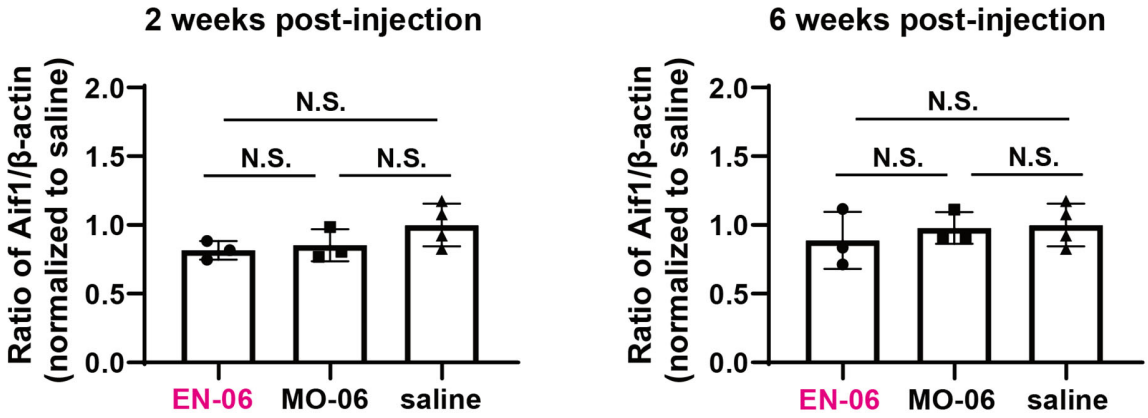


Figure S10. *Aif1* expression in the brain after ICV administration of ENA- or MOE-modified ASO-06.

The expression level of *Aif1*, which encodes the Iba1 protein, was assessed in brain homogenates of mice administered either EN-06 or MO-06. Data are shown as *Aif1* per *β-actin*; n = 3 for EN-06 and MO-06 and n = 4 for the saline control; one-way ANOVA). N.S. denotes not significant. The data shown represent the mean ± SD.

Table S1. ASOs used in this study.

Label	Sequence	Target	Function
EN-01	agCCagaaaaaaggaTga	Intron 9	Exon 10 skipping
EN-02	TggaCgTTgCTaagaTCC	Exon 10	Exon 10 skipping
EN-03	gCCaCaCTTggaCTggaC	Exon 10	Exon 10 skipping
EN-04	CCTTTgagCCaCaCTTgg	Exon 10	Exon 10 skipping
EN-05	TTaTCCTTTgagCCaCaC	Exon 10	Exon 10 skipping
EN-06	TaTTaTCCTTTgagCCaC	Exon 10	Exon 10 skipping
EN-07	gaTaTTaTCCTTTgagCC	Exon 10	Exon 10 skipping
EN-08	ggaCgTgTTTgaTaTTaT	Exon 10	Exon 10 skipping
EN-09	gCaTgggaCgTgTgaagg	Intron 10	Exon 10 inclusion
EN-10	gCaCggCgCaTgggaCgT	Intron 10	Exon 10 inclusion
EN-11	TTTaTTCTaTgCagTgTC	Intron 10	Exon 10 inclusion
EN-12	gCCCaagaaggaTTTaTT	Intron 10	Exon 10 inclusion
EN-13	ggCgCaTgggaCgTgTga	Intron 10	Exon 10 inclusion
EN-C1	CaTCTaagCaaCaaTTga	Non-specific	Control ASO
EN-C2 (EN-Ctrl)	CTCTTgaCgCaCaTCTgg	Non-specific	Control ASO
EN-C3	TTCCCTgaaggTTCCTCC	Non-specific	Control ASO
EN-C4	TCagTaaaCTTgaCaCCa	Non-specific	Control ASO

Uppercase letters: ENA-modified nucleotides

Lowercase letters: 2′-*O*-methylation-modified nucleotides

Table S2. Primers used for RT-PCR.

Gene	Forward	Reverse
mouse Fus	GGCTACTCCCAACAGAGCAG	GCTGTTTTGGGTCTGTCCAT
mouse β-actin	GCAAGTGCTTCTAGGCGGAC	AAGAAAGGGTGTAAAACGCAGC
mouse Aif1	AAGAGAGGCTGGAGGGGATC	GCTTCAAGTTTGGACGGCAG
human MAPT (exon10)	CCATGCCAGACCTGAAGAAT	TGCTCAGGTCAACTGGTTTG
human MAPT (whole)	GCAACATCCATCATAAACCAGGA	AGGGACCCAATCTTCGACTG
human FUS	AGCTCCCAATCGTCTTACGG	TTGCTGCTGTCCACCATAGC
human β-actin	GATCAAGATCATTGCTCCTCCT	GGGTGTAACGCAACTAAGTCA
human βIII-Tubulin	CATTCTGGTGGACCTGGAAC	ATACTCCTCACGCACCTTGC
human PSD95	ATATGTGAACGGGACCGAGG	TCACCGATGTGTGGGTTGTC
human SYN	GCCAACAAGACCGAGAGTGA	GAATTCGGCTGACGAGGAGT

Table S3. shRNAs used in this study.

shRNA Name	Target Sequence
FUS	GCAACAAAGCTACGGACAA
Ctrl	AATTCTCCGAACGTGTCACGT