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Roy et al.

(10) **Patent No.: US 11,173,216 B2**
(45) **Date of Patent: Nov. 16, 2021**

(54) **GENE EDITING-BASED METHOD OF ATTENUATING THE BETA-AMYLOID PATHWAY**

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(73) Assignee: **WISCONSIN ALUMNI RESEARCH FOUNDATION**, Madison, WI (US)

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(51) **Int. Cl.**

A61P 25/28	(2006.01)
A61K 48/00	(2006.01)
A61K 9/00	(2006.01)
C12N 15/90	(2006.01)
C07K 14/47	(2006.01)
C12N 9/22	(2006.01)
C12N 15/10	(2006.01)

(52) **U.S. Cl.**

CPC ***A61K 48/0066*** (2013.01); ***A61K 9/0019*** (2013.01); ***A61K 48/0008*** (2013.01); ***A61K 48/0058*** (2013.01); ***A61K 48/0075*** (2013.01); ***A61P 25/28*** (2018.01); ***C07K 14/4711*** (2013.01); ***C12N 9/22*** (2013.01); ***C12N 15/102*** (2013.01); ***C12N 15/907*** (2013.01); *C12N 2310/20* (2017.05); *C12N 2740/16043* (2013.01); *C12N 2750/14143* (2013.01)

(58) **Field of Classification Search**

CPC . A61P 25/28; A61K 48/0066; A61K 48/0058; A61K 48/0075; C07K 14/4711; C12N 9/22; C12N 15/102; C12N 15/907; C12N 2310/20

See application file for complete search history.

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(57) **ABSTRACT**

Described herein are CRISPR/Cas9 constructs designed for the C-terminal truncation of human amyloid precursor protein (APP) as well as methods of making and using such a construct. A Cas9 nuclease/gRNA ribonucleoprotein directs cleavage of an APP gene to provide a C-terminal truncated APP having a length of 659, 670, 676, or 686 amino acids, relative to the human or mouse APP sequence.

10 Claims, 53 Drawing Sheets
(38 of 53 Drawing Sheet(s) Filed in Color)

Specification includes a Sequence Listing.

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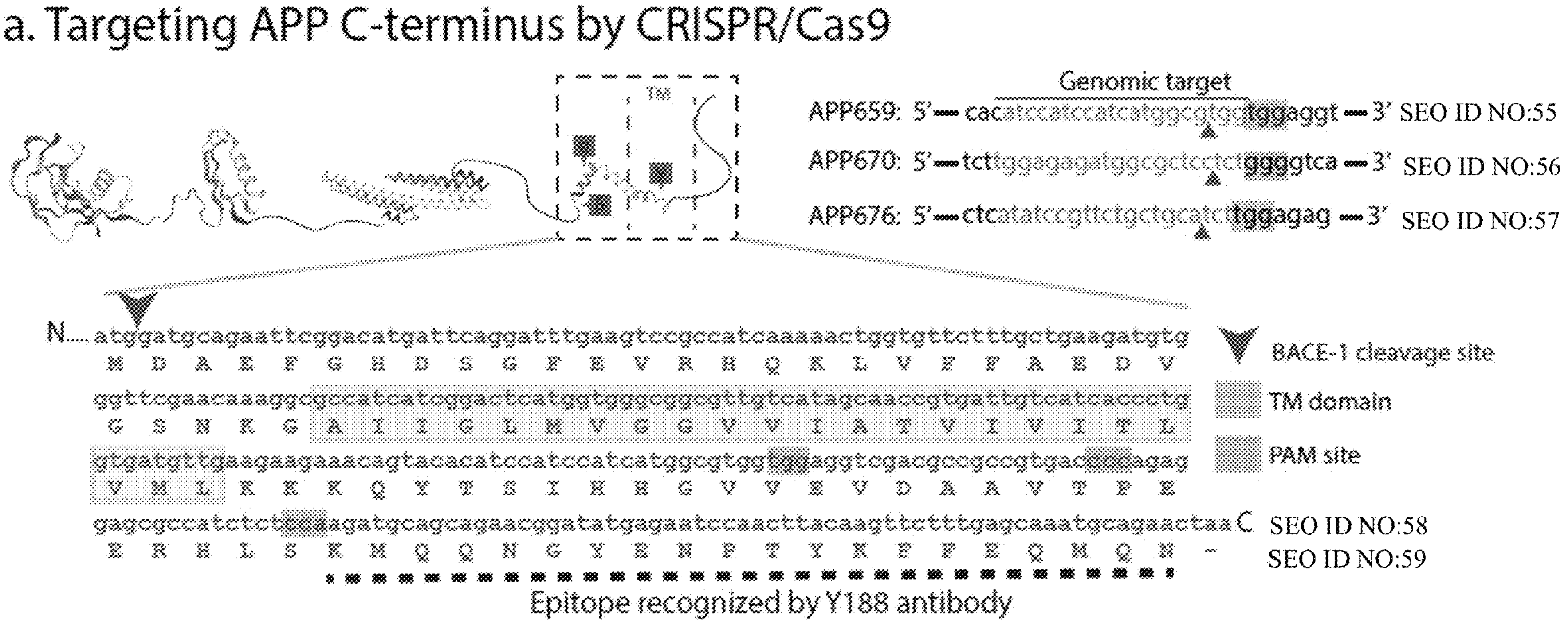
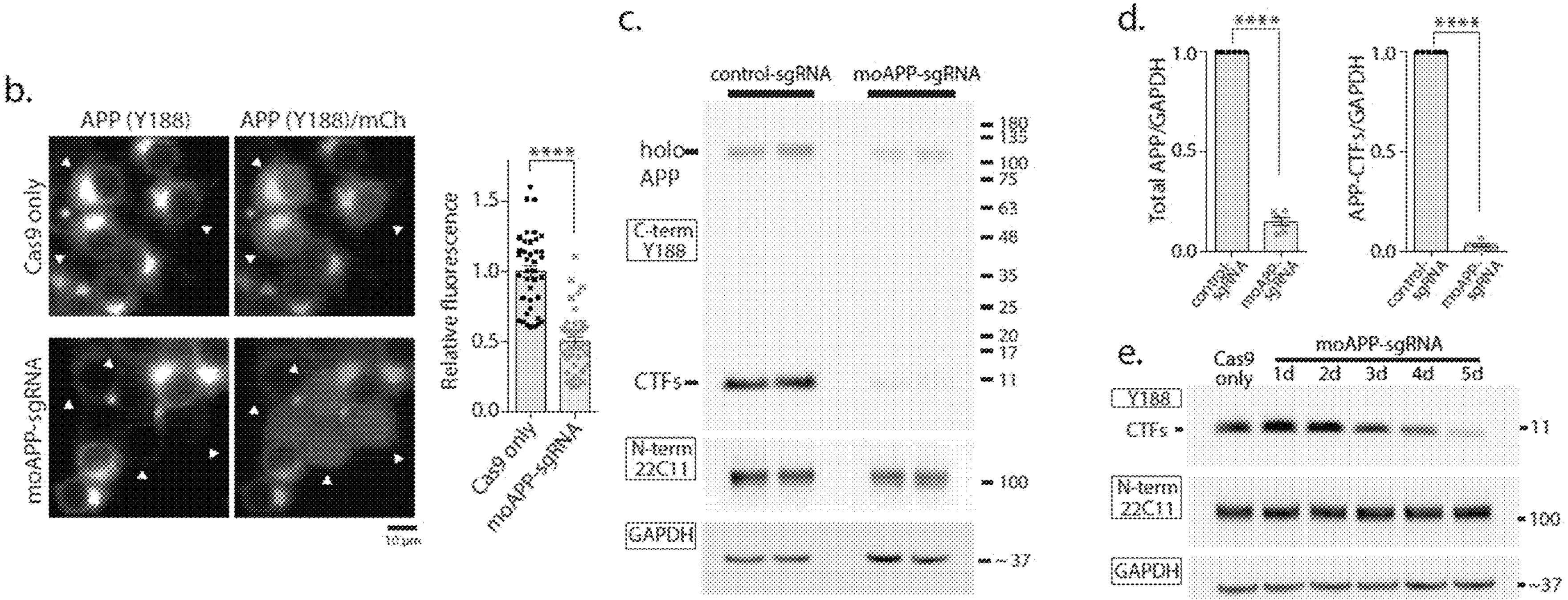
FIGS. 1A-1F

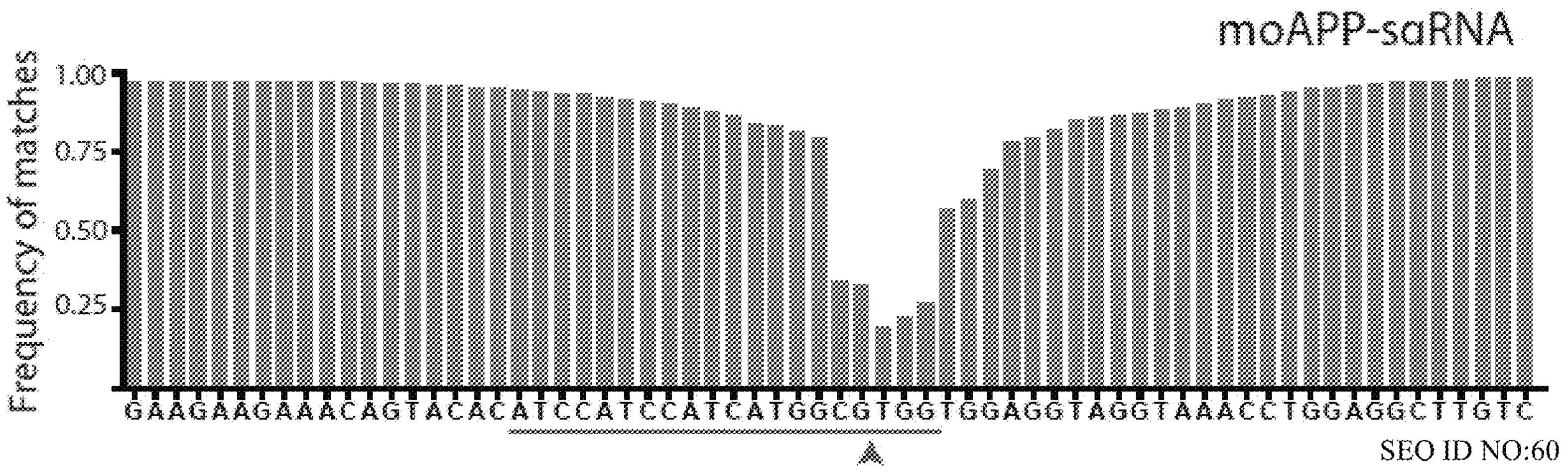

a. Targeting APP C-terminus by CRISPR/Cas9

FIGS. 1A-1F CONTINUED



FIGS. 1A-1F CONTINUED

f. Genomic analyses



SEO ID NO:61

		Freq (%)	
WT	AACAGTACACATCCATCCATCATGGCGTGGTGGAGGTAGGTAAACC		
C1	AACAGTACACATCCATCCATCATGG-----TGGAGGTAGGTAAACC	22.4	SEO ID NO:62
C2	AACAGTACACATCCATCCATCATGGCG-----GAGGTAGGTAAACC	6.8	SEO ID NO:63
C3	AACAGTACACATCCATCCATCATGG--------AGGTAGGTAAACC	5.9	SEO ID NO:64
C4	AACAGTACACATCCATCCATCATGGCG--GTGGAGGTAGGTAAACC	3.8	SEO ID NO:65
C5	AACAGTACACATCCATCCATCATGG--TGGTGGAGGTAGGTAAACC	3.4	SEO ID NO:66

Mutated locus

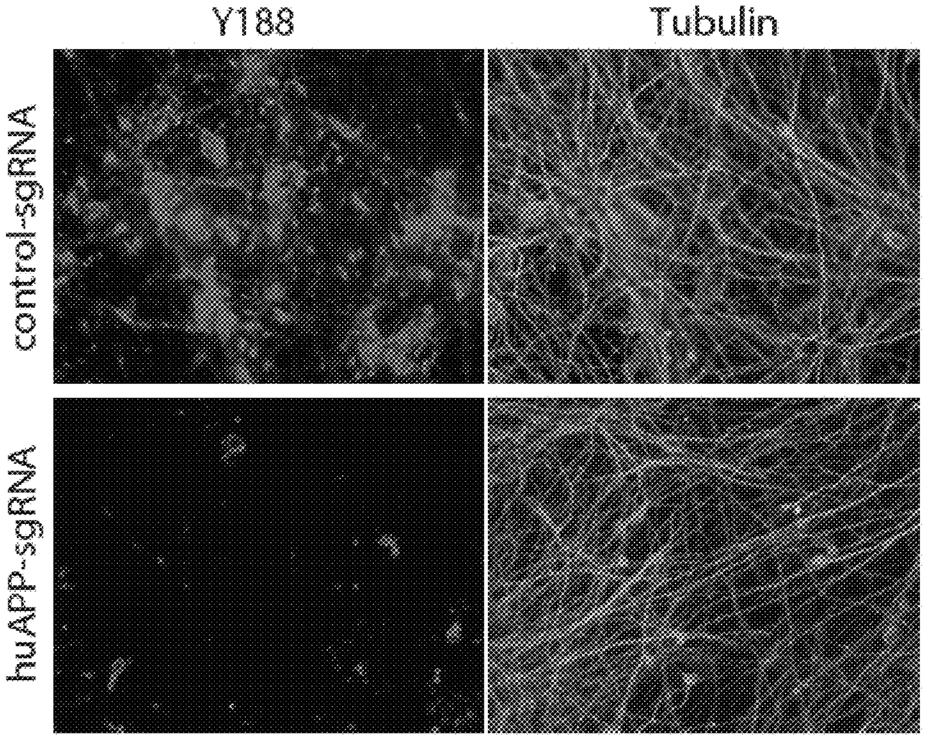
FIGS. 2A-2H

a. Genomic targets:

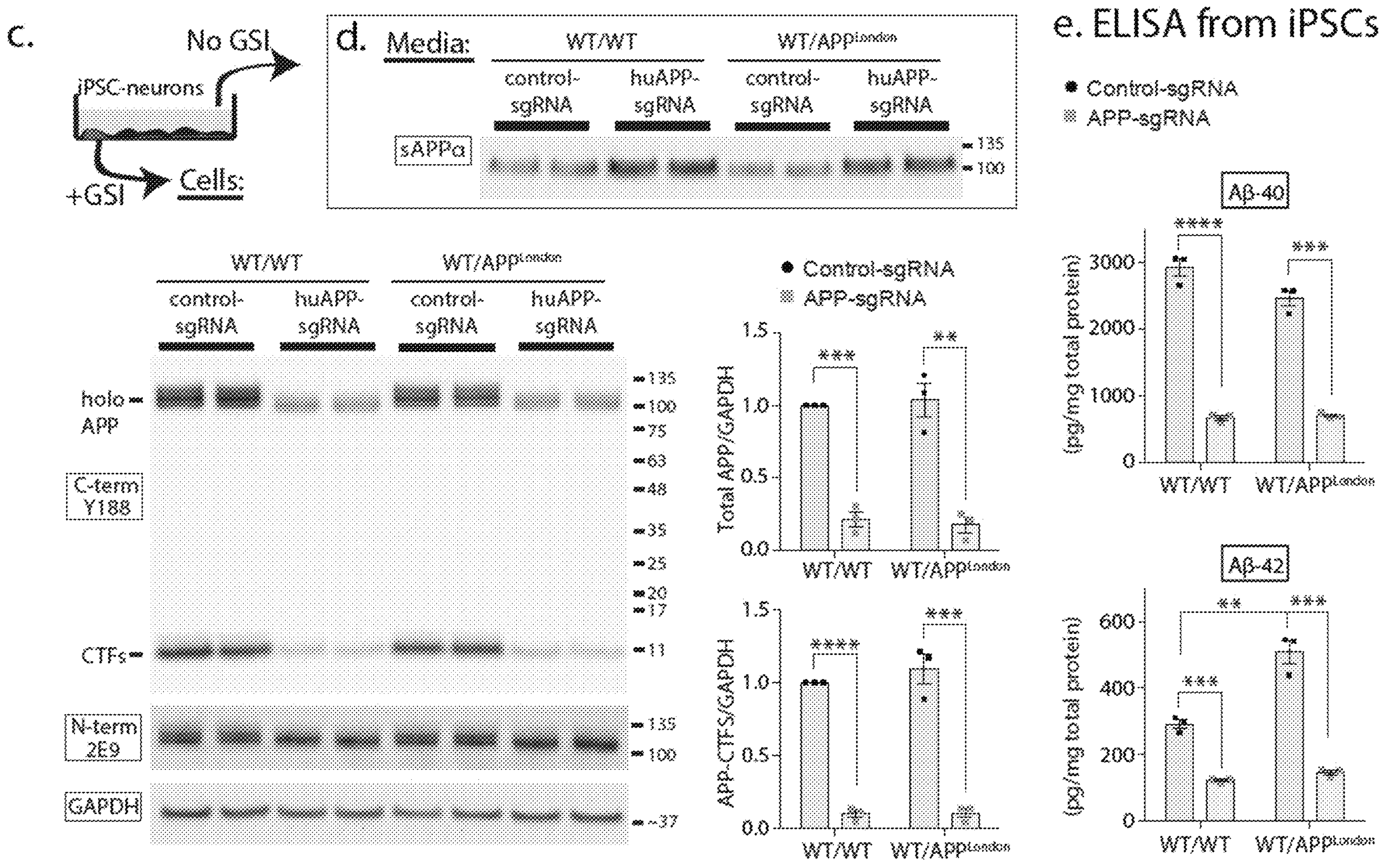
Mouse: atccatccatcatggcgtggtgg SEO ID NO:67

Human: atccattcatcatggtgtggtgg SEO ID NO:68

b. Human iPSC-derived neurons

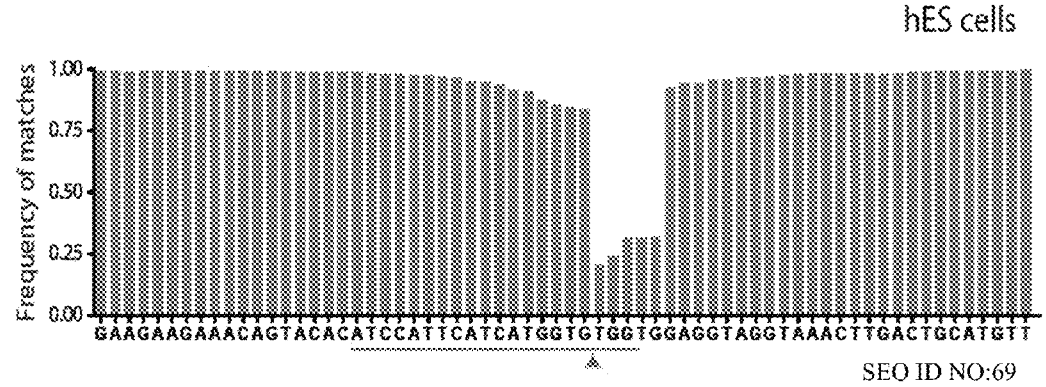


FIGS. 2A-2H CONTINUED

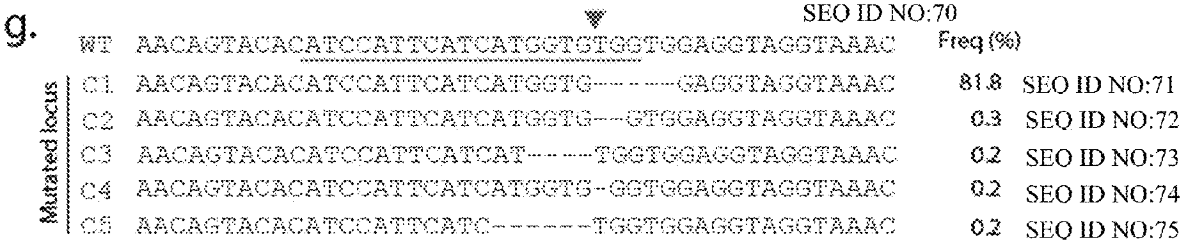


FIGS. 2A-2H CONTINUED

f.



g.



SEO ID NO:70

WT AACAGTACACATCCATTCATCATGGTGTGGTGGAGGTAGGTAAAC Freq (%)

Mutated locus

C1 AACAGTACACATCCATTCATCATGGTG-----GAGGTAGGTAAAC 81.8 SEO ID NO:71

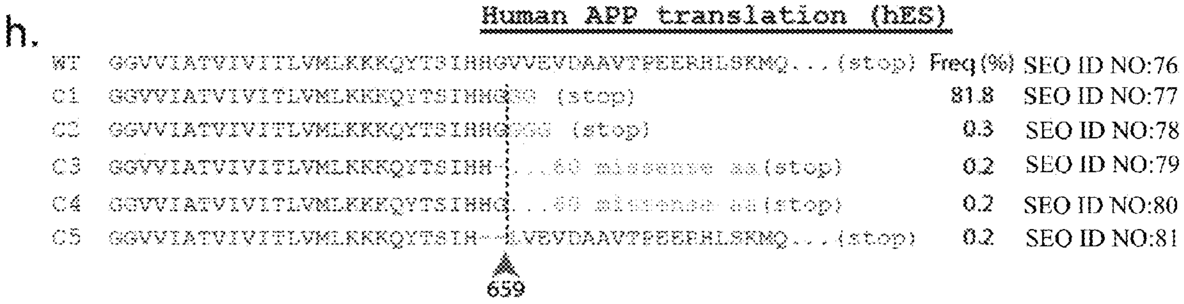
C2 AACAGTACACATCCATTCATCATGGTG--GTGGAGGTAGGTAAAC 0.3 SEO ID NO:72

C3 AACAGTACACATCCATTCATCAT----TGGTGGAGGTAGGTAAAC 0.2 SEO ID NO:73

C4 AACAGTACACATCCATTCATCATGGTG-GGTGGAGGTAGGTAAAC 0.2 SEO ID NO:74

C5 AACAGTACACATCCATTCATC------TGGTGGAGGTAGGTAAAC 0.2 SEO ID NO:75

h.



Human APP translation (hES)

WT GGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQ...(stop) Freq (%) SEO ID NO:76

C1 GGVVIATVIVITLVMLKKKQYTSIHHGGG (stop) 81.8 SEO ID NO:77

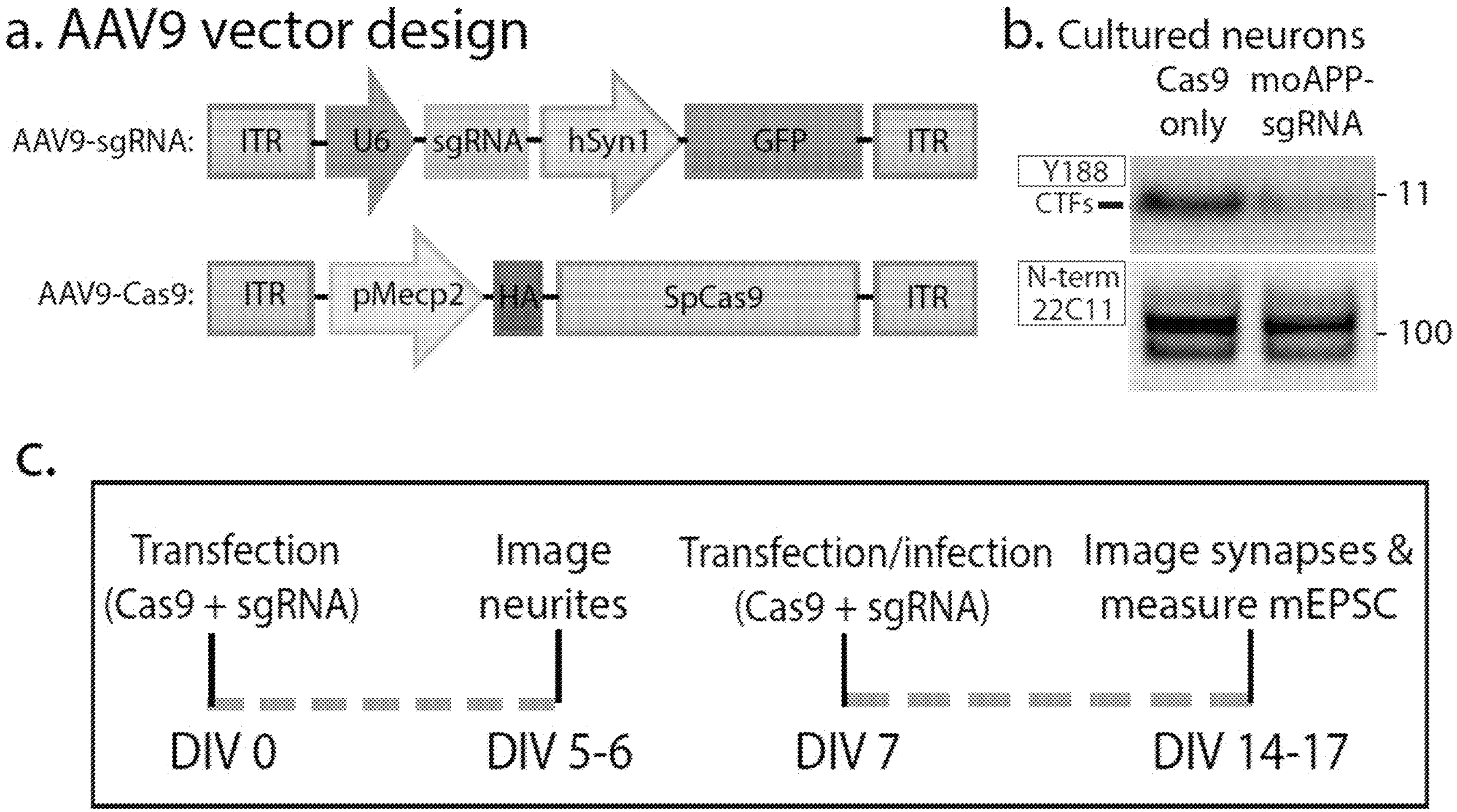
C2 GGVVIATVIVITLVMLKKKQYTSIHHGGGG (stop) 0.3 SEO ID NO:78

C3 GGVVIATVIVITLVMLKKKQYTSIHH-...60 missense aa(stop) 0.2 SEO ID NO:79

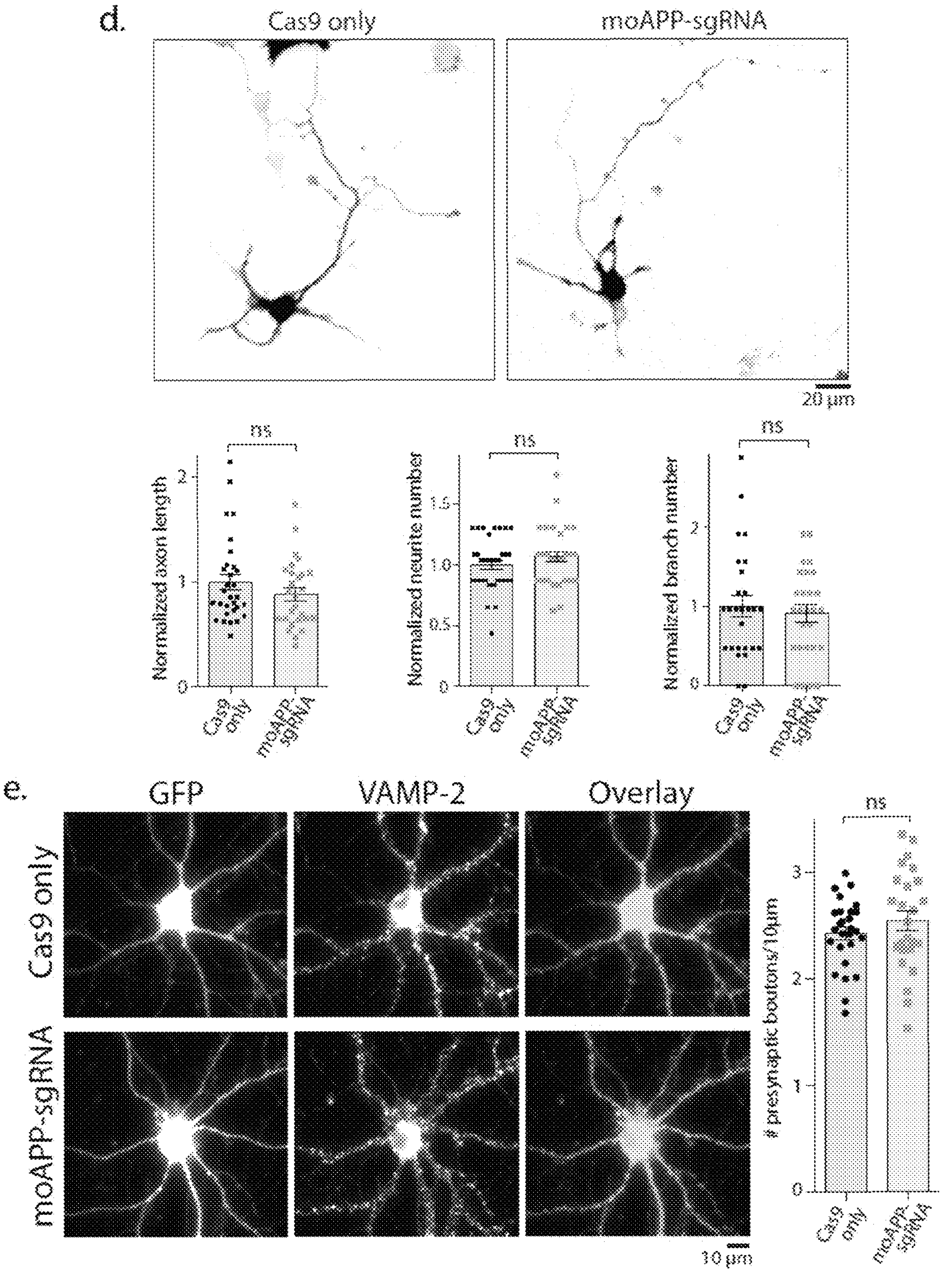
C4 GGVVIATVIVITLVMLKKKQYTSIHHG...60 missense aa(stop) 0.2 SEO ID NO:80

C5 GGVVIATVIVITLVMLKKKQYTSIH--LVEVDAAVTPEERHLSKMQ...(stop) 0.2 SEO ID NO:81

FIGS. 3A-3G

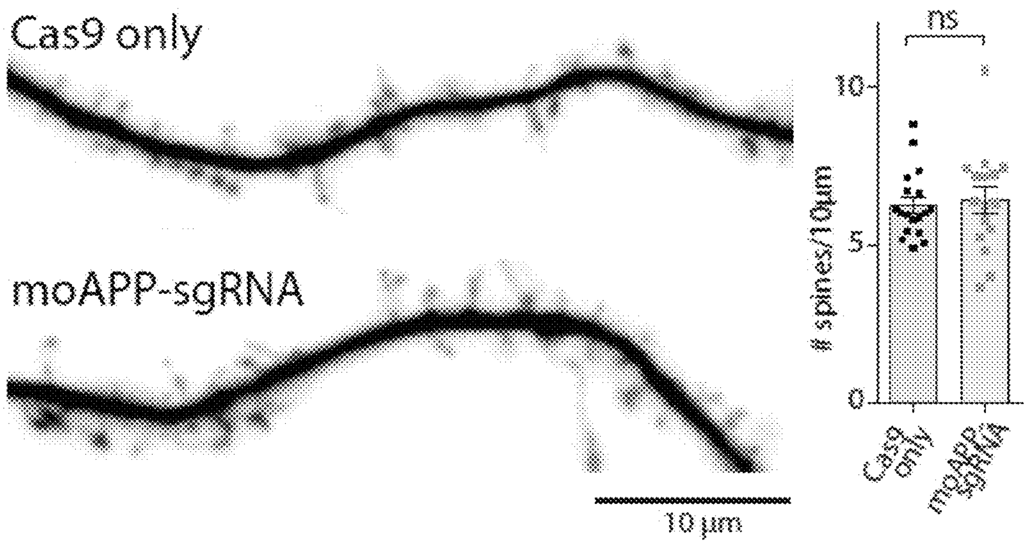

a. AAV9 vector design
AAV9-sgRNA:
ITR
U6
sgRNA
hSyn1
GFP
ITR
AAV9-Cas9:
ITR
pMecp2
HA
SpCas9
ITR
b. Cultured neurons
Cas9 only
moAPP-sgRNA
Y188
CTFs
11
N-term 22C11
100
c.
Transfection (Cas9 + sgRNA)
Image neurites
Transfection/infection (Cas9 + sgRNA)
Image synapses & measure mEPSC
DIV 0
DIV 5-6
DIV 7
DIV 14-17

FIGS. 3A-3G CONTINUED

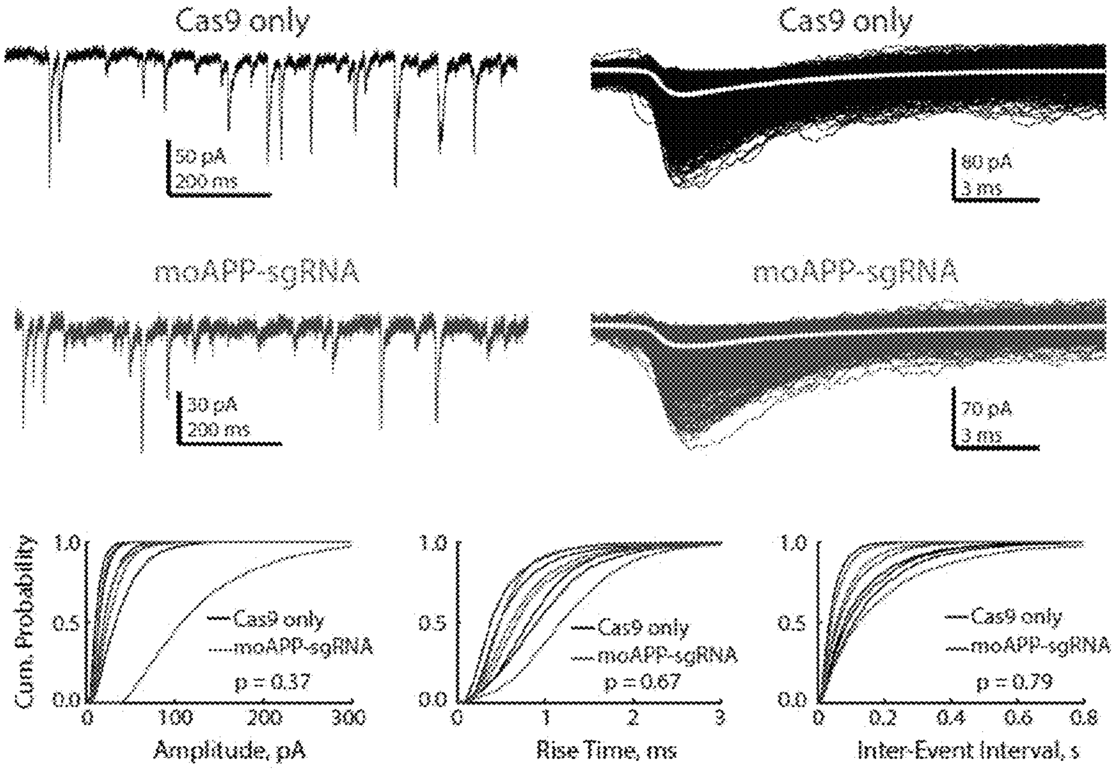


FIGS. 3A-3G CONTINUED

f.

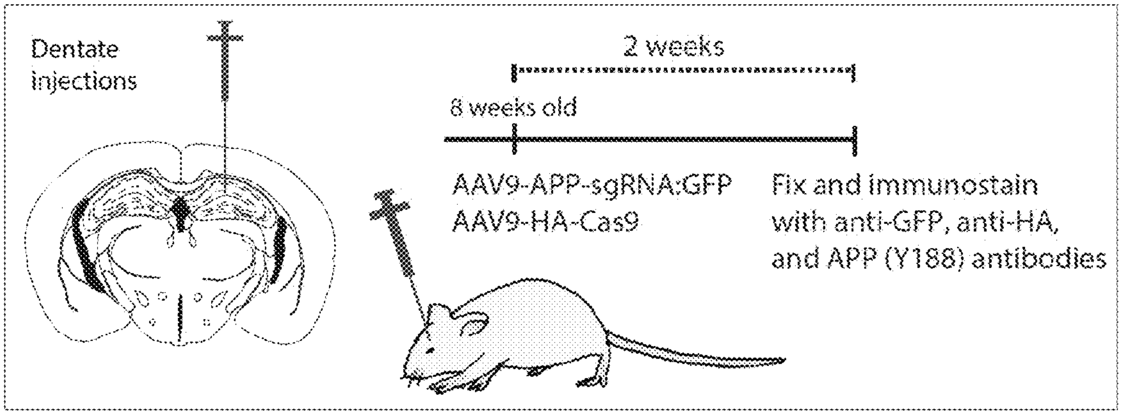


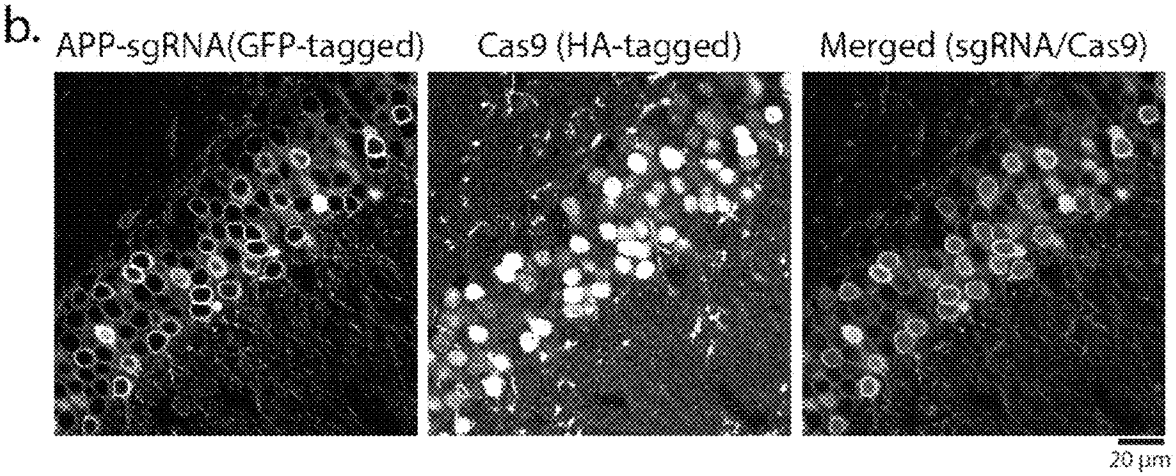
g.



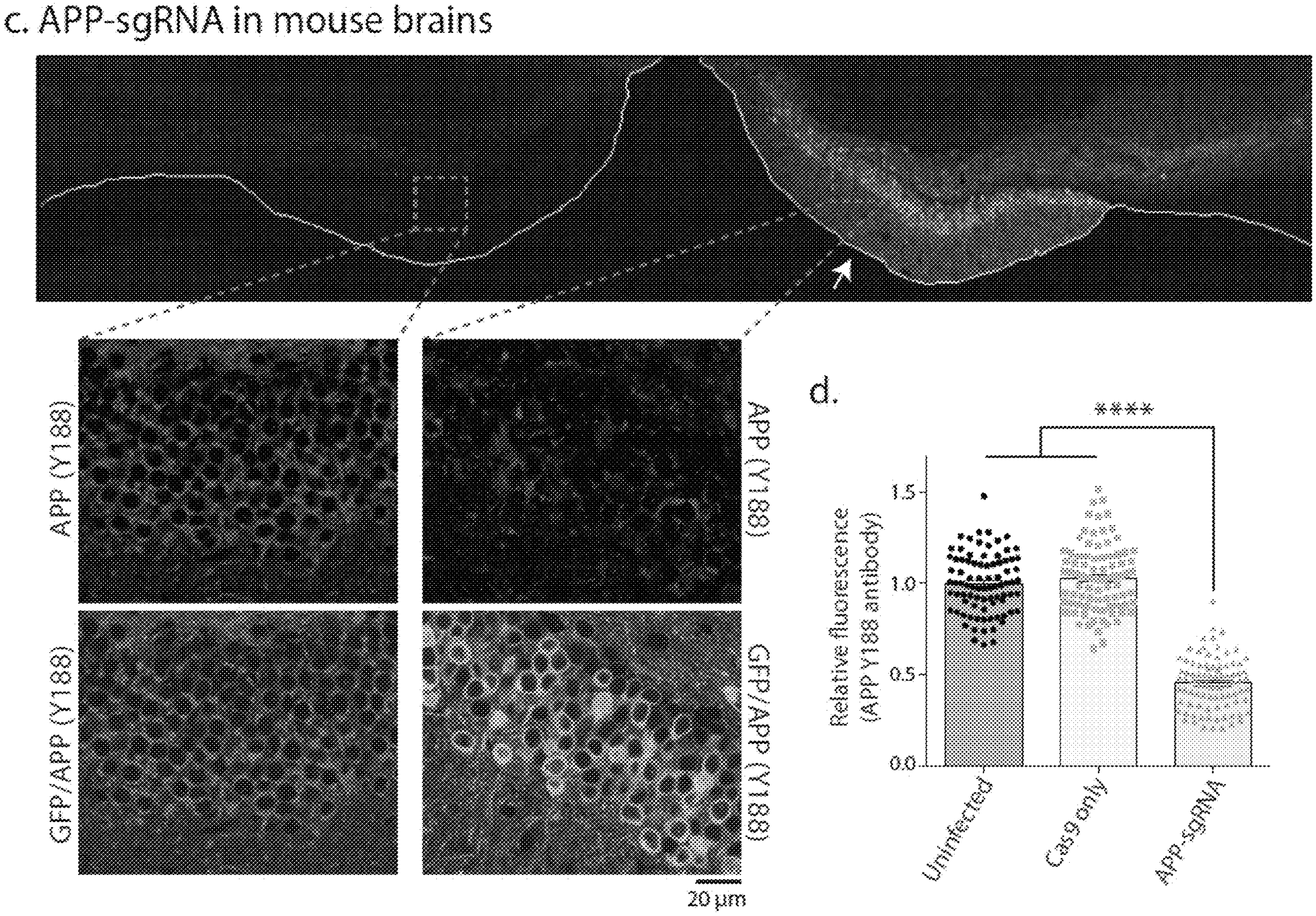
FIGS. 4A-4G

a. Hippocampal AAV9 injections (schematic)



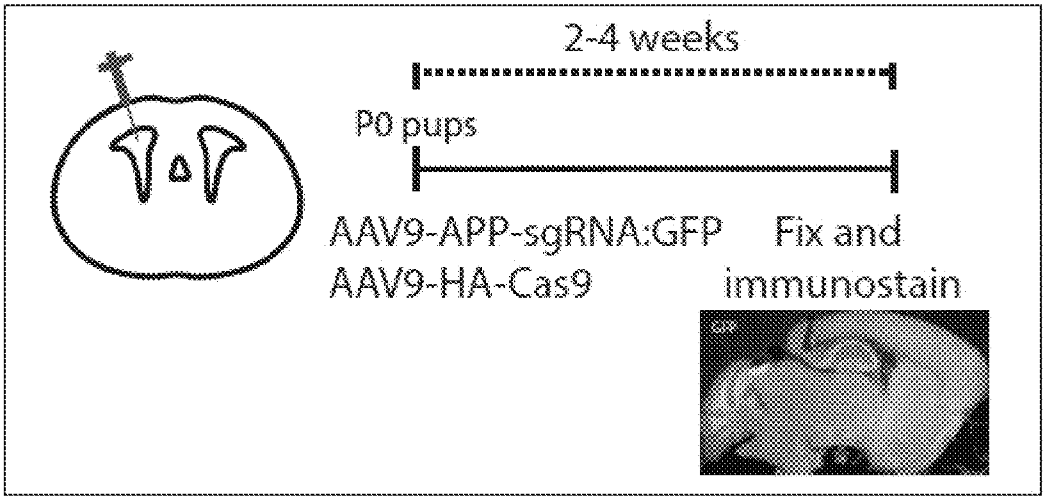


FIGS. 4A-4G CONTINUED

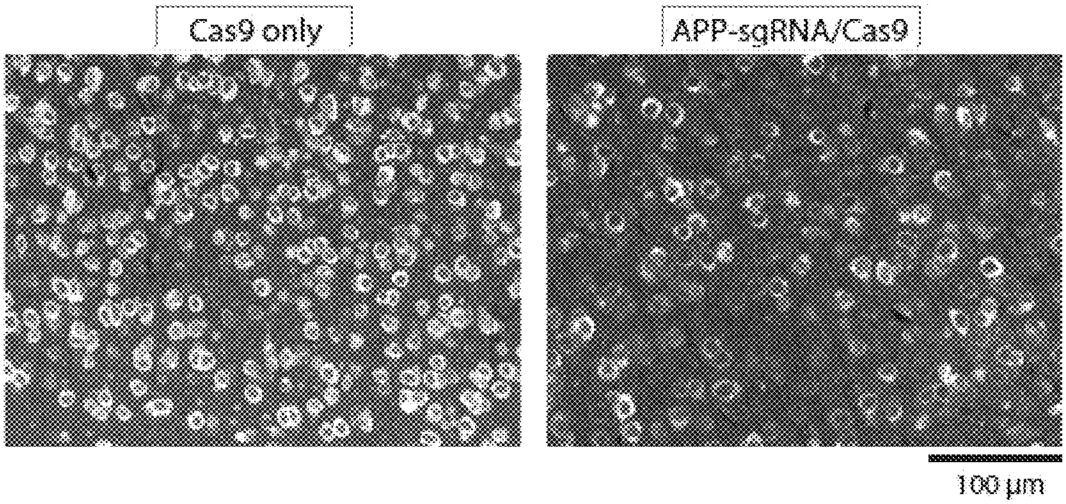


FIGS. 4A-4G CONTINUED

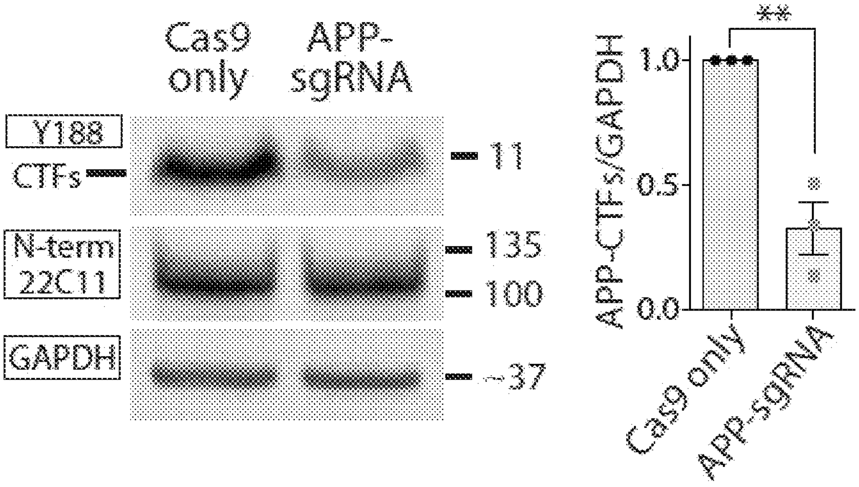
e. ICV AAV9 injections (schematic)



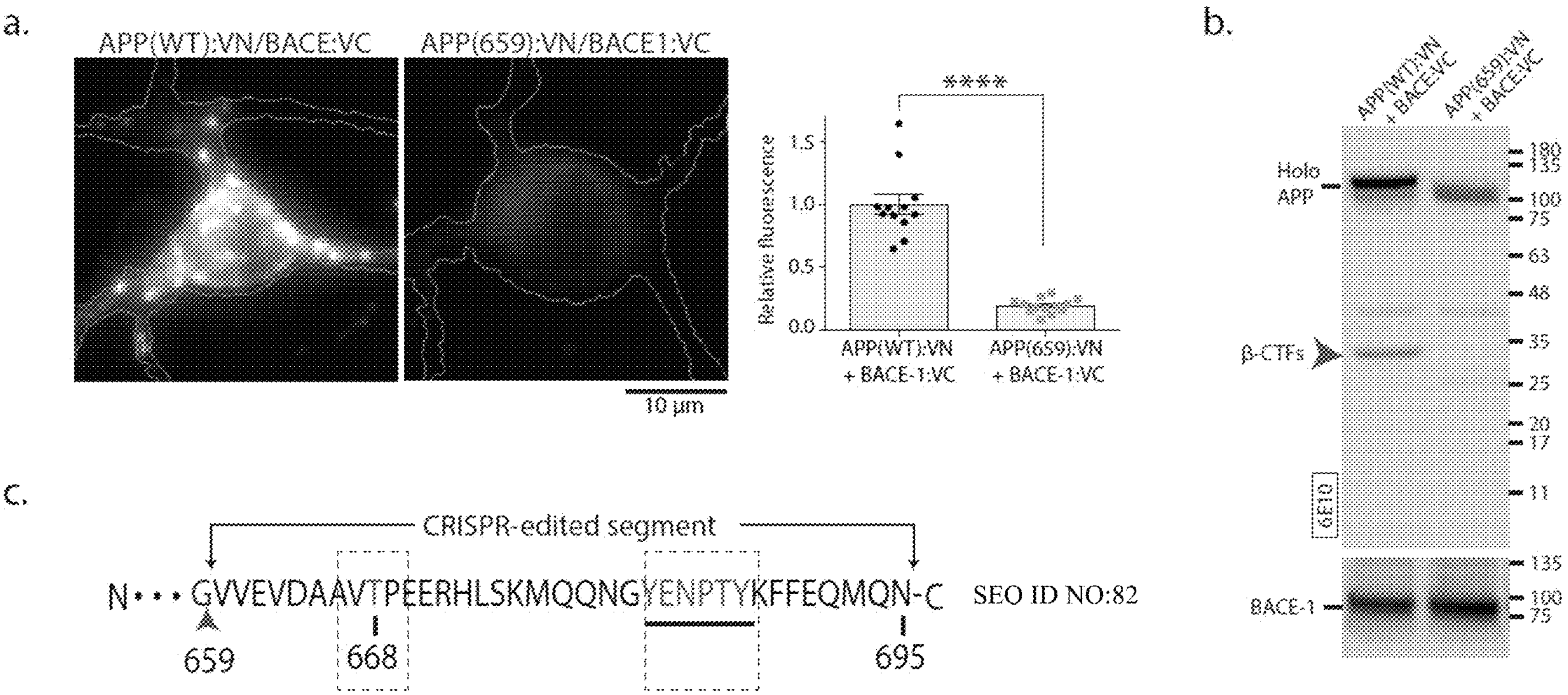
f. APP (Y188) staining



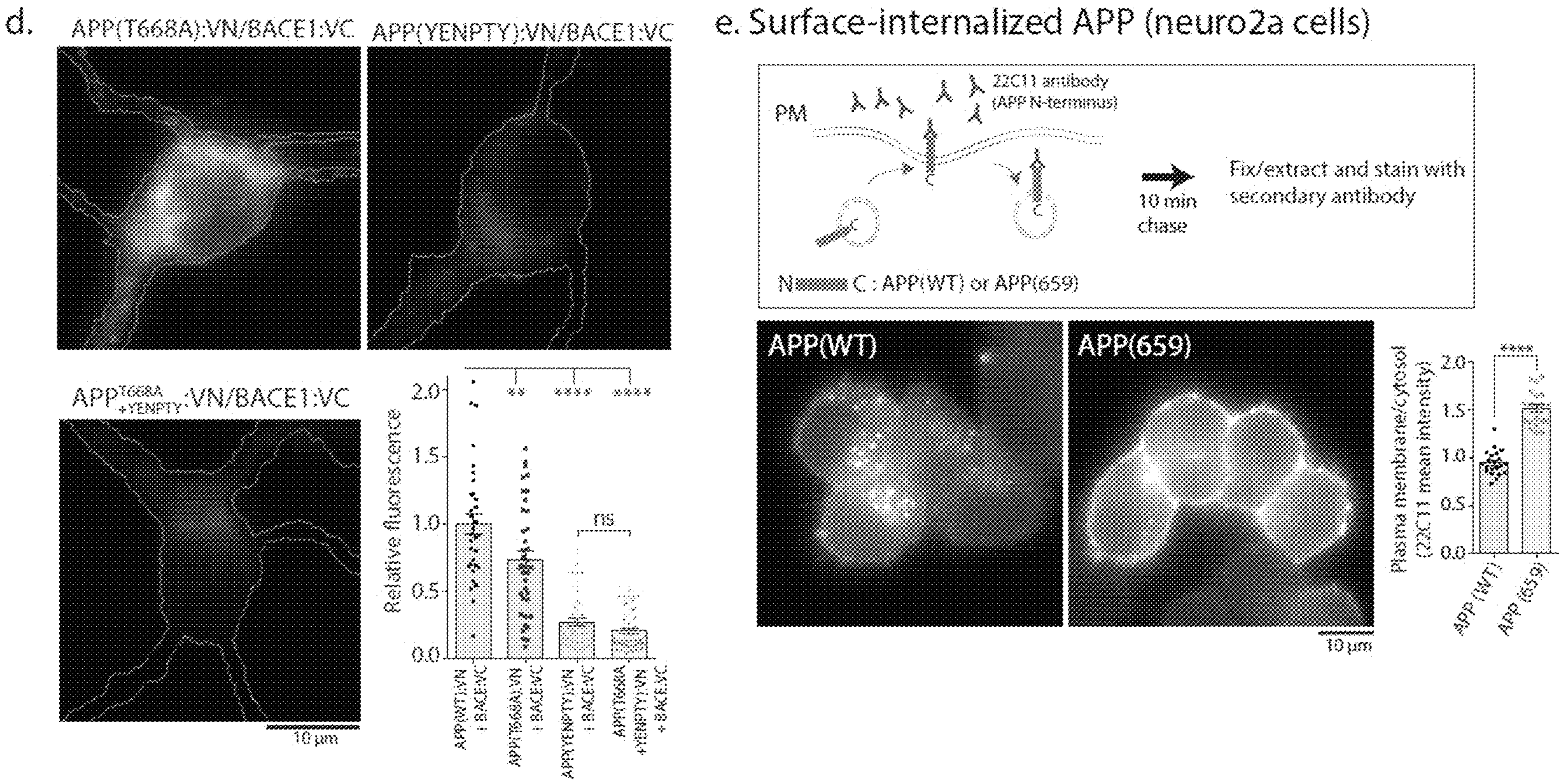
g.



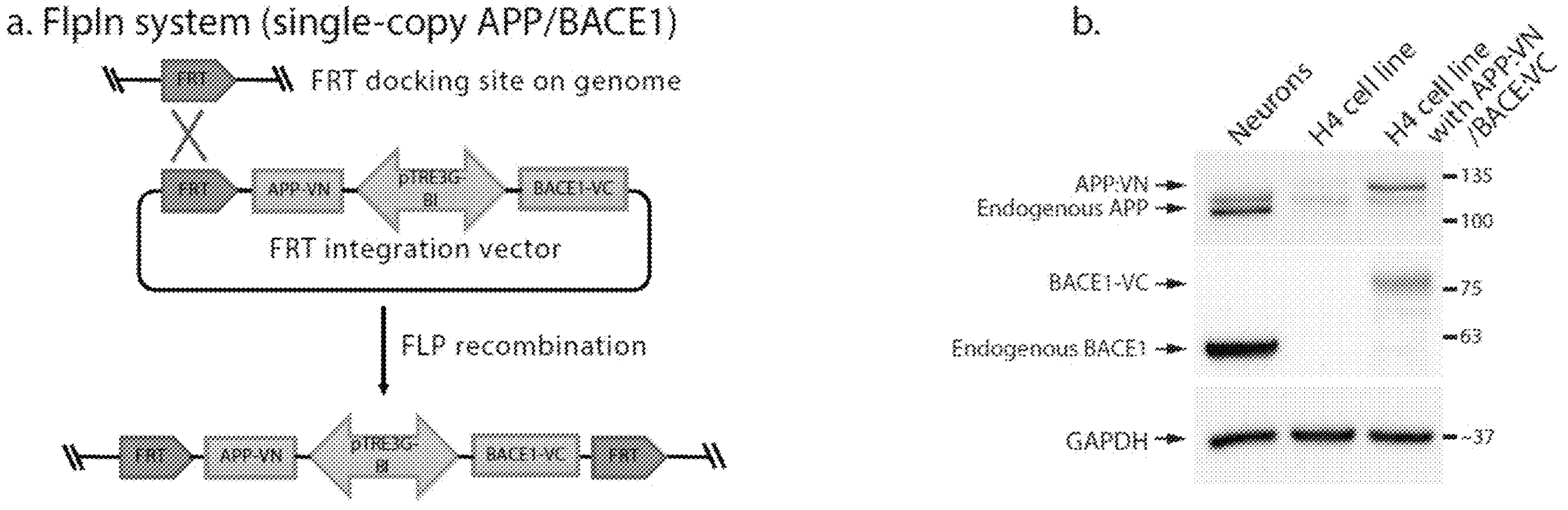
FIGS. 5A-5E


a.
APP(WT):VN/BACE:VC
APP(659):VN/BACE1:VC
10 µm
****
Relative fluorescence
1.5
1.0
0.5
0.0
APP(WT):VN + BACE-1:VC
APP(659):VN + BACE-1:VC
b.
APP(WT):VN + BACE:VC
APP(659):VN + BACE:VC
Holo APP
β-CTFs
6E10
BACE-1
180
135
100
75
63
48
35
25
20
17
11
135
100
75
c.
CRISPR-edited segment
N • • • GVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN-C
659
668
695
SEQ ID NO:82

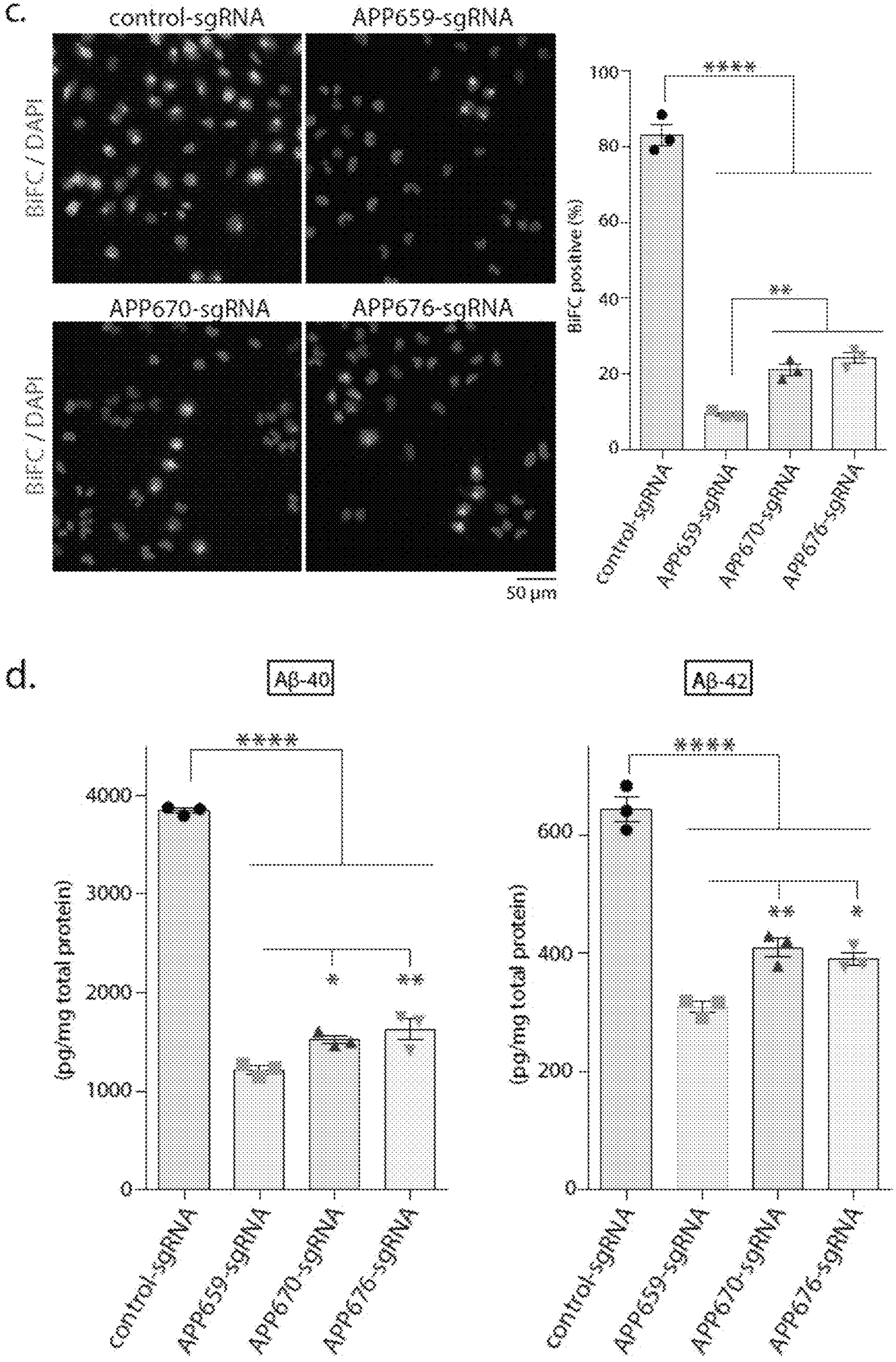
FIGS. 5A-5E CONTINUED



FIGS. 6A-6E


a. FlpIn system (single-copy APP/BACE1)
FRT
FRT docking site on genome
FRT
APP-VN
pTRE3G-BI
BACE1-VC
FRT integration vector
FLP recombination
FRT
APP-VN
pTRE3G-BI
BACE1-VC
FRT
b.
Neurons
H4 cell line
H4 cell line with APP:VN /BACE:VC
APP:VN
Endogenous APP
BACE1-VC
Endogenous BACE1
GAPDH
135
100
75
63
~37

FIGS. 6A-6E CONTINUED



FIGS. 6A-6E CONTINUED

e. Left

HEK APP659-sgRNA

Human APP genomic sequence

WT	AACAGTACACATCCATTCATCATGGTGTGGTGGAGGTAGGTAAAC	Freq (%)	SEO ID NO:83
C1	AACAGTACACATCCATTCATCATGGTG-----GAGGTAGGTAAAC	42.9	SEO ID NO:84
C2	AACAGTACACATCCATTCATCATGGTG--GTGGAGGTAGGTAAAC	10.2	SEO ID NO:85
C3	AACAGTACACATCCATTCATCATGGTG-GGTGGAGGTAGGTAAAC	3.6	SEO ID NO:86
C4	AACAGTACACATCCATTCATCAT----TGGTGGAGGTAGGTAAAC	2.3	SEO ID NO:87
C5	AACAGTACACATCCATTCATCATG---TGGTGGAGGTAGGTAAAC	1.9	SEO ID NO:88

Mutated locus

HEK APP670-sgRNA

Human APP genomic sequence

WT	TGCTGCATCTTGGACAGGTGGCGCTCCTCTGGGGTGACAGCGGCG	Freq (%)	SEO ID NO:89
C1	TGCTGCATCTTGGACAGGTGGCGCTCCNTCTGGGGTGACAGCGGCG	17.8	SEO ID NO:90
C2	TGCTGCATCTTGGACAGGTGGCGCT--TCTGGGGTGACAGCGGCG	12.5	SEO ID NO:91
C3	TGCTGCATCTTGGACAGGTGGC-----TCTGGGGTGACAGCGGCG	8.4	SEO ID NO:92
C4	TGCTGCATCTTGGACAGGTGGCGC---TCTGGGGTGACAGCGGCG	8.1	SEO ID NO:93
C5	TGCTGCATCTTGGACAGGTGGCGCTC-TCTGGGGTGACAGCGGCG	5.7	SEO ID NO:94

Mutated locus

HEK APP676-sgRNA

Human APP genomic sequence

WT	TTGGATTTTCGTAGCCGTTCTGCTGCATCTTGGACAGGTGGCGCT	Freq (%)	SEO ID NO:95
C1	TTGGATTTTCGTAGCCGTTCTGCTGCANTCTTGGACAGGTGGCGCT	24.1	SEO ID NO:96
C2	TTGGATTTTCGTAGCCGTTCT---------TGGACAGGTGGCGCT	19.2	SEO ID NO:97
C3	TTGGATTTTCGTAGCCGTTCTGCTGC-TCTTGGACAGGTGGCGCT	8.8	SEO ID NO:98
C4	TTGGATTTTCGTAGCCGTTCTGCT---TCTTGGACAGGTGGCGCT	8.4	SEO ID NO:99
C5	TTGGATTTTCGTAGCCGTTCT------TCTTGGACAGGTGGCGCT	4.4	SEQ ID NO:100

Mutated locus

FIGS. 6A-6E CONTINUED

e. CONTINUED Right

Human APP translational products

WT	GGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQ...(stop)	Freq (%)	SEQ ID NO:101
C1	GGVVIATVIVITLVMLKKKQYTSIHHGSS (stop)	42.9	SEQ ID NO:102
C2	GGVVIATVIVITLVMLKKKQYTSIHHGPPP (stop)	10.2	SEQ ID NO:103
C3	GGVVIATVIVITLVMLKKKQYTSIHHG...60 missense aa (stop)	3.6	SEQ ID NO:104
C4	GGVVIATVIVITLVMLKKKQYTSIHH-...60 missense aa (stop)	2.3	SEQ ID NO:105
C5	GGVVIATVIVITLVMLKKKQYTSIHH-VVEVDAAVTPEERHLSKMQ...(stop)	1.9	SEQ ID NO:106

659

Human APP translational products

WT	KKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN(stop)	Freq (%)	SEQ ID NO:107
C1	KKQYTSIHHGVVEVDAAVTPEEAPPVQDAAEELKKKSNLQVL(stop)	17.8	SEQ ID NO:108
C2	KKQYTSIHHGVVEVDAAVTPEAPPVQDAAEELEKKSNLQVL(stop)	12.5	SEQ ID NO:109
C3	KKQYTSIHHGVVEVDAAVTPEPPVQDAAEELEKKSNLQVL(stop)	8.4	SEQ ID NO:110
C4	KKQYTSIHHGVVEVDAAVTPE-RHLSKMQQNGYENPTYKFFEQMQN(stop)	8.1	SEQ ID NO:111
C5	KKQYTSIHHGVVEVDAAVTP...30 missense aa (stop)	5.7	SEQ ID NO:112

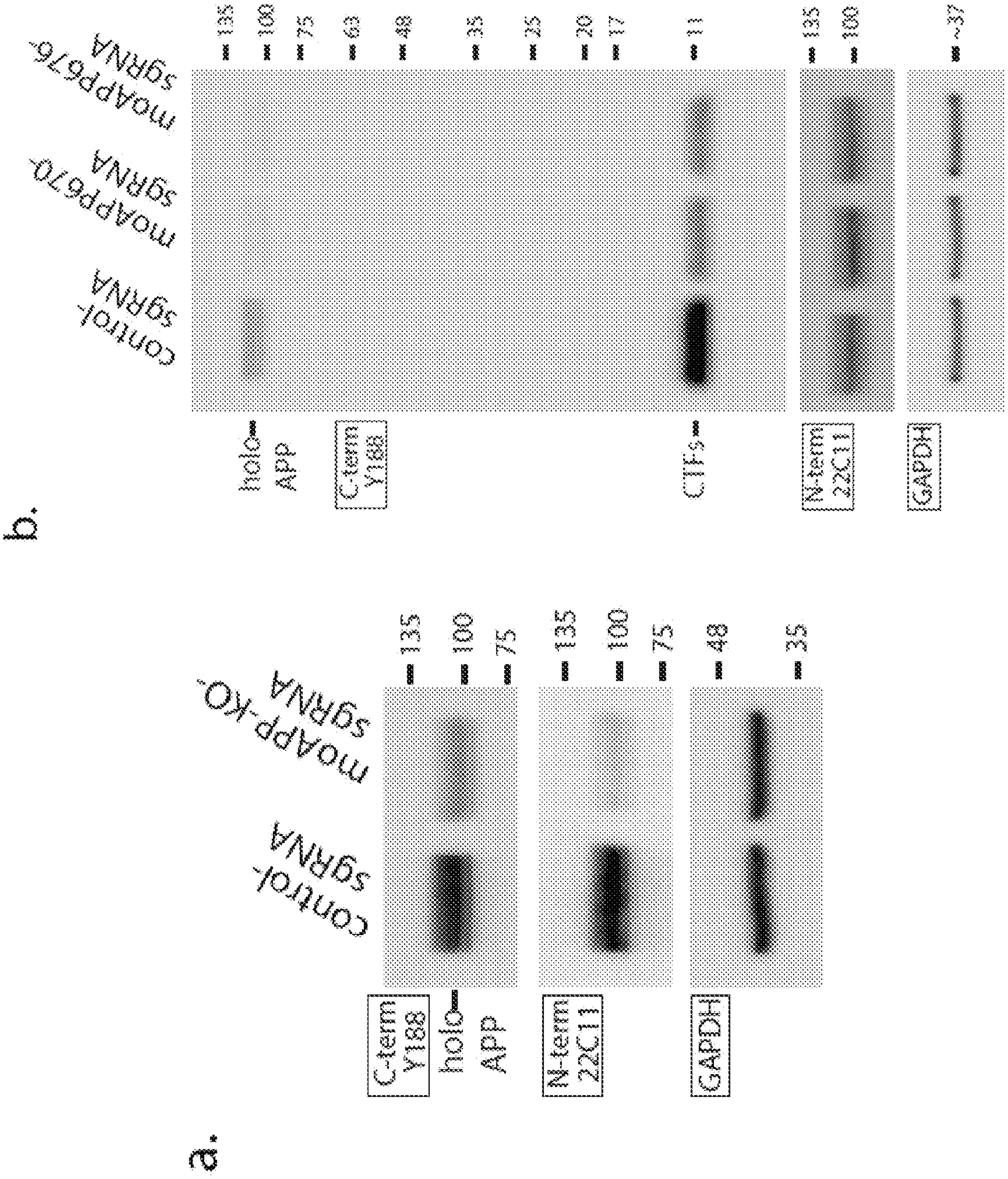
670

Human APP translational products

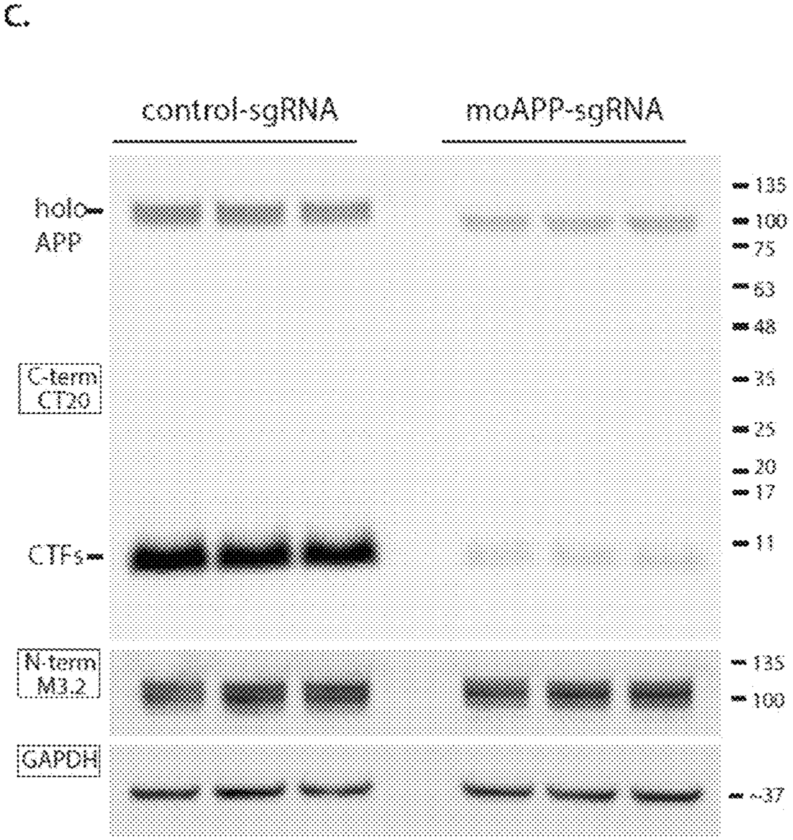
WT	KKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN(stop)	Freq (%)	SEQ ID NO:107
C1	KKQYTSIHHGVVEVDAAVTPEERHLSK...14 missense aa(stop)	24.1	SEQ ID NO:113
C2	KKQYTSIHHGVVEVDAAVTPEERHLSK----NGYENPTYKFFEQMQN(stop)	19.2	SEQ ID NO:114
C3	KKQYTSIHHGVVEVDAAVTPEERHLSK...43 missense aa(stop)	8.8	SEQ ID NO:115
C4	KKQYTSIHHGVVEVDAAVTPEERHLSKM-QNGYENPTYKFFEQMQN(stop)	8.4	SEQ ID NO:116
C5	KKQYTSIHHGVVEVDAAVTPEERHLSKM--NGYENPTYKFFEQMQN(stop)	4.4	SEQ ID NO:117

676

FIGS. 7A-7D


a.
control-sgRNA
moAPP-KO-sgRNA
C-term Y188
holo APP
N-term 22C11
GAPDH
135
100
75
135
100
75
48
35
b.
control-sgRNA
moAPP670-sgRNA
moAPP676-sgRNA
holo APP
C-term Y188
CTFs
N-term 22C11
GAPDH
135
100
75
63
48
35
25
20
17
11
135
100
~37

FIGS. 7A-7D CONTINUED



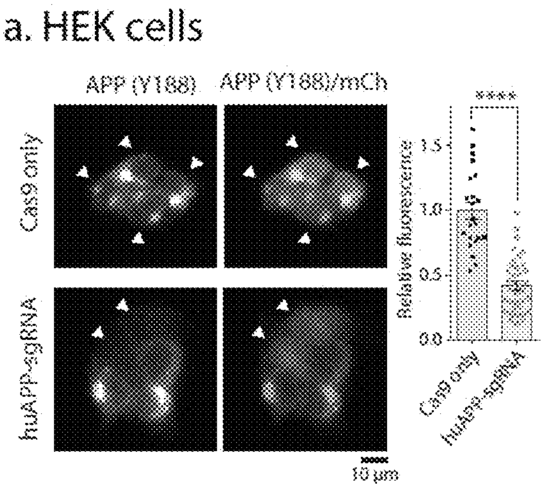
d. Mouse translational products (post-editing)

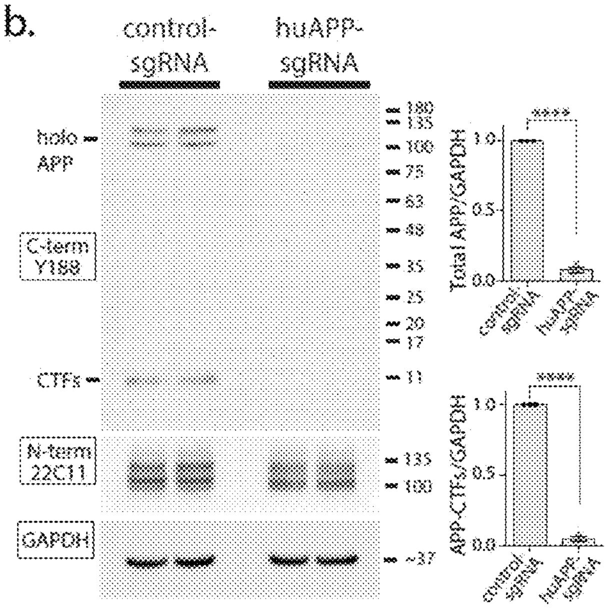
Mouse APP translation (neuro2a)

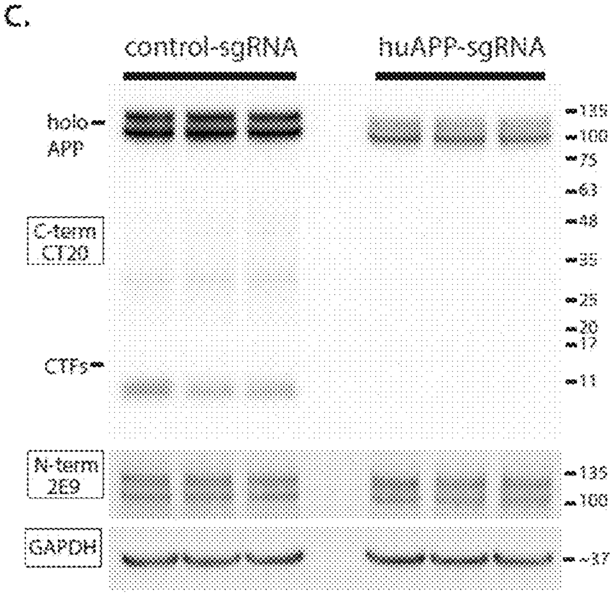
WT	GGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQ...	(stop)	Freq (%)	SEQ ID NO:118
C1	GGVVIATVIVITLVMLKKKQYTSIHHGHKKKRRDPRGAPSLQDAAEKI	(stop)	22.4	SEQ ID NO:119
C2	GGVVIATVIVITLVMLKKKQYTSIHHGKRRKKRDPRGAPSLQDAAERI	(stop)	6.8	SEQ ID NO:119
C3	GGVVIATVIVITLVMLKKKQYTSIHHGHKKKRDPRGAPSLQDAAEKI	(stop)	5.9	SEQ ID NO:120
C4	GGVVIATVIVITLVMLKKKQYTSIHHGKGKRRRDPRGAPSLQDAAEKI	(stop)	3.8	SEQ ID NO:121
C5	GGVVIATVIVITLVMLKKKQYTSIHHGGGKKRRRDPRGAPSLQDAAEKI	(stop)	3.4	SEQ ID NO:121

659

FIGS. 8A-8G

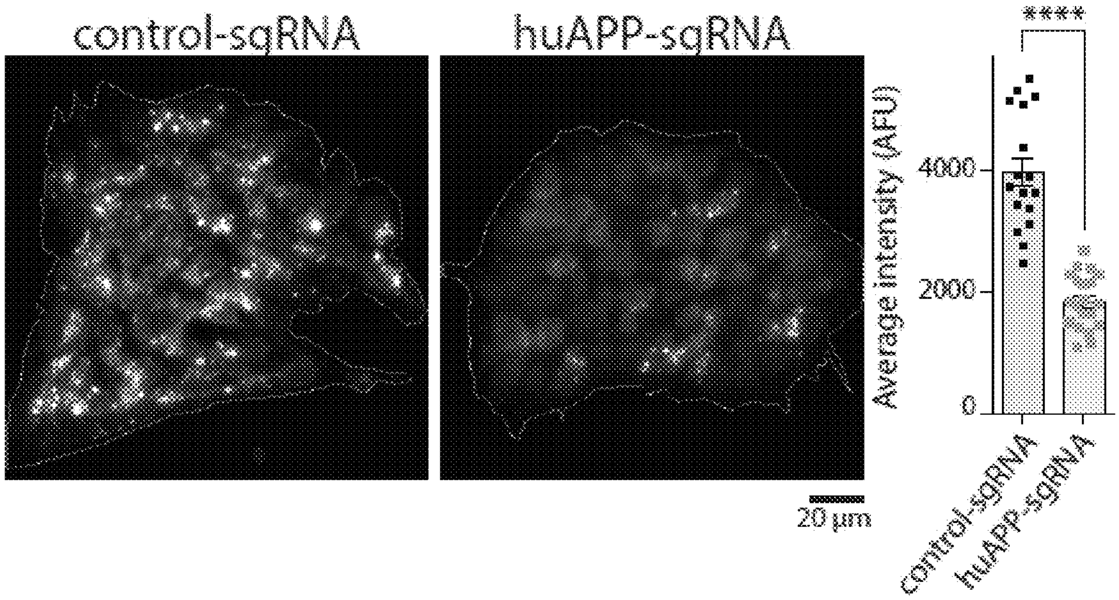

a. HEK cells
APP (Y188)
APP (Y188)/mCh
Cas9 only
huAPP-sgRNA
10 μm
****
Relative fluorescence
1.5
1.0
0.5
0.0
Cas9 only
huAPP-sgRNA


b.
control-sgRNA
huAPP-sgRNA
holo APP
C-term Y188
CTFs
N-term 22C11
GAPDH
180
135
100
75
63
48
35
25
20
17
11
135
100
~37
****
Total APP/GAPDH
1.0
0.5
0.0
control-sgRNA
huAPP-sgRNA
****
APP-CTFs/GAPDH
1.0
0.5
0.0
control-sgRNA
huAPP-sgRNA

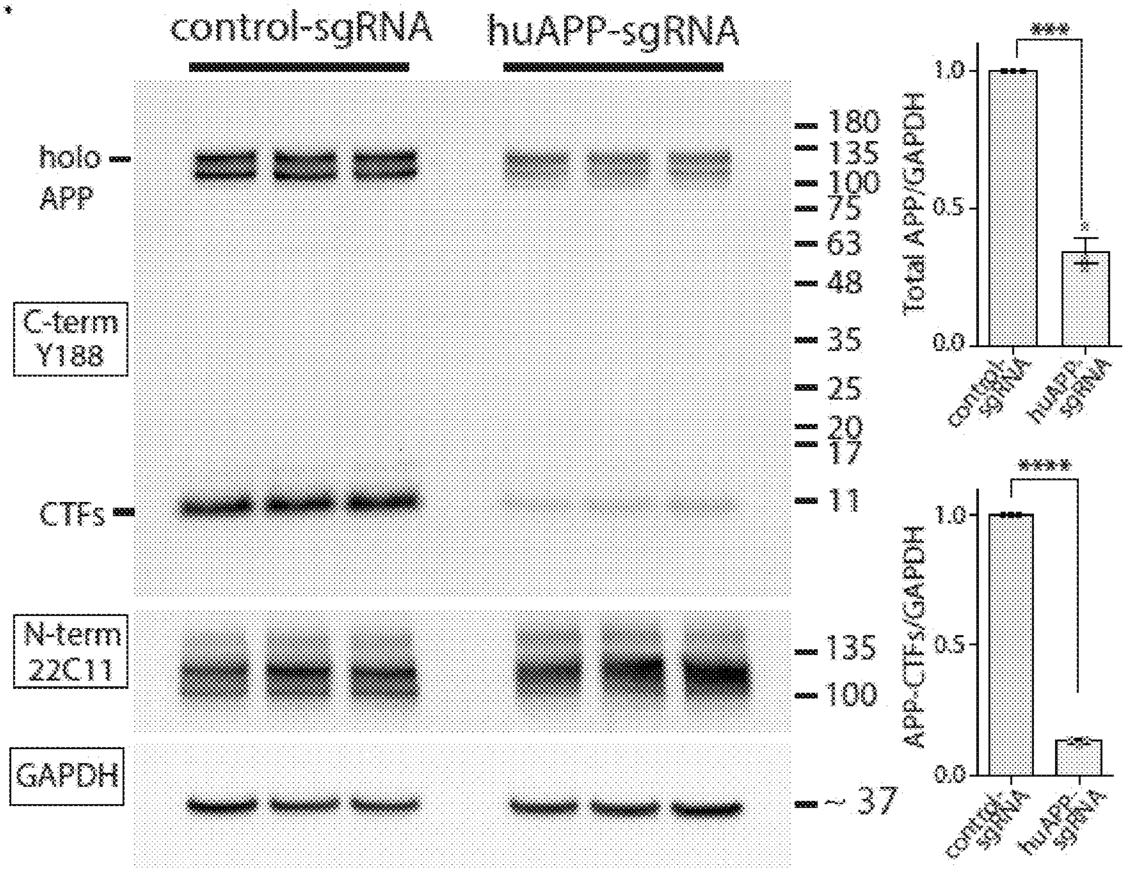

c.
control-sgRNA
huAPP-sgRNA
holo APP
C-term CT20
CTFs
N-term 2E9
GAPDH
135
100
75
63
48
35
25
20
17
11
135
100
~37

FIGS. 8A-8G CONTINUED

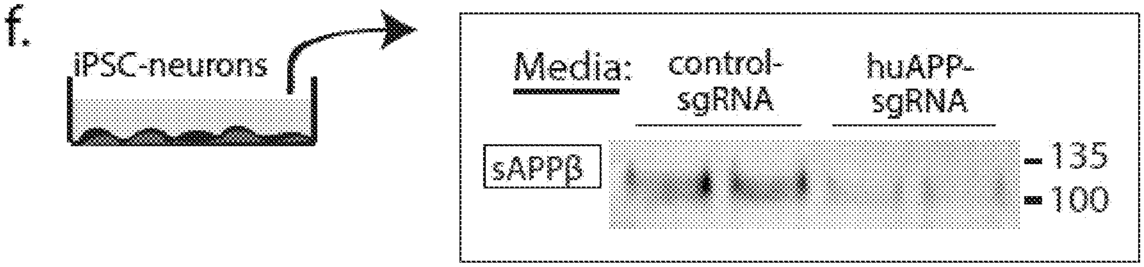
d. Human embryonic stem cells

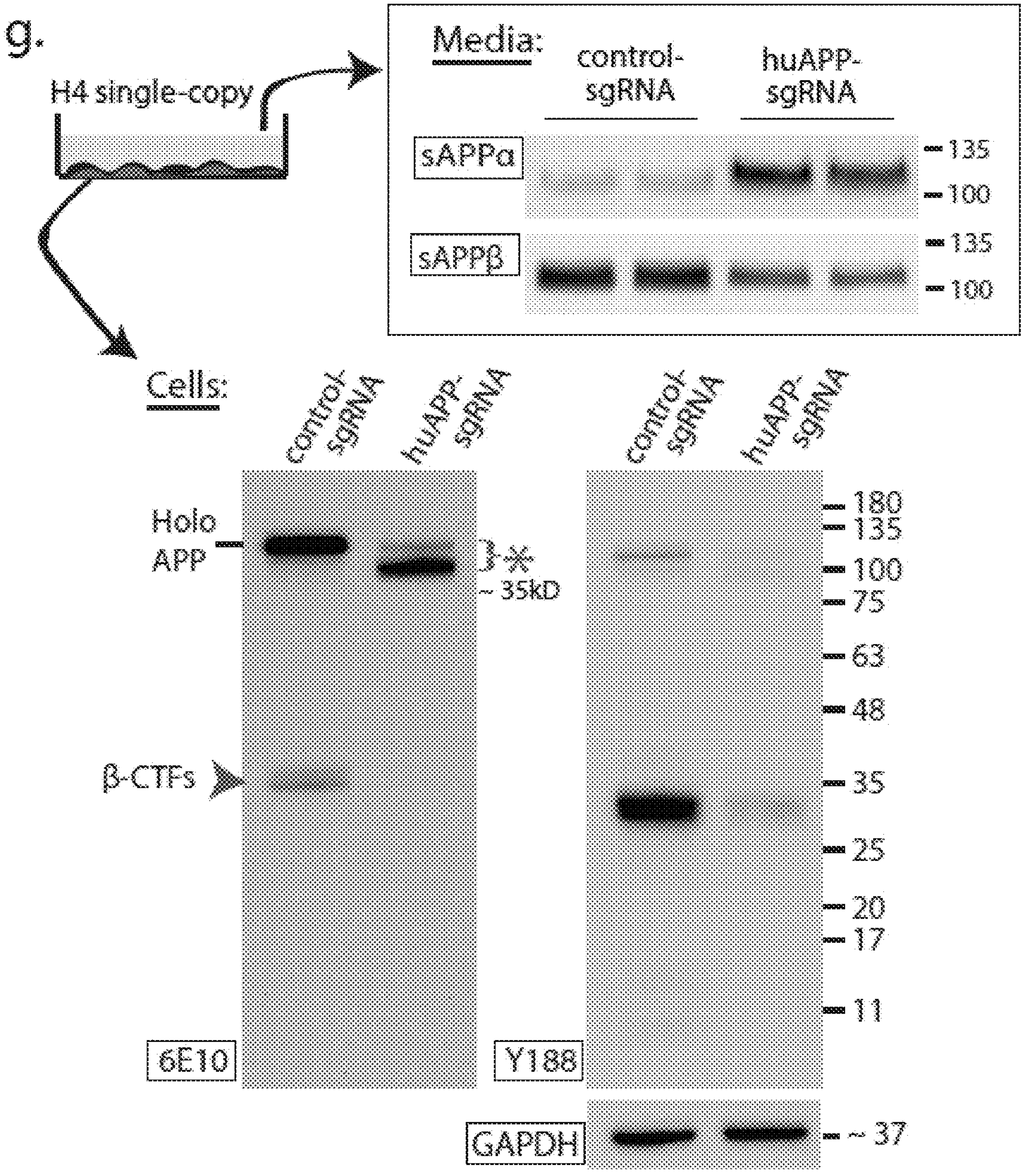


e.

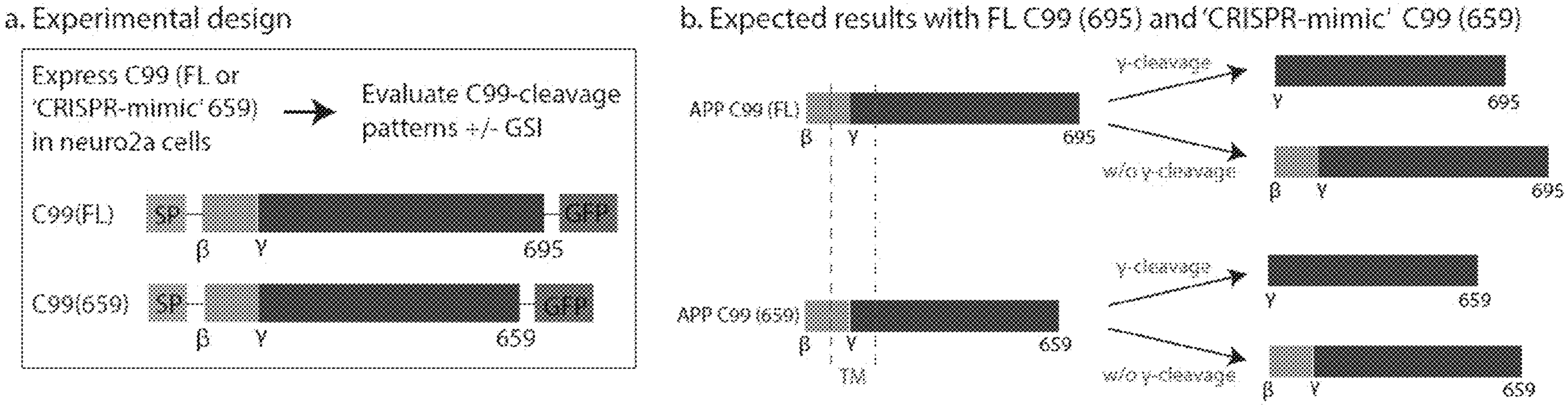


FIGS. 8A-8G CONTINUED



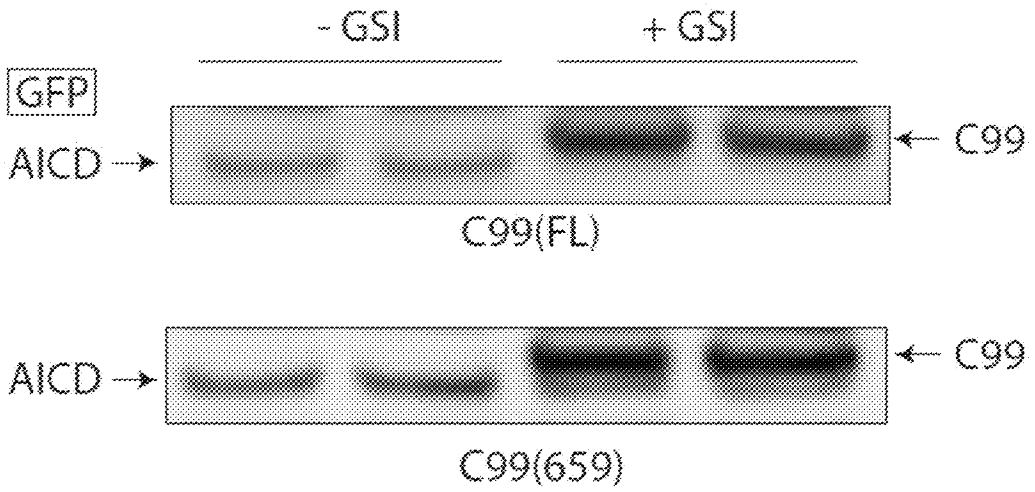


FIGS. 9A-9C


a. Experimental design

b. Expected results with FL C99 (695) and 'CRISPR-mimic' C99 (659)

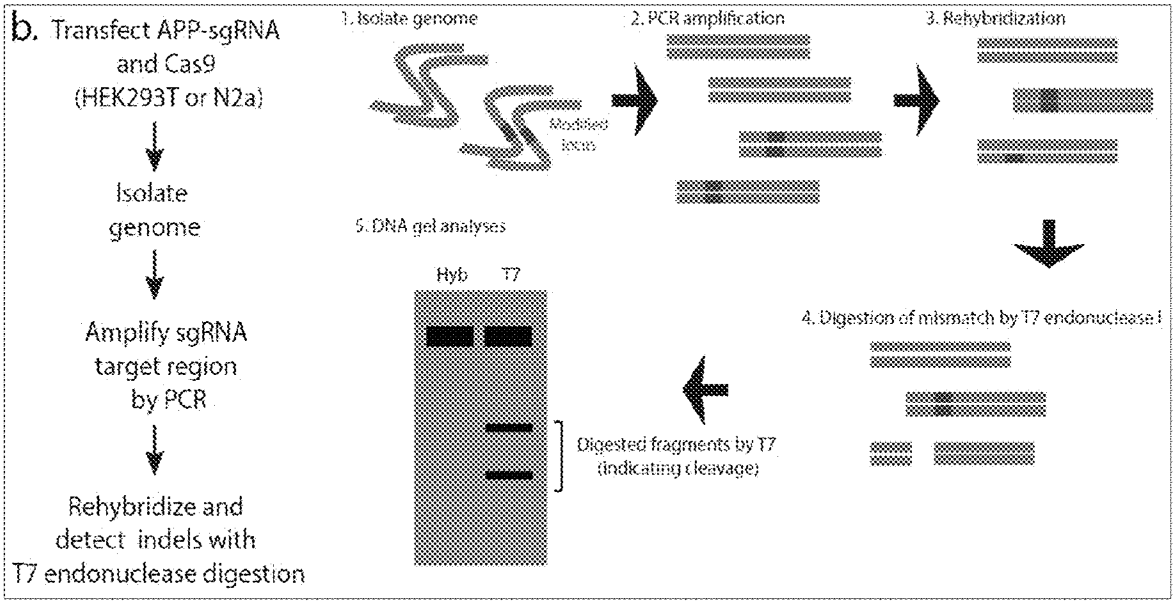
c. Identical g-cleavage pattern of FL and 'CRISPR-mimic' C99



FIGS. 10A-10G

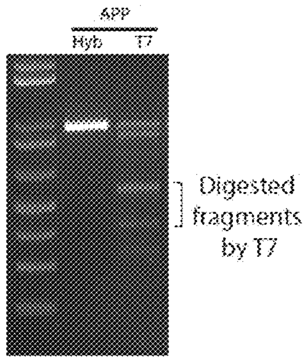
a. Off-target sites

		Sequence	Location	Coding region	
Mouse	OT1	GTCCATCCATCATGGCCTGG	chr18:+82500985	No	SEQ ID NO:122
	OT2	TTCCATCCATCATGGCTTGG	chr15:+83079515	No	SEQ ID NO:123
	OT3	GTCCCTCCATCATGGCCTGG	chr8:+54570804	No	SEQ ID NO:124
	OT4	GGCCATCATTCATGGCGTGG	chr12:-30225859	No	SEQ ID NO:125
	OT5	ATAAATATATCATGGCGTGG	chr2:+85075854	No	SEQ ID NO:126
Human	OT1	CTCCCTTCATCTTGGTGTGG	chr17:+42059975	No	SEQ ID NO:127
	OT2	ATGCAATCAGCATGGTGTGG	chr11:+45935195	Yes, PHF21A	SEQ ID NO:128
	OT3	TTCTGTTCAGCATGGTGTGG	chr19:+13830088	No	SEQ ID NO:129
	OT4	TTAAATTCAACATGGTGTGG	chrX:+78686020	No	SEQ ID NO:130
	OT5	AGCCATTTATCAAGGTGTGG	chr8:+61517706	No	SEQ ID NO:131

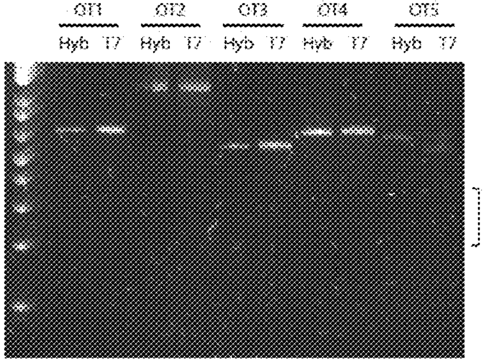


FIGS. 10A-10G CONTINUED

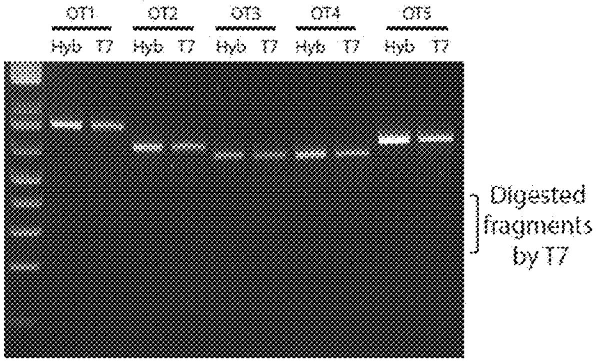
c. huAPP-sgRNA



d. moAPP-sgRNA



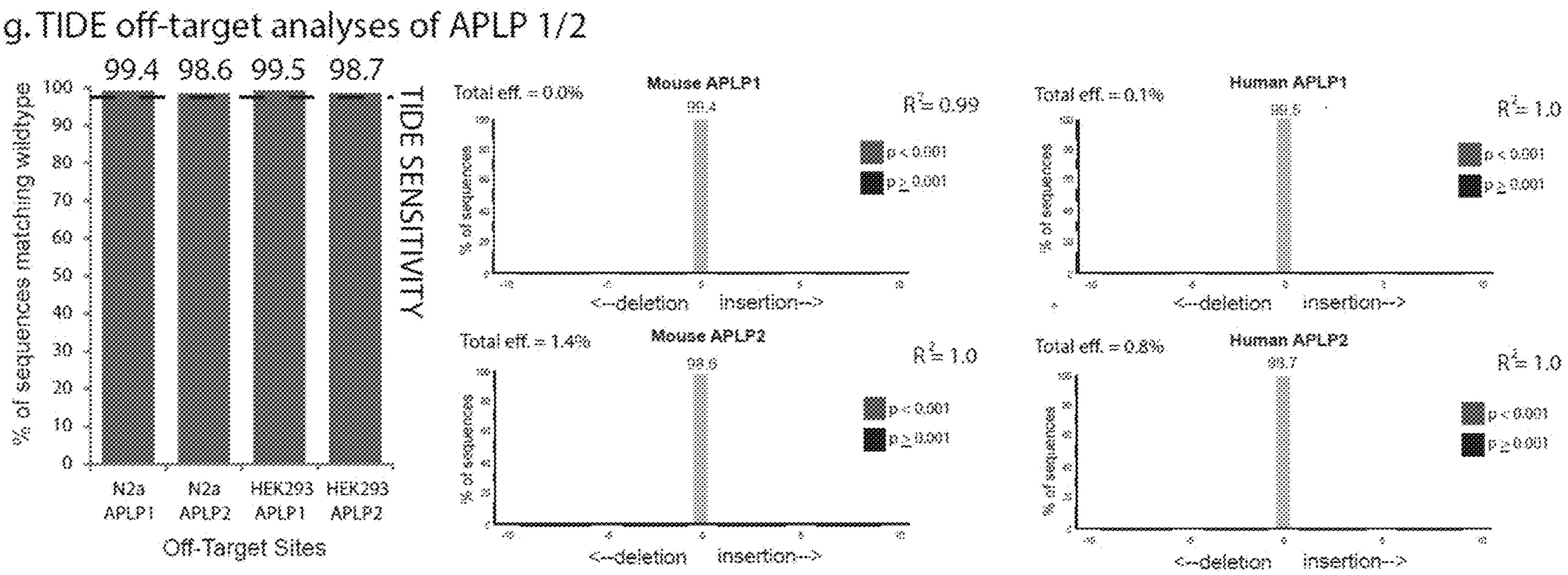
e. huAPP-sgRNA



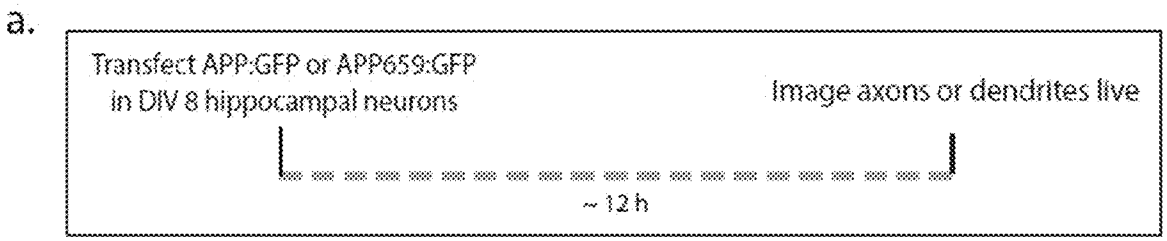
f. PAM/targeting sequence allignment of APP and APLP 1/2

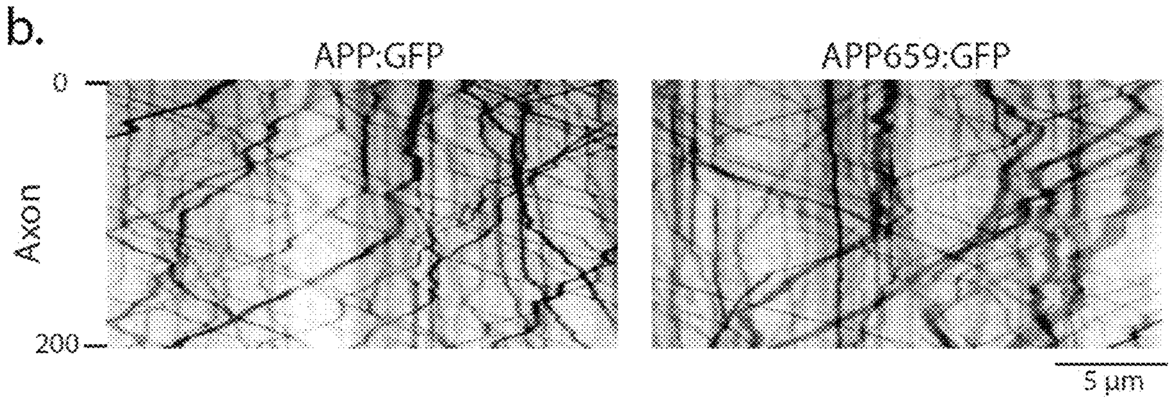
```
SEQ ID NO:132 Mouse  APP     atccatccatcatggcgtggtgg     Mouse  APP    atccatccatcatggcgtggtgg SEQ ID NO:134
SEQ ID NO:133 Mouse  APLP1   gactatcagccatggagtggtgg     Mouse  APLP2  caccatcagccacgggattgtgg SEQ ID NO:135
                               * ***   ***** ****                        *****   ** **  * *

SEQ ID NO:136 Human  APP     atccattcatcatggtgtggtgg     Human  APP    atccattcatcatggtgtggtgg SEQ ID NO:138
SEQ ID NO:137 Human  APLP1   ggctatcagccatggcgtggtgg     Human  APLP2  caccatcagccacgggatcgtgg SEQ ID NO:139
                               * **    ***** ****                        ****    ** **  * *
```

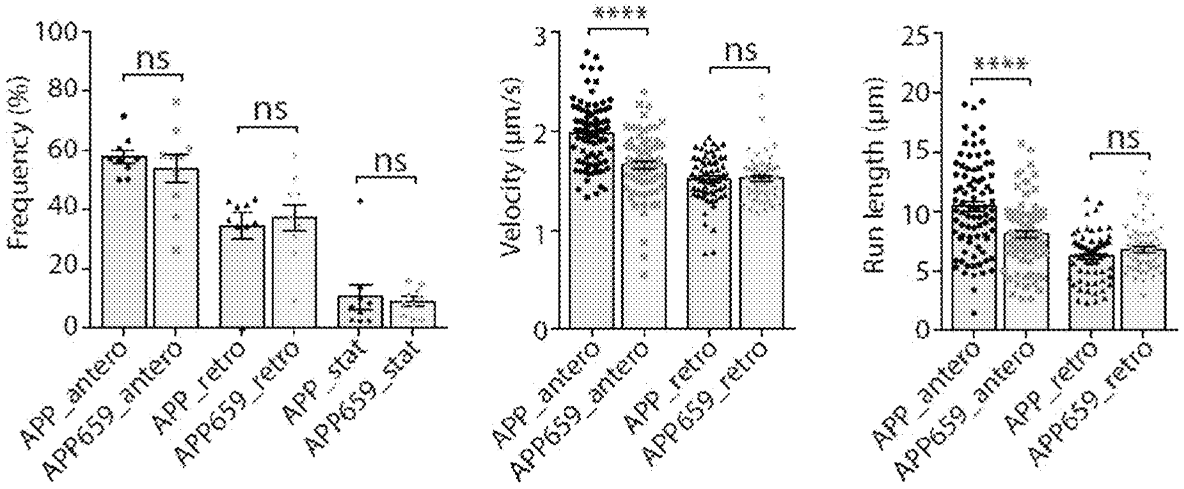
FIGS. 10A-10G CONTINUED



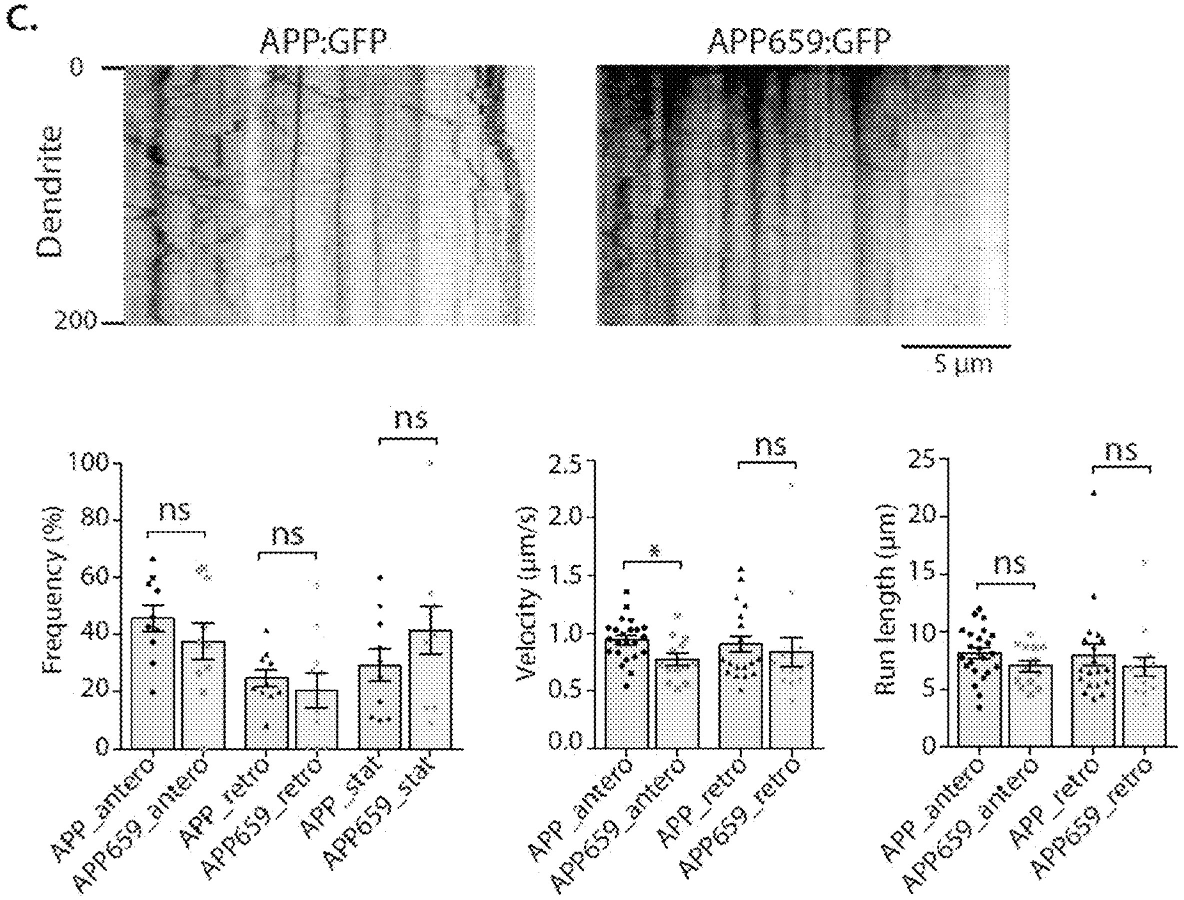
FIGS. 11A-11C


a.
Transfect APP:GFP or APP659:GFP
in DIV 8 hippocampal neurons
Image axons or dendrites live
~ 12 h


b.
APP:GFP
APP659:GFP
0
Axon
200
5 µm


Frequency (%)
100
80
60
40
20
0
ns
ns
ns
APP_antero
APP659_antero
APP_retro
APP659_retro
APP_stat
APP659_stat
Velocity (µm/s)
3
2
1
0
****
ns
APP_antero
APP659_antero
APP_retro
APP659_retro
Run length (µm)
25
20
15
10
5
0
****
ns
APP_antero
APP659_antero
APP_retro
APP659_retro

FIGS. 11A-11C CONTINUED



FIGS. 12A-12C

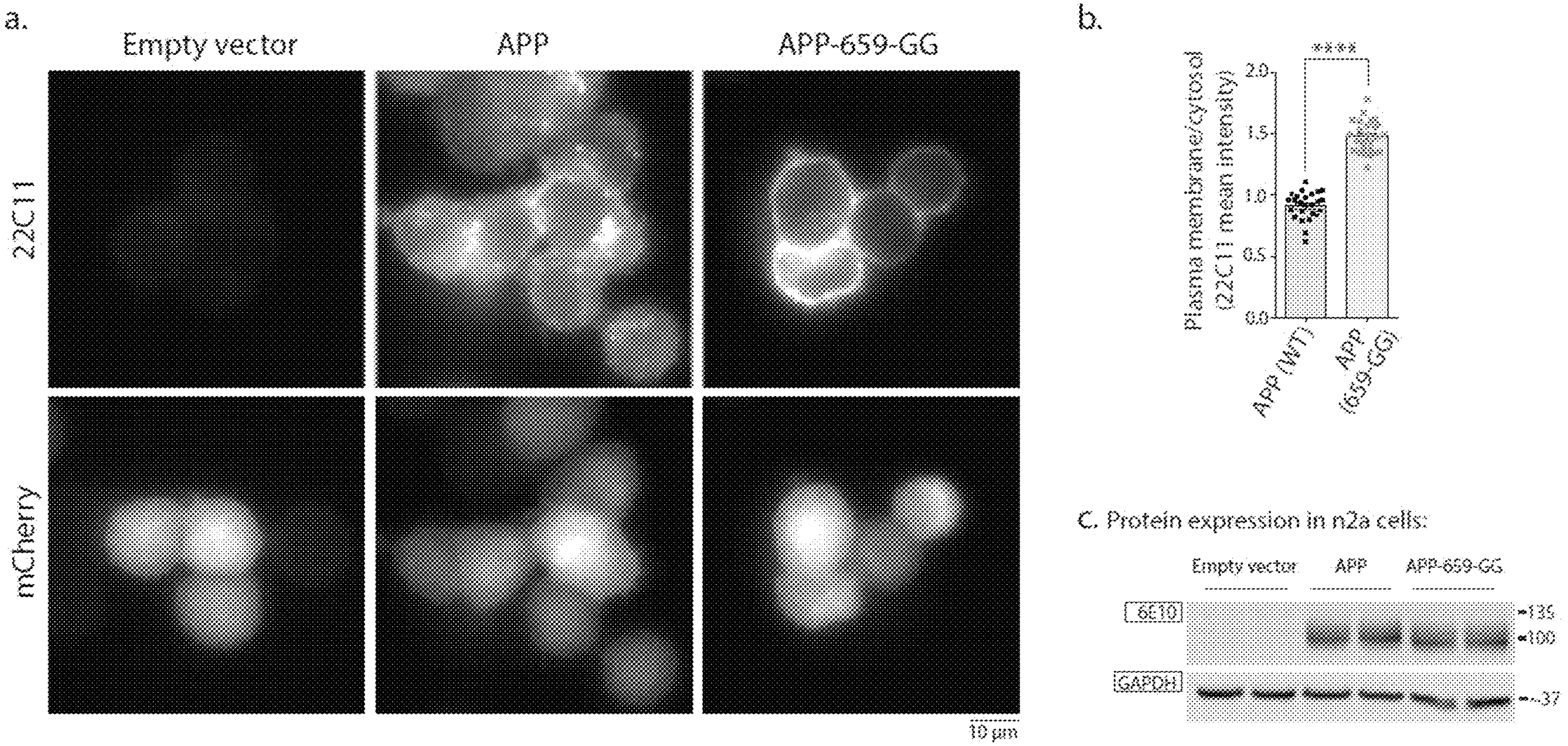

a.
Empty vector
APP
APP-659-GG
22C11
mCherry
10 μm
b.
Plasma membrane/cytosol (22C11 mean intensity)
2.0
1.5
1.0
0.5
0.0
****
APP (WT)
APP (659-GG)
C. Protein expression in n2a cells:
Empty vector
APP
APP-659-GG
6E10
GAPDH
135
100
~37

FIG. 13

>human APP (nucleotide sequence SEQ ID NO:11, amino acid sequence SEQ ID NO:12)

```
atgctgcccggtttggcactgctcctgctggccgcctggacggctcgggcgctggaggta
 M  L  P  G  L  A  L  L  L  L  A  A  W  T  A  R  A  L  E  V
cccactgatggtaatgctggcctgctggctgaaccccagattgccatgttctgtggcaga
 P  T  D  G  N  A  G  L  L  A  E  P  Q  I  A  M  F  C  G  R
ctgaacatgcacatgaatgtccagaatgggaagtgggattcagatccatcagggaccaaa
 L  N  M  H  M  N  V  Q  N  G  K  W  D  S  D  P  S  G  T  K
acctgcattgataccaaggaaggcatcctgcagtattgccaagaagtctaccctgaactg
 T  C  I  D  T  K  E  G  I  L  Q  Y  C  Q  E  V  Y  P  E  L
cagatcaccaatgtggtagaagccaaccaaccagtgaccatccagaactggtgcaagcgg
 Q  I  T  N  V  V  E  A  N  Q  P  V  T  I  Q  N  W  C  K  R
ggccgcaagcagtgcaagacccatccccactttgtgattccctaccgctgcttagttggt
 G  R  K  Q  C  K  T  H  P  H  F  V  I  P  Y  R  C  L  V  G
gagtttgtaagtgatgcccttctcgttcctgacaagtgcaaattcttacaccaggagagg
 E  F  V  S  D  A  L  L  V  P  D  K  C  K  F  L  H  Q  E  R
atggatgtttgcgaaactcatcttcactggcacaccgtcgccaaagagacatgcagtgag
 M  D  V  C  E  T  H  L  H  W  H  T  V  A  K  E  T  C  S  E
aagagtaccaacttgcatgactacggcatgttgctgccctgcggaattgacaagttccga
 K  S  T  N  L  H  D  Y  G  M  L  L  P  C  G  I  D  K  F  R
ggggtagagtttgtgtgttgcccactggctgaagaaagtgacaatgtggattctgctgat
 G  V  E  F  V  C  C  P  L  A  E  E  S  D  N  V  D  S  A  D
gcggaggaggatgactcggatgtctggtggggcggagcagacacagactatgcagatggg
 A  E  E  D  D  S  D  V  W  W  G  G  A  D  T  D  Y  A  D  G
agtgaagacaaagtagtagaagtagcagaggaggaagaagtggctgaggtggaagaagaa
 S  E  D  K  V  V  E  V  A  E  E  E  E  V  A  E  V  E  E  E
gaagccgatgatgacgaggacgatgaggatggtgatgaggtagaggaagaggctgaggaa
 E  A  D  D  D  E  D  D  E  D  G  D  E  V  E  E  E  A  E  E
ccctacgaagaagccacagagagaaccaccagcattgccaccaccaccaccaccaccaca
 P  Y  E  E  A  T  E  R  T  T  S  I  A  T  T  T  T  T  T  T
gagtctgtggaagaggtggttcgagttcctacaacagcagccagtacccctgatgccgtt
 E  S  V  E  E  V  V  R  V  P  T  T  A  A  S  T  P  D  A  V
gacaagtatctcgagacacctggggatgagaatgaacatgcccatttccagaaagccaaa
 D  K  Y  L  E  T  P  G  D  E  N  E  H  A  H  F  Q  K  A  K
gagaggcttgaggccaagcaccgagagagaatgtcccaggtcatgagagaatgggaagag
 E  R  L  E  A  K  H  R  E  R  M  S  Q  V  M  R  E  W  E  E
gcagaacgtcaagcaaagaacttgcctaaagctgataagaaggcagttatccagcatttc
 A  E  R  Q  A  K  N  L  P  K  A  D  K  K  A  V  I  Q  H  F
caggagaaagtggaatctttggaacaggaagcagccaacgagagacagcagctggtggag
 Q  E  K  V  E  S  L  E  Q  E  A  A  N  E  R  Q  Q  L  V  E
acacacatggccagagtggaagccatgctcaatgaccgccgccgcctggccctggagaac
 T  H  M  A  R  V  E  A  M  L  N  D  R  R  R  L  A  L  E  N
tacatcaccgctctgcaggctgttcctcctcggcctcgtcacgtgttcaatatgctaaag
 Y  I  T  A  L  Q  A  V  P  P  R  P  R  H  V  F  N  M  L  K
aagtatgtccgcgcagaacagaaggacagacagcacaccctaaagcatttcgagcatgtg
 K  Y  V  R  A  E  Q  K  D  R  Q  H  T  L  K  H  F  E  H  V
cgcatggtggatcccaagaaagccgctcagatccggtcccaggttatgacacacctccgt
 R  M  V  D  P  K  K  A  A  Q  I  R  S  Q  V  M  T  H  L  R
gtgatttatgagcgcatgaatcagtctctctccctgctctacaacgtgcctgcagtggcc
 V  I  Y  E  R  M  N  Q  S  L  S  L  L  Y  N  V  P  A  V  A
gaggagattcaggatgaagttgatgagctgcttcagaaagagcaaaactattcagatgac
 E  E  I  Q  D  E  V  D  E  L  L  Q  K  E  Q  N  Y  S  D  D
gtcttggccaacatgattagtgaaccaaggatcagttacggaaacgatgctctcatgcca
 V  L  A  N  M  I  S  E  P  R  I  S  Y  G  N  D  A  L  M  P
tctttgaccgaaacgaaaaccaccgtggagctccttcccgtgaatggagagttcagcctg
```

FIG. 13 CONTINUED

```
 S   L   T   E   T   K   T   T   V   E   L   L   P   V   N   G   E   F   S   L
gacgatctccagccgtggcattcttttggggctgactctgtgccagccaacacagaaaac
 D   D   L   Q   P   W   H   S   F   G   A   D   S   V   P   A   N   T   E   N
gaagttgagcctgttgatgcccgccctgctgccgaccgaggactgaccactcgaccaggt
 E   V   E   P   V   D   A   R   P   A   A   D   R   G   L   T   T   R   P   G
tctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatgcagaattc
 S   G   L   T   N   I   K   T   E   E   I   S   E   V   K   M   D   A   E   F
cgacatgactcaggatatgaagttcatcatcaaaaattggtgttctttgcagaagatgtg
 R   H   D   S   G   Y   E   V   H   H   Q   K   L   V   F   F   A   E   D   V
ggttcaaacaaaggtgcaatcattggactcatggtgggcggtgttgtcatagcgacagtg
 G   S   N   K   G   A   I   I   G   L   M   V   G   G   V   V   I   A   T   V
atcgtcatcaccttggtgatgctgaagaagaaacagtacacatccattcatcatggtgtg
 I   V   I   T   L   V   M   L   K   K   K   Q   Y   T   S   I   H   H   G   V
gtggaggttgacgccgctgtcaccccagaggagcgccacctgtcca[illegible]
 V   E   V   D   A   A   V   T   P   E   E   R   H   L   S   K   M   Q   Q   N
[illegible]gaaaatccaacctacaagttctttgagcagatgcagaactag
 G   Y   E   N   P   T   Y   K   F   F   E   Q   M   Q   N   -
```

Sense:
▨ 659 gRNA
Antisense:
▨ 669 gRNA
▨ 676 gRNA
▨ 686 gRNA
670 gRNA

>mouse APP (nucleotide sequence SEQ ID NO:13, amino acid sequence SEQ ID NO:14)

```
atgctgcccagcttggcactgctcctgctggccgcctggacggttcgggctctggaggta
 M   L   P   S   L   A   L   L   L   L   A   A   W   T   V   R   A   L   E   V
cccactgatggcaacgccgggctgctggcagaaccccagatcgccatgttctgtggtaaa
 P   T   D   G   N   A   G   L   L   A   E   P   Q   I   A   M   F   C   G   K
ctcaacatgcacatgaatgtgcagaatggaaagtgggagtcagacccgtcagggaccaaa
 L   N   M   H   M   N   V   Q   N   G   K   W   E   S   D   P   S   G   T   K
acctgcattggcaccaaggagggcatcttgcagtactgccaagaggtctaccctgaactg
 T   C   I   G   T   K   E   G   I   L   Q   Y   C   Q   E   V   Y   P   E   L
cagatcacaaacgtggtggaagccaaccagccagtgaccatccagaactggtgcaagcgg
 Q   I   T   N   V   V   E   A   N   Q   P   V   T   I   Q   N   W   C   K   R
ggccgcaagcagtgcaagacacacacccacatcgtgattccttaccgttgcctagttggt
 G   R   K   Q   C   K   T   H   T   H   I   V   I   P   Y   R   C   L   V   G
gagtttgtgagcgacgcccttctcgtgcccgacaagtgcaagttcctacaccaggagcgg
 E   F   V   S   D   A   L   L   V   P   D   K   C   K   F   L   H   Q   E   R
atggatgtttgtgagacccatcttcactggcacaccgtcgccaaagagacatgcagcgag
 M   D   V   C   E   T   H   L   H   W   H   T   V   A   K   E   T   C   S   E
aagagcactaacttgcacgactatggcatgctgctgccctgcggcatcgacaagttccga
 K   S   T   N   L   H   D   Y   G   M   L   L   P   C   G   I   D   K   F   R
ggggtagagtttgtatgctgcccgttggccgaggaaagcgacagcgtggattctgcggat
 G   V   E   F   V   C   C   P   L   A   E   E   S   D   S   V   D   S   A   D
gcagaggaggatgactctgatgtctggtggggtggagcggacacagactacgctgatggc
 A   E   E   D   D   S   D   V   W   W   G   G   A   D   T   D   Y   A   D   G
ggtgaagacaaagtagtagaagtcgccgaagaggaggaagtggctgatgttgaggaagag
 G   E   D   K   V   V   E   V   A   E   E   E   E   V   A   D   V   E   E   E
gaagctgatgatgatgaggatgtggaggatggggacgaggtggaggaggaggccgaggag
 E   A   D   D   D   E   D   V   E   D   G   D   E   V   E   E   E   A   E   E
```

FIG. 13 CONTINUED

```
ccctacgaagaggccaccgagagaacaaccagcactgccaccaccaccacaaccaccact
 P  Y  E  E  A  T  E  R  T  T  S  T  A  T  T  T  T  T  T  T
gagtccgtggaggaggtggtccgagttcccacgacagcagccagcacccccgacgccgtc
 E  S  V  E  E  V  V  R  V  P  T  T  A  A  S  T  P  D  A  V
gacaagtacctggagacacccggggacgagaacgagcatgcccatttccagaaagccaaa
 D  K  Y  L  E  T  P  G  D  E  N  E  H  A  H  F  Q  K  A  K
gagaggctggaagccaagcaccgagagagaatgtcccaggtcatgagagaatgggaagag
 E  R  L  E  A  K  H  R  E  R  M  S  Q  V  M  R  E  W  E  E
gcagagcgtcaagccaagaacttgcccaaagctgacaagaaggccgttatccagcatttc
 A  E  R  Q  A  K  N  L  P  K  A  D  K  K  A  V  I  Q  H  F
caggagaaagtggaatctctggaacaggaagcagccaatgagagacagcagcttgtagag
 Q  E  K  V  E  S  L  E  Q  E  A  A  N  E  R  Q  Q  L  V  E
acacacatggccagagttgaagccatgctcaatgaccgccgccgcctggccctcgagaat
 T  H  M  A  R  V  E  A  M  L  N  D  R  R  R  L  A  L  E  N
tacatcactgcactgcaggcggtgcccccaaggcctcatcatgtgttcaacatgctgaag
 Y  I  T  A  L  Q  A  V  P  P  R  P  H  H  V  F  N  M  L  K
aagtacgtccgtgcggagcagaaagacagacagcacaccctaaagcattttgaacatgtg
 K  Y  V  R  A  E  Q  K  D  R  Q  H  T  L  K  H  F  E  H  V
cgcatggtggaccccaagaaagctgctcagatccggtcccaggttatgacacacctccgt
 R  M  V  D  P  K  K  A  A  Q  I  R  S  Q  V  M  T  H  L  R
gtgatctacgagcgcatgaaccagtctctgtccctgctctacaatgtccctgcggtggct
 V  I  Y  E  R  M  N  Q  S  L  S  L  L  Y  N  V  P  A  V  A
gaggagattcaagatgaagtcgatgagctgcttcagaaggagcagaactactccgacgat
 E  E  I  Q  D  E  V  D  E  L  L  Q  K  E  Q  N  Y  S  D  D
gtcttggccaacatgatcagtgagcccagaatcagctacggaaacgacgctctcatgcct
 V  L  A  N  M  I  S  E  P  R  I  S  Y  G  N  D  A  L  M  P
tcgctgacggaaaccaagaccaccgtggagctccttcccgtgaatggggaattcagcctg
 S  L  T  E  T  K  T  T  V  E  L  L  P  V  N  G  E  F  S  L
gatgacctccagccgtggcacccctttggggtggactctgtgccagccaataccgaaaat
 D  D  L  Q  P  W  H  P  F  G  V  D  S  V  P  A  N  T  E  N
gaagtcgagcctgttgacgcccgccccgctgctgaccgaggactgaccactcgaccaggt
 E  V  E  P  V  D  A  R  P  A  A  D  R  G  L  T  T  R  P  G
tctgggctgacaaacatcaagacggaagagatctcggaagtgaagatggatgcagaattc
 S  G  L  T  N  I  K  T  E  E  I  S  E  V  K  M  D  A  E  F
ggacatgattcaggatttgaagtccgccatcaaaaactggtgttctttgctgaagatgtg
 G  H  D  S  G  F  E  V  R  H  Q  K  L  V  F  F  A  E  D  V
ggttcgaacaaaggcgccatcatcggactcatggtgggcggcgttgtcatagcaaccgtg
 G  S  N  K  G  A  I  I  G  L  M  V  G  G  V  V  I  A  T  V
attgtcatcaccctggtgatgttgaagaagaaacagtacacatccatccatcatggcgtg
 I  V  I  T  L  V  M  L  K  K  K  Q  Y  T  S  I  H  H  G  V
gtggaggtcgacgccgccgtgaccccgaggagcggcatctctcca[illegible]
 V  E  V  D  A  A  V  T  P  E  E  R  H  L  S  K  M  Q  Q  N
[illegible]gagaatccaacttacaagttctttgagcaaatgcagaactaa
 G  Y  E  N  P  T  Y  K  F  F  E  Q  M  Q  N  -
```

Sense:

659 gRNA

Antisense:

670 gRNA

676 gRNA

686 gRNA

670 gRNA

FIG. 13 CONTINUED

>mouse APP

```
atgctgcccagtttggcactgctcctgctggccgcctggacggttcgggctctggaggta
 M  L  P  S  L  A  L  L  L  L  A  A  W  T  V  R  A  L  E  V
cccactgatggcaacgccgggctgctggcagaaccccagatcgccatgttctgtggtaaa
 P  T  D  G  N  A  G  L  L  A  E  P  Q  I  A  M  F  C  G  K
ctcaacatgcacatgaatgtgcagaatgggaagtgggagtcagacccgtcagggaccaaa
 L  N  M  H  M  N  V  Q  N  G  K  W  E  S  D  P  S  G  T  K
acctgcattggcaccaaggagggcatcttgcagtactgccaagaggtctaccctgaactg
 T  C  I  G  T  K  E  G  I  L  Q  Y  C  Q  E  V  Y  P  E  L
cagatcacaaacgtggtggaagccaaccagccagtgaccatccagaactggtgcaagcgg
 Q  I  T  N  V  V  E  A  N  Q  P  V  T  I  Q  N  W  C  K  R
ggccgcaagcagtgcaagacacacacccacatcgtgattccttaccgttgcctagttggt
 G  R  K  Q  C  K  T  H  T  H  I  V  I  P  Y  R  C  L  V  G
gagtttgtgagcgacgcccttctcgtgcccgacaagtgcaagttcctacaccaggagcgg
 E  F  V  S  D  A  L  L  V  P  D  K  C  K  F  L  H  Q  E  R
atggatgtttgtgagacccatcttcactggcacaccgtcgccaaagagacatgcagcgag
 M  D  V  C  E  T  H  L  H  W  H  T  V  A  K  E  T  C  S  E
aagagcactaacttgcacgactatggcatgctgctgccctgcggcatcgacaagttccga
 K  S  T  N  L  H  D  Y  G  M  L  L  P  C  G  I  D  K  F  R
ggggtagagtttgtatgctgcccgttggccgaggaaagcgacagcgtggattctgcggat
 G  V  E  F  V  C  C  P  L  A  E  E  S  D  S  V  D  S  A  D
gcagaggaggatgactctgatgtctggtggggtggagcggacacagactacgctgatggc
 A  E  E  D  D  S  D  V  W  W  G  G  A  D  T  D  Y  A  D  G
ggtgaagacaaggtagtagaagtcgccgaagaggaggaagtggctgatgttgaggaagag
 G  E  D  K  V  V  E  V  A  E  E  E  E  V  A  D  V  E  E  E
gaagctgatgatgatgaggatgtggaggatggggacgaggtggaggaggaggccgaggag
 E  A  D  D  D  E  D  V  E  D  G  D  E  V  E  E  E  A  E  E
ccctacgaagaggccaccgagagaaccaccagcactgccaccaccaccaccaccaccact
 P  Y  E  E  A  T  E  R  T  T  S  T  A  T  T  T  T  T  T  T
gagtccgtggaggaggtggttcgagttcccacgacagcagccagcaccccggacgccgtt
 E  S  V  E  E  V  V  R  V  P  T  T  A  A  S  T  P  D  A  V
gacaagtacctggagacacccggggacgagaacgagcatgcccatttccagaaagccaaa
 D  K  Y  L  E  T  P  G  D  E  N  E  H  A  H  F  Q  K  A  K
gagaggctggaggccaagcaccgagagagaatgtcccaggtcatgagagaatgggaagag
 E  R  L  E  A  K  H  R  E  R  M  S  Q  V  M  R  E  W  E  E
gcagagcgtcaagccaagaacttgcccaaagctgacaagaaggccgttatccagcatttc
 A  E  R  Q  A  K  N  L  P  K  A  D  K  K  A  V  I  Q  H  F
caggagaaagtggaatctctggaacaggaagcagccaatgagagacagcagctggtagag
 Q  E  K  V  E  S  L  E  Q  E  A  A  N  E  R  Q  Q  L  V  E
acacacatggccagagttgaagccatgctcaatgaccgccgccgcctggccctggagaat
 T  H  M  A  R  V  E  A  M  L  N  D  R  R  R  L  A  L  E  N
tacatcactgcactgcaggctgttcctccaaggcctcatcatgtgttcaacatgctgaag
 Y  I  T  A  L  Q  A  V  P  P  R  P  H  H  V  F  N  M  L  K
aagtacgtccgtgcggagcagaaagacagacagcacaccctaaagcattttgaacatgtg
 K  Y  V  R  A  E  Q  K  D  R  Q  H  T  L  K  H  F  E  H  V
cgcatggtggaccccaagaaagctgctcagatccggtcccaggttatgacacacctccgt
 R  M  V  D  P  K  K  A  A  Q  I  R  S  Q  V  M  T  H  L  R
gtgatctacgagcgcatgaaccagtctctgtccctgctctacaatgtccctgcggtggct
 V  I  Y  E  R  M  N  Q  S  L  S  L  L  Y  N  V  P  A  V  A
gaggagattcaagatgaagtcgatgagctgcttcagaaggagcagaactactccgacgat
 E  E  I  Q  D  E  V  D  E  L  L  Q  K  E  Q  N  Y  S  D  D
gtcctggccaacatgatcagtgagcccagaatcagctacggaaacgacgctctcatgcct
 V  L  A  N  M  I  S  E  P  R  I  S  Y  G  N  D  A  L  M  P
tcgctgacggaaaccaagaccaccgtggagctccttcccgtgaatggggagttcagcctg
 S  L  T  E  T  K  T  T  V  E  L  L  P  V  N  G  E  F  S  L
gatgacctccagccgtggcaccctttcggggtggactctgtgccagccaatacggaaaat
 D  D  L  Q  P  W  H  P  F  G  V  D  S  V  P  A  N  T  E  N
gaagttgagcctgttgatgcccgccctgctgccgaccgaggactgaccactcgaccaggt
 E  V  E  P  V  D  A  R  P  A  A  D  R  G  L  T  T  R  P  G
tctgggctgaccaacatcaagacggaagagatctcggaagtgaagatggatgcagaattc
 S  G  L  T  N  I  K  T  E  E  I  S  E  V  K  M  D  A  E  F
ggacatgattcaggatttgaagtccgccatcaaaaactggtgttctttgctgaagatgtg
 G  H  D  S  G  F  E  V  R  H  Q  K  L  V  F  F  A  E  D  V
ggttcgaacaaaggcgccatcatcggactcatggtgggcggcgttgtcatagcaaccgtg
 G  S  N  K  G  A  I  I  G  L  M  V  G  G  V  V  I  A  T  V
attgtcatcaccctggtgatgctgaagaagaaacagtacacatccatccatcatggcgtg
 I  V  I  T  L  V  M  L  K  K  K  Q  Y  T  S  I  H  H  G  V
gtggaggtcgacgccgccgtgaccccagaggagcgccacctctccaagatgcagcagaac
 V  E  V  D  A  A  V  T  P  E  E  R  H  L  S  K  M  Q  Q  N
ggatatgagaatccaacctacaagttctttgagcagatgcagaactag
 G  Y  E  N  P  T  Y  K  F  F  E  Q  M  Q  N  *
```

(nucleotide sequence SEQ ID NO:13, amino acid sequence SEQ ID NO:14)

······ 653 gRNA
······ 669 gRNA
······ 670 gRNA
······ 676 gRNA
······ 686 gRNA

>human APP

FIG. 13 CONTINUED

(nucleotide sequence SEQ ID NO:11, amino acid sequence SEQ ID NO:12)

```
atgctgcccggtttggcactgctcctgctggccgcctggacggctcgggcgctggaggta
 M  L  P  G  L  A  L  L  L  L  A  A  W  T  A  R  A  L  E  V 
cccactgatggtaatgctggcctgctggctgaaccccagattgccatgttctgtggcaga
 P  T  D  G  N  A  G  L  L  A  E  P  Q  I  A  M  F  C  G  R 
ctgaacatgcacatgaatgtccagaatgggaagtgggattcagatccatcagggaccaaa
 L  N  M  H  M  N  V  Q  N  G  K  W  D  S  D  P  S  G  T  K 
acctgcattgataccaaggaaggcatcctgcagtattgccaagaagtctaccctgaactg
 T  C  I  D  T  K  E  G  I  L  Q  Y  C  Q  E  V  Y  P  E  L 
cagatcaccaatgtggtagaagccaaccaaccagtgaccatccagaactggtgcaagcgg
 Q  I  T  N  V  V  E  A  N  Q  P  V  T  I  Q  N  W  C  K  R 
ggccgcaagcagtgcaagacccatccccactttgtgattccctaccgctgcttagttggt
 G  R  K  Q  C  K  T  H  P  H  F  V  I  P  Y  R  C  L  V  G 
gagtttgtaagtgatgcccttctcgttcctgacaagtgcaaattcttacaccaggagagg
 E  F  V  S  D  A  L  L  V  P  D  K  C  K  F  L  H  Q  E  R 
atggatgtttgcgaaactcatcttcactggcacaccgtcgccaaagagacatgcagtgag
 M  D  V  C  E  T  H  L  H  W  H  T  V  A  K  E  T  C  S  E 
aagagtaccaacttgcatgactacggcatgttgctgccctgcggaattgacaagttccga
 K  S  T  N  L  H  D  Y  G  M  L  L  P  C  G  I  D  K  F  R 
ggggtagagtttgtgtgttgcccactggctgaagaaagtgacaatgtggattctgctgat
 G  V  E  F  V  C  C  P  L  A  E  E  S  D  N  V  D  S  A  D 
gcggaggaggatgactcggatgtctggtggggcggagcagacacagactatgcagatggg
 A  E  E  D  D  S  D  V  W  W  G  G  A  D  T  D  Y  A  D  G 
agtgaagacaaagtagtagaagtagcagaggaggaagaagtggctgaggtggaagaagaa
 S  E  D  K  V  V  E  V  A  E  E  E  E  V  A  E  V  E  E  E 
gaagccgatgatgacgaggacgatgaggatggtgatgaggtagaggaagaggctgaggaa
 E  A  D  D  D  E  D  D  E  D  G  D  E  V  E  E  E  A  E  E 
ccctacgaagaagccacagagagaaccaccagcattgccaccaccaccaccaccacaca
 P  Y  E  E  A  T  E  R  T  T  S  I  A  T  T  T  T  T  T  T 
gagtctgtggaagaggtggttcgagttcctacaacagcagccagtacccctgatgccgtt
 E  S  V  E  E  V  V  R  V  P  T  T  A  A  S  T  P  D  A  V 
gacaagtatctcgagacacctggggatgagaatgaacatgcccatttccagaaagccaaa
 D  K  Y  L  E  T  P  G  D  E  N  E  H  A  H  F  Q  K  A  K 
gagaggcttgaggccaagcaccgagagagaatgtcccaggtcatgagagaatgggaagag
 E  R  L  E  A  K  H  R  E  R  M  S  Q  V  M  R  E  W  E  E 
gcagaacgtcaagcaaagaacttgcctaaagctgataagaaggcagttatccagcatttc
 A  E  R  Q  A  K  N  L  P  K  A  D  K  K  A  V  I  Q  H  F 
caggagaaagtggaatctttggaacaggaagcagccaacgagagacagcagctggtggag
 Q  E  K  V  E  S  L  E  Q  E  A  A  N  E  R  Q  Q  L  V  E 
acacacatggccagagtggaagccatgctcaatgaccgccgccgcctggccctggagaac
 T  H  M  A  R  V  E  A  M  L  N  D  R  R  R  L  A  L  E  N 
tacatcaccgctctgcaggctgttcctcctcggcctcgtcacgtgttcaatatgctaaag
 Y  I  T  A  L  Q  A  V  P  P  R  P  R  H  V  F  N  M  L  K 
aagtatgtccgcgcagaacagaaggacagacagcacaccctaaagcatttcgagcatgtg
 K  Y  V  R  A  E  Q  K  D  R  Q  H  T  L  K  H  F  E  H  V 
cgcatggtggatcccaagaaagccgctcagatccggtcccaggttatgacacacctccgt
 R  M  V  D  P  K  K  A  A  Q  I  R  S  Q  V  M  T  H  L  R 
gtgatttatgagcgcatgaatcagtctctctccctgctctacaacgtgcctgcagtggcc
 V  I  Y  E  R  M  N  Q  S  L  S  L  L  Y  N  V  P  A  V  A 
gaggagattcaggatgaagttgatgagctgcttcagaaagagcaaaactattcagatgac
 E  E  I  Q  D  E  V  D  E  L  L  Q  K  E  Q  N  Y  S  D  D 
gtcttggccaacatgattagtgaaccaaggatcagttacggaaacgatgctctcatgcca
 V  L  A  N  M  I  S  E  P  R  I  S  Y  G  N  D  A  L  M  P 
tctttgaccgaaacgaaaaccaccgtggagctccttcccgtgaatggagagttcagcctg
 S  L  T  E  T  K  T  T  V  E  L  L  P  V  N  G  E  F  S  L 
gacgatctccagccgtggcattcttttggggctgactctgtgccagccaacacagaaaac
 D  D  L  Q  P  W  H  S  F  G  A  D  S  V  P  A  N  T  E  N 
gaagttgagcctgttgatgcccgccctgctgccgaccgaggactgaccactcgaccaggt
 E  V  E  P  V  D  A  R  P  A  A  D  R  G  L  T  T  R  P  G 
tctgggttgacaaatatcaagacggaggagatctctgaagtgaagatggatgcagaattc
 S  G  L  T  N  I  K  T  E  E  I  S  E  V  K  M  D  A  E  F 
cgacatgactcaggatatgaagttcatcatcaaaaattggtgttctttgcagaagatgtg
 R  H  D  S  G  Y  E  V  H  H  Q  K  L  V  F  F  A  E  D  V 
ggttcaaacaaaggtgcaatcattggactcatggtgggcggtgttgtcatagcgacagtg
 G  S  N  K  G  A  I  I  G  L  M  V  G  G  V  V  I  A  T  V 
atcgtcatcaccttggtgatgctgaagaagaaacagtacacatccattcatcatggtgtg
 I  V  I  T  L  V  M  L  K  K  K  Q  Y  T  S  I  H  H  G  V 
gtggaggttgacgccgctgtcaccccagaggagcgccacctgtccaagatgcagcagaac
 V  E  V  D  A  A  V  T  P  E  E  R  H  L  S  K  M  Q  Q  N 
ggctacgaaaatccaacctacaagttctttgagcagatgcagaactag
 G  Y  E  N  P  T  Y  K  F  F  E  Q  M  Q  N  - 
```

653 gRNA

669 gRNA

670 gRNA

676 gRNA

686 gRNA

FIG. 14

>SpCas9 (SEQ ID NO:15)
ATGGGTATCCACGGAGTCCCAGCAGCCGACAAGAAGTACAGCATCGGCCTGGACAT
CGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGACGAGTACAAGGTGCCCAGCA
AGAAATTCAAGGTGCTGGGCAACACCGACCGGCACAGCATCAAGAAGAACCTGATC
GGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAAC
CGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCT
TCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAGTCC
TTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGCAACATCGT
GGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAGAAAC
TGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCAC
ATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAG
CGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGG
AAAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTG
AGCAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGAGAAGAAGA
ATGGCCTGTTCGGAAACCTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGA
GCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCAAGGACACCTACGAC
GACGACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTG
GCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGCGACATCCTGAGAGTGAACAC
CGAGATCACCAAGGCCCCCCTGAGCGCCTCTATGATCAAGAGATACGACGAGCACC
ACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGGCAGCAGCTGCCTGAGAAGTAC
AAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCCGGCTACATTGACGGCGG
AGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTGGAAAAGATGGACG
GCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGGAAGCAGCG
GACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCTGCACGCCA
TTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATC
GAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAAC
AGCAGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTT
CGAGGAAGTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCA
ACTTCGATAAGAACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTAC
GAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTGACCGAGGGAAT
GAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGT
TCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAAA
ATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGATCGGTTCAACGCCTCC
CTGGGCACATACCACGATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAA
TGAGGAAAACGAGGACATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGG
ACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCCCACCTGTTCGACGACAAA
GTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGCAGGCTGAGCCGGA
AGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTGGATTTCCTG
AAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCTGATCCACGACGACAGCCT

FIG. 14 CONTINUED

GACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTGC
ACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAG
ACAGTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGA
ACATCGTGATCGAAATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAA
CAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAG
ATCCTGAAAGAACACCCCGTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCT
GTACTACCTGCAGAATGGGCGGGATATGTACGTGGACCAGGAACTGGACATCAACC
GGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGCTTTCTGAAGGACGACT
CCATCGACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGGGCAAGAGCGACAA
CGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAGAACTACTGGCGGCAGCTGCTGA
ACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGACCAAGGCCGAGAGAGGC
GGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAGCTGGTGGAAACCCG
GCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAACACTAAGTACG
ACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCCTGAAGTCCAAGCTG
GTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAACTAC
CACCACGCCCACGACGCCTACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAA
GTACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGC
GGAAGATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTC
TTCTACAGCAACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAG
ATCCGGAAGCGGCCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGA
TAAGGGCCGGGATTTTGCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATA
TCGTGAAAAAGACCGAGGTGCAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCC
AAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGGACCCTAAGAAGT
ACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGTGGTGGCCAAAGTGG
AAAAGGGCAAGTCCAAGAAACTGAAGAGTGTGAAAGAGCTGCTGGGGATCACCATC
ATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGACTTTCTGGAAGCCAAGGGCTA
CAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTCGAGC
TGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTGCAGAAGGGAAA
CGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCAGCCACTATGA
GAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAGCTGTTTGTGGAACAGC
ACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTG
ATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGGGA
TAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCT
GGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACA
CCAGCACCAAAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTG
TACGAGACACGGATCGACCTGTCTCAGCTGGGAGGCGACAAAAGGCCGGCGGCCAC
GAAAAAGGCCGGCCAGGCAAAAAAGAAAAAG

FIG. 15

APP 659 gRNA Cas9 vector (SEQ ID NO:17)

CCCCACGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCT
GTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAA
TACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTT
AAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT
ATATCTTGTGGAAAGGACGAAACACCG*atccattcatcatggtgtgg*GTTTTAGAGCTAGAAAT
AGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG
TGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAG
CGCGTGCGCCAATTCTGCAGACAAATGGCTCTAGAGGTACCCGTTACATAACTTACG
GTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAGTA
ACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCC
CACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAAT
GACGGTAAATGGCCCGCCTGGCATTGTGCCCAGTACATGACCTTATGGGACTTTCCT
ACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCC
ACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATT
TATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGGGGCGCGCGC
CAGGCGGGGCGGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGG
CGGCAGCCAATCAGAGCGGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGG
CGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGGCGGGCGGGAGTCGCTGCGACGCT
GCCTTCGCCCCGTGCCCCGCTCCGCCGCCGCCTCGCGCCGCCCGCCCCGGCTCTGAC
TGACCGCGTTACTCCCACAGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTA
ATTAGCTGAGCAAGAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATG
TTTAATTACCTGGAGCACCTGCCTGAAATCACTTTTTTTCAGGTTGGACCGGTGCCAC
CATGGACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACAAAG
ACGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTC
CCAGCAGCCGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGG
CTGGGCCGTGATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGG
GCAACACCGACCGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGAC
AGCGGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACA
CCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCC
AAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAGTCCTTCCTGGTGGAAGAGGA
TAAGAAGCACGAGCGGCACCCCATCTTCGGCAACATCGTGGACGAGGTGGCCTACC
ACGAGAAGTACCCCACCATCTACCACCTGAGAAAGAAACTGGTGGACAGCACCGAC
AAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCACATGATCAAGTTCCGGGGC
CACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCGACGTGGACAAGCTGTT
CATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCCATCAACGCCA
GCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTG
GAAAATCTGATCGCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGAAACCT

FIG. 15 CONTINUED

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GATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGA
GGATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGACAACCTGC
TGGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCG
ACGCCATCCTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCC
CTGAGCGCCTCTATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCT
GAAAGCTCTCGTGCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACC
AGAGCAAGAACGGCTACGCCGGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTC
TACAAGTTCATCAAGCCCATCCTGGAAAAGATGGACGGCACCGAGGAACTGCTCGT
GAAGCTGAACAGAGAGGACCTGCTGCGGAAGCAGCGGACCTTCGACAACGGCAGC
ATCCCCCACCAGATCCACCTGGGAGAGCTGCACGCCATTCTGCGGCGGCAGGAAGA
TTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCCG
CATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGCAGATTCGCCTGGATGA
CCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAAGTGGTGGACAA
GGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAGAACCTGC
CCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTGTAT
AACGAGCTGACCAAAGTGAAATACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCT
GAGCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAA
GTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTC
CGTGGAAATCTCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACG
ATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGAC
ATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGA
GGAACGGCTGAAAACCTATGCCCACCTGTTCGACGACAAAGTGATGAAGCAGCTGA
AGCGGCGGAGATACACCGGCTGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCATC
CGGGACAAGCAGTCCGGCAAGACAATCCTGGATTTCCTGAAGTCCGACGGCTTCGC
CAACAGAAACTTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACA
TCCAGAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTGCACGAGCACATTGCCAAT
CTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACAGTGAAGGTGGTGGA
CGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACATCGTGATCGAAATGG
CCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAGAGAATGAA
GCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAAAGAACACCCC
GTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGG
GCGGGATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGATG
TGGACCATATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACAACAAGGTGC
TGACCAGAAGCGACAAGAACCGGGGCAAGAGCGACAACGTGCCCTCCGAAGAGGT
CGTGAAGAAGATGAAGAACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCC
AGAGAAAGTTCGACAATCTGACCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGAT
AAGGCCGGCTTCATCAAGAGACAGCTGGTGGAAACCCGGCAGATCACAAAGCACGT
GGCACAGATCCTGGACTCCCGGATGAACACTAAGTACGACGAGAATGACAAGCTGA
TCCGGGAAGTGAAAGTGATCACCCTGAAGTCCAAGCTGGTGTCCGATTTCCGGAAG
GATTTCCAGTTTTACAAAGTGCGCGAGATCAACAACTACCACCACGCCCACGACGCC
```

FIG. 15 CONTINUED

TACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAAGTACCCTAAGCTGGAAAG
CGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAAGA
GCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGCAACATCATG
AACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGGCCTCT
GATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTTG
CCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAGACCGAG
GTGCAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAA
GCTGATCGCCAGAAAGAAGGACTGGGACCCTAAGAAGTACGGCGGCTTCGACAGCC
CCACCGTGGCCTATTCTGTGCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAG
AAACTGAAGAGTGTGAAAGAGCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTT
CGAGAAGAATCCCATCGACTTTCTGGAAGCCAAGGGCTACAAAGAAGTGAAAAAGG
ACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTCGAGCTGGAAAACGGCCGGAAG
AGAATGCTGGCCTCTGCCGGCGAACTGCAGAAGGGAAACGAACTGGCCCTGCCCTC
CAAATATGTGAACTTCCTGTACCTGGCCAGCCACTATGAGAAGCTGAAGGGCTCCCC
CGAGGATAATGAGCAGAAACAGCTGTTTGTGGAACAGCACAAGCACTACCTGGACG
AGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAAT
CTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAGCCCATCAGAGAGCA
GGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCCCCTGCCGCCTT
CAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACCAAAGAGGTGC
TGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATCGAC
CTGTCTCAGCTGGGAGGCGACAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGG
CAAAAAAGAAAAAGCTTGAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTGGA
GGAGAATCCCGGCCCTGCTAGCATGGTGAGCAAGGGCGAGGAGGATAACATGGCCA
TCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCAC
GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCG
CCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCC
CTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACT
ACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGG
ACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATC
TACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCAGACGGCCCCGTAATGCAGAA
GAAAACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCC
TGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGC
TGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACA
ACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAA
CAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAA
GTAAGAATTCCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCA
TCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTG
TCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTAT
TCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAGAATAGC
AGGCATGCTGGGGAGCGGCCGCAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTC

FIG. 15 CONTINUED

TCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGG
GCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGGGGCGC
CTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATACGTCAAA
GCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTA
CGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTT
CCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTC
CCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTTG
GGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACG
TTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAAC
CCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGT
TAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGT
TTACAATTTTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCA
GCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGC
ATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTC
ACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATA
GGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAA
TGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTC
ATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAG
TATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTT
TTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA
CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGC
CCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTA
TTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAG
AATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGAC
AGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACT
TACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGG
GGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCA
AACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACT
ATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGA
GGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTAT
TGCTGATAAATCTGGAGCCGGTGAGCGTGGAAGCCGCGGTATCATTGCAGCACTGG
GGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCA
ACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCA
TTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCAT
TTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATC
CCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGA
TCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCAC
CGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGG
TAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGT
TAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCC

FIG. 15 CONTINUED

TGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAA
GACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACA
CAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCT
ATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGC
GGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT
ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATG
CTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGT
TCCTGGCCTTTTGCTGGCCTTTTGCTCACATGT

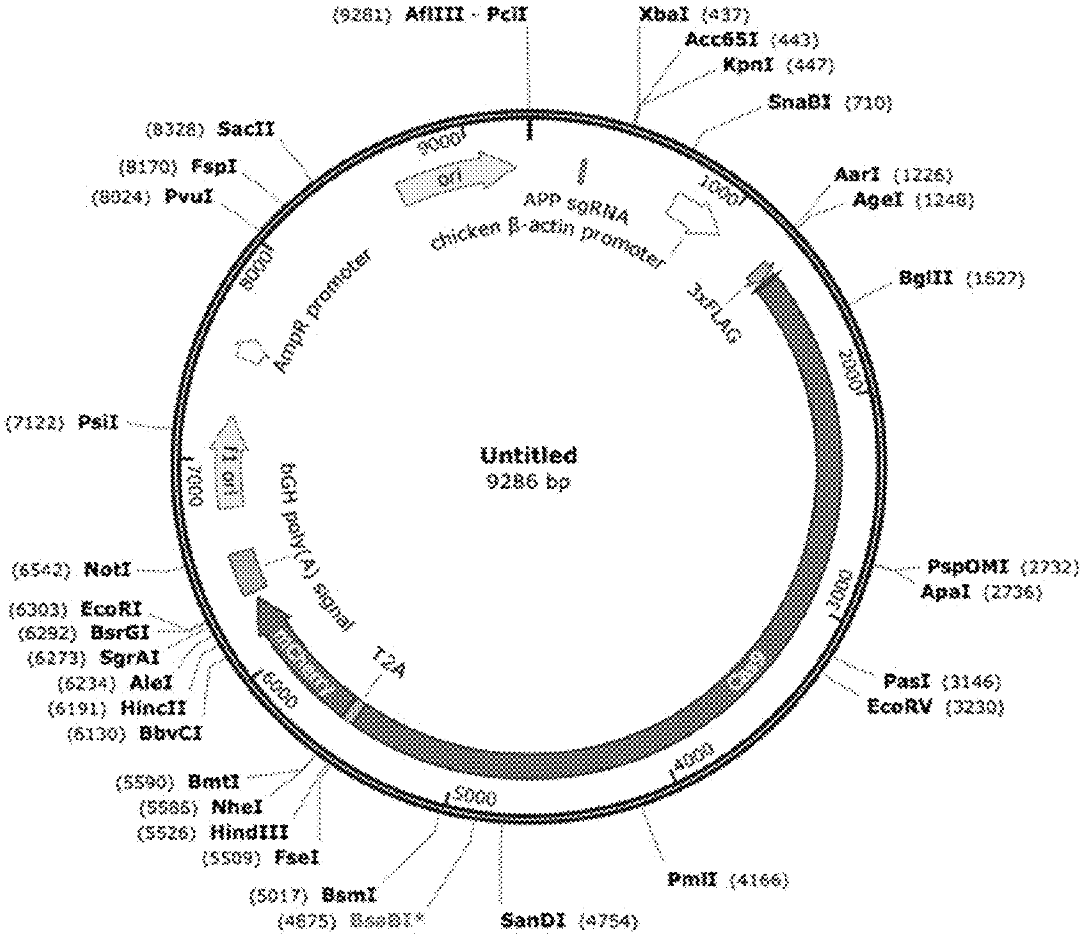

FIG. 16

PX551 Cas9 vector (SEQ ID NO:18)

cctgcaggcagctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccgggcgtcgggcgacctttggtcgcccggcctcagt
gagcgagcgagcgcgcagagagggagtggccaactccatcactaggggttcctgcggcctctagaaagcttAGCTGAATGGG
GTCCGCCTCTTTTCCCTGCCTAAACAGACAGGAACTCCTGCCAATTGAGGGCGTCAC
CGCTAAGGCTCCGCCCCAGCCTGGGCTCCACAACCAATGAAGGGTAATCTCGACAA
AGAGCAAGGGGTGGGGCGCGGGCGCGCAGGTGCAGCAGCACACAGGCTGGTCGGG
AGGGCGGGGCGCGACGTCTGCCGTGCGGGGTCCCGGCATCGGTTGCGCGCaccggtgcca
ccatgtacccatacgatgttccagattacgcttcgccgaagaaaaagcgcaaggtcgaagcgtccgacaagaagtacagcatcggcctgg
acatcggcaccaactctgtgggctgggccgtgatcaccgacgagtacaaggtgcccagcaagaaattcaaggtgctgggcaacaccgac
cggcacagcatcaagaagaacctgatcggagccctgctgttcgacagcggcgaaacagccgaggccacccggctgaagagaaccgcc
agaagaagatacaccagacggaagaaccggatctgctatctgcaagagatcttcagcaacgagatggccaaggtggacgacagcttcttc
cacagactggaagagtccttcctggtggaagaggataagaagcacgagcggcaccccatcttcggcaacatcgtggacgaggtggccta
ccacgagaagtaccccaccatctaccacctgagaaagaaactggtggacagcaccgacaaggccgacctgcggctgatctatctggccct
ggcccacatgatcaagttccggggccacttcctgatcgagggcgacctgaaccccgacaacagcgacgtggacaagctgttcatccagct
ggtgcagacctacaaccagctgttcgaggaaaaccccatcaacgccagcggcgtggacgccaaggccatcctgtctgccagactgagca
agagcagacggctggaaaatctgatcgcccagctgcccggcgagaagaagaatggcctgttcggcaacctgattgccctgagcctgggc
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FIG. 16 CONTINUED

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FIG. 16 CONTINUED

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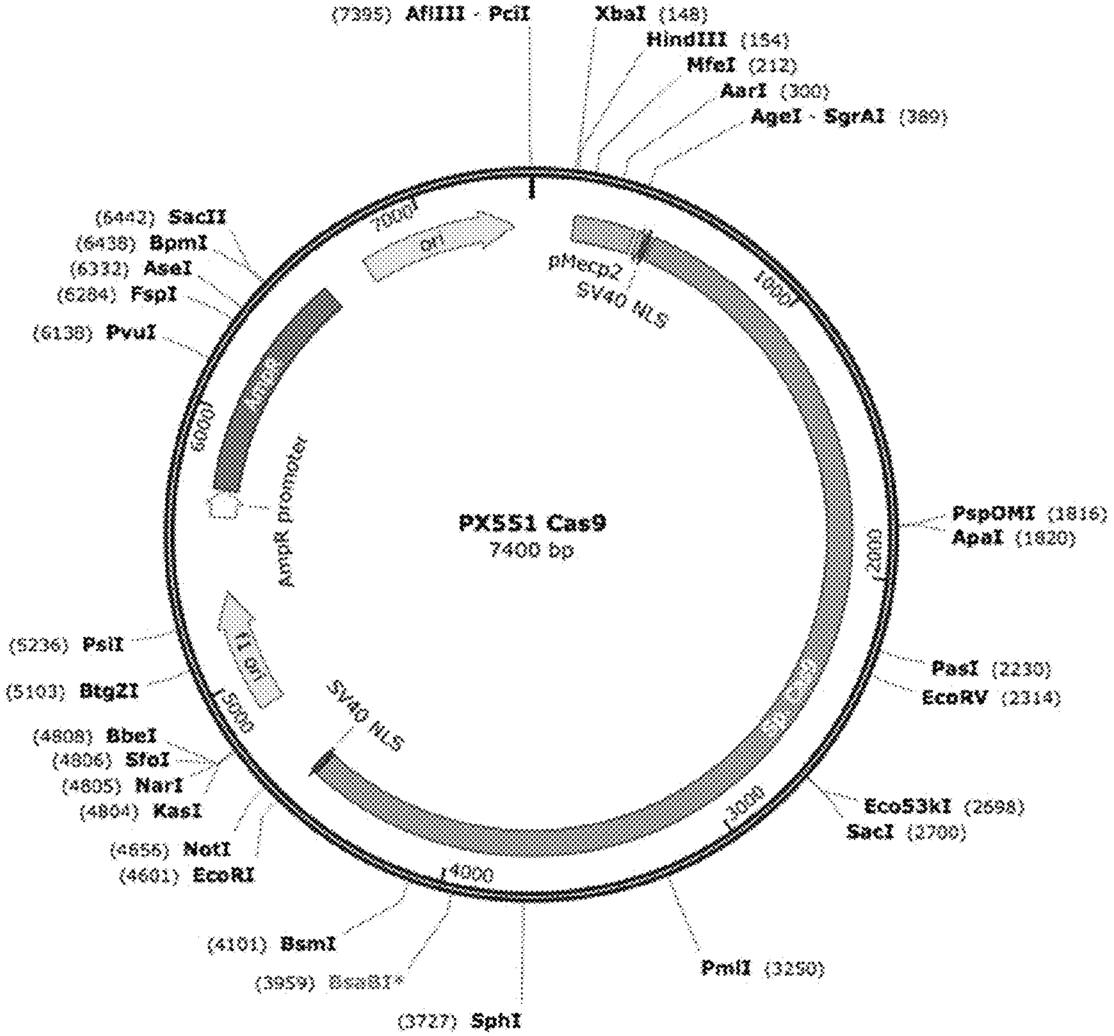

FIG. 17

PX552 APP sgRNA vector (SEQ ID NO:19)

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FIG. 17 CONTINUED

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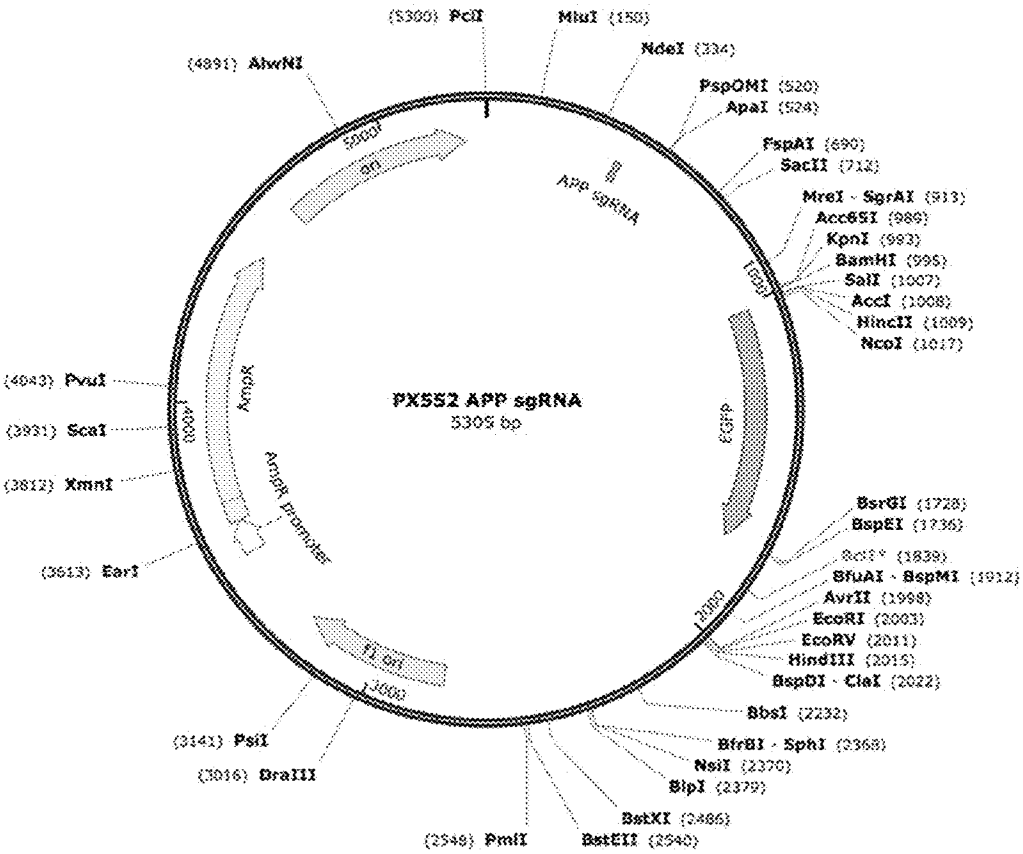

FIG. 18

LentiCRISPRv2 APP sgRNA vector (SEQ ID NO:20)

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FIG. 18 CONTINUED

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CGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGACCAA

FIG. 18 CONTINUED

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GCCCTGATCAAAAAGTACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAA
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FIG. 18 CONTINUED

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FIG. 18 CONTINUED

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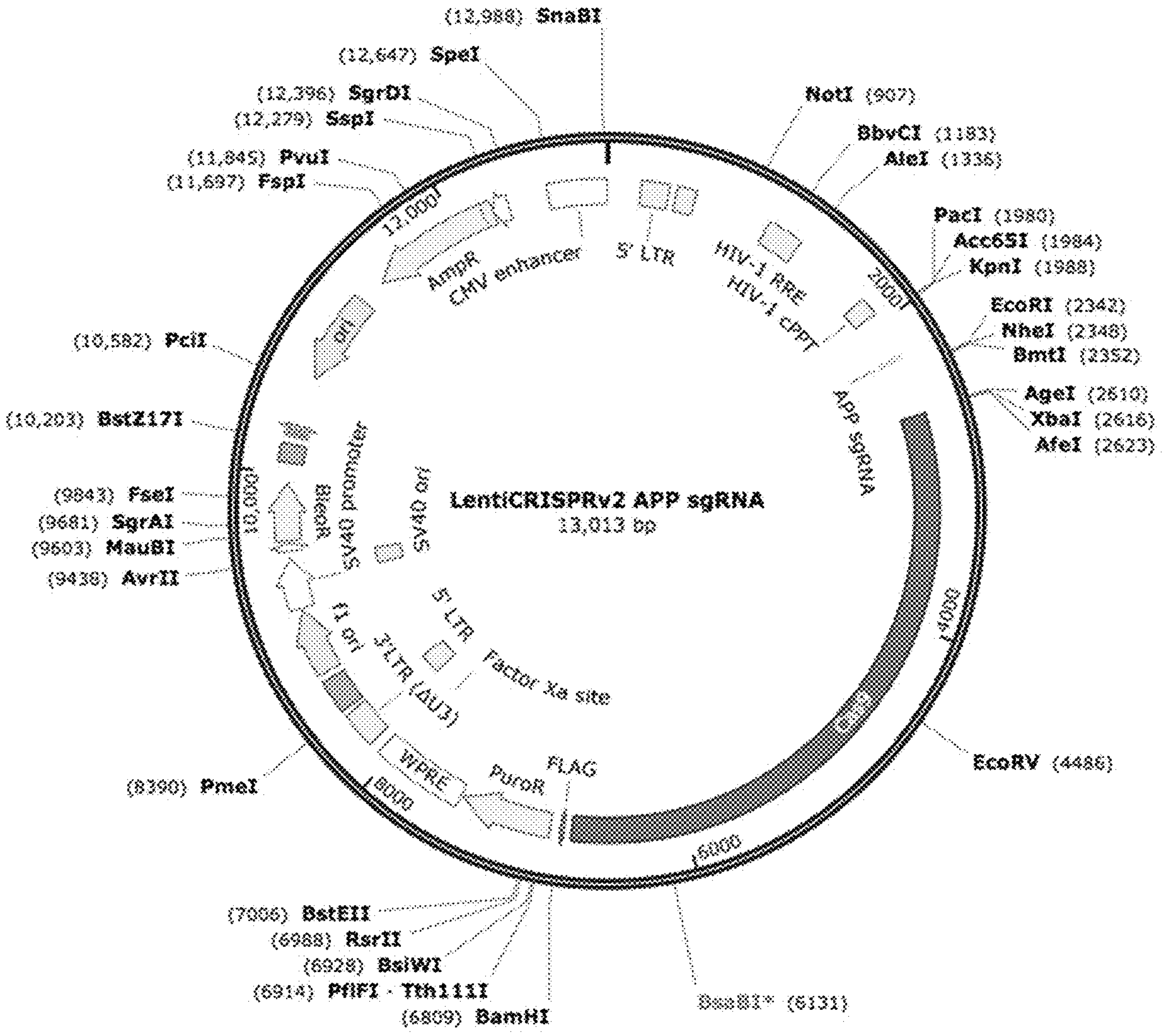
FIG. 18 CONTINUED



GENE EDITING-BASED METHOD OF ATTENUATING THE BETA-AMYLOID PATHWAY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 62/618,694, filed Jan. 18, 2018, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under AG048218 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Jan. 18, 2019, is named 960296_02327_ST25.txt and is 101,371 bytes in size.

BACKGROUND

The gradual accumulation of Aβ in brains is a neuropathologic hallmark of Alzheimer's disease (AD). Aβ is generated by the sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (β-secretase aka BACE-1, and γ-secretase), with BACE-1-cleavage as the rate-limiting step. Substantial evidence indicates that accrual of APP-cleavage products play a key role in AD, making the "amyloidogenic pathway" an important therapeutic target (1-3).

CRISPR/Cas9 gene editing is emerging as a promising tool to disrupt the expression of disease-causing genes or edit pathogenic mutations (4). Originally discovered in bacteria as part of a natural self-defense mechanism, the Cas9 nuclease—guided by a short guide RNA (sgRNA)—generates double-stranded breaks (DSB) at targeted genomic loci (5).

However, to date, the application of gene editing to neurologic diseases has been limited (6). For instance, CRISPR/Cas9 has been used in cell-based models to edit triplet-repeat expansions of Huntington's and Fragile X syndrome (7, 8). Besides significant technical caveats such as low editing efficiency and limited in vivo validation (6), such canonical approaches would only be applicable to the small fraction of cases that are inherited (i.e. <10% of AD, Parkinson's, ALS); with a different approach required for each gene. Moreover, the feasibility of CRISPR/Cas9 as a therapeutic possibility in AD has not been reported.

Needed in the art of Alzheimer's disease treatment is an improved method of using gene editing methods to treat or prevent the disease.

SUMMARY OF THE INVENTION

In a first aspect, provided herein is a method of treating or preventing Alzheimer's disease (AD) caused by formation of amyloid plaques composed of amyloid beta (Aβ) peptides, wherein the method comprises the steps of (a) obtaining a gene-editing construct specific for the amyloid precursor protein (APP), wherein the construct facilitates truncation of the APP C-terminus when combined with a Cas9 nuclease, and (b) delivering the construct and a construct encoding the Cas9 nuclease to a patient in need of AD therapy, wherein the APP molecule is truncated and production of Aβ peptides is decreased in the patient's brain. In some embodiments, the truncation of the APP C-terminus occurs at an APP residue selected from the group consisting of 659, 670, 676, and 686. In some embodiments, the gene-editing construct comprises a gRNA sequence selected from the group consisting of SEQ ID NOs:1-10. In some embodiments, the construct and the nuclease are delivered in a composition comprising an adeno-associated viral vector and a nanocarrier delivery vehicle. In some embodiments, the composition is delivered intravenously or intrathecally.

In a second aspect, provided herein is a method of reducing the formation of amyloid plaques in a patient's brain, wherein the plaques comprise amyloid beta (Aβ) peptides, the method comprises the steps of (a) obtaining a gene-editing construct specific for the amyloid precursor protein (APP), wherein the construct facilitates truncation of the APP C-terminus when combined with a Cas9 nuclease, and (b) delivering the construct and nuclease to a patient in need of AD therapy, wherein the APP molecule is truncated and production of Aβ peptides is decreased in the patient's brain. In some embodiments, the truncation of the APP C-terminus occurs at an APP residue selected from the group consisting of 659, 670, 676, and 686. In some embodiments, the gene-editing construct comprises a gRNA sequence selected from the group consisting of SEQ ID NO:1-10. In some embodiments, the construct and the nuclease are delivered in a composition comprising an adeno-associated viral vector and a nanocarrier delivery vehicle. In some embodiments, the composition is delivered intravenously or intrathecally.

In a third aspect, provided herein is a genetic construct comprising, a sequence encoding for a Cas9 nuclease and a sequence encoding a gRNA specific to amyloid precursor protein (APP). In some embodiments, the construct is packaged in a viral vector selected from the group consisting of a lentiviral vector and an adeno-associated viral (AAV) vector. In some embodiments, the construct further comprises at least one neuron specific promoter. In some embodiments, the neuron specific promoter is selected from the group consisting of human synapsin 1 (hSyn1) promoter, and mouse Mecp2 promoter (pMecp2). In some embodiments, the construct further comprises an RNA Pol III promoter. In some embodiments, the RNA Pol III promoter is a U6 promoter. In some embodiments, the sequence of the gRNA is selected from the group consisting of SEQ ID NOs:1-10. In some embodiments, the sequence of the Cas9 nuclease consists of SEQ ID NO:15. In some embodiments, the construct comprises the sequence of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, or SEQ ID NO:20. In some embodiments, the sequence encoding for a Cas9 nuclease in packaged on a first AAV vector and the sequence encoding a gRNA specific to amyloid precursor protein (APP) is packaged on a second AAV vector.

In a forth aspect, provided herein is a kit for reducing the formation of amyloid plaques in a patient's brain, the kit comprising a first viral vector encoding a gRNA selected from the group consisting of SEQ ID NOs:1-10 and a second viral vector encoding a Cas9 nuclease. In some embodiments, the viral vector is selected from the group consisting of a lentiviral vector and an adeno-associated viral (AAV) vector. In some embodiments, the first or second viral vector further comprises at least one neuron specific promoter. In some embodiments, the neuron specific promoter is selected from the group consisting of human synapsin 1 (hSyn1) promoter, and mouse Mecp2 promoter (pMecp2). In some embodiments, the first or second viral vector further comprises an RNA Pol III promoter. In some embodiments, the RNA Pol III promoter is a U6 promoter. In some embodiments, the kit comprises a viral vector encoding both a gRNA selected from the group consisting of SEQ ID NOs: 1-10 and a Cas9 nuclease.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

FIGS. 1A-1F show manipulation of the amyloid pathway by CRISPR/Cas9 editing. (FIG. 1A) Schematic and C-terminal sequence of mouse APP showing PAM sites (yellow) and genomic targets for the three APP-sgRNAs (APP-659 sgRNA used henceforth and referred to as 'APP-sgRNA'—see text). Note that the C-terminal antibody Y188 recognizes the last 20 amino acids of APP. (FIG. 1B) Neuro2A cells were transfected with APP-sgRNA and Cas9 (or Cas9 only), and immunostained with the Y188 antibody (after 5 days; mCherry labels transfected cells). Note decreased APP (Y188) fluorescence, indicating APP editing (quantified on right, mean±SEM of 39 cells from two independent experiments per condition, $p<0.0001$). (FIGS. 1C-1D) Neuro2A cells were transduced by lentiviral vectors carrying APP-sgRNA and Cas9 (or non-targeting control-sgRNA/Cas9 as control) and immunoblotted with Y188 and 22C11 antibodies (latter recognizes APP N-terminus). A gamma secretase inhibitor (GSI) was added to allow detection of accumulated APP CTF's (see methods, GAPDH used as loading controls). Note attenuated signal with the Y188 antibody in APP-sgRNA treated samples, but no change in 22C11 signal. Blots quantified in (d), mean±SEM of six independent experiments, $p<0.0001$. (FIG. 1E) Time course of APP-editing in neuro2a cells. Cells were transfected with a vector carrying APP-sgRNA and Cas9, and APP-CTFs were analyzed by Western blotting (in the presence of GSI). (FIG. 1F) Deep sequencing of APP C-terminus in neuro2A cells. Top: Frequency of base-pair matches between gRNA-edited and WT mouse sequence. Red underline marks the sgRNA target sequence and arrowhead denotes predicted cut-site. Note extensive mismatch around predicted cut-site, indicating robust editing. Bottom: Major mutated APP loci resulting from sgRNA-editing, and their frequencies.

FIGS. 2A-2H show gene editing of APP C-terminus and effects on APP processing in human cells. (FIG. 2A) Comparison of mouse and human APP-sgRNA targeting sequences (red arrowheads indicate differences; yellow bar denotes the PAM site). (FIG. 2B) Human iPSC-derived NPCs were transduced by lentiviral vectors carrying APP-sgRNA and Cas9 (or non-targeting control-sgRNA/Cas9 as control) and differentiated into neurons. After 3 weeks of differentiation, cells were immunostained with the Y188 and Tuj 1 (tubulin) antibodies. Note decreased APP (Y188) fluorescence, indicating APP editing. (FIG. 2C) The iPSC-derived neurons above (or isogenic APPV717I London-mutant knock-in iPSC-neurons) were transduced and differentiated as above and immunoblotted with C- and N-terminus antibodies (GSI was added to allow detection of accumulated APP CTFs). Note attenuation of APP signal with Y188 after APP-sgRNA treatment in both wild type and isogenic APP London iPSCs (quantified on right, mean±SEM of three independent experiments, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$). (FIG. 2D) Media from the iPSC-derived neurons above was immunoblotted for secreted sAPPa (6E10 antibody). Note increased sAPPa in sgRNA-treated samples, indicating upregulation of the non-amyloidogenic pathway. (FIG. 2E) ELISA of media from iPSC derived neurons. Note decreased Aβ in the sgRNA-treated samples (mean±SEM of three independent experiments, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$). (FIG. 2F) Deep sequencing of APP C-terminus in human ESCs. Red underline marks the sgRNA target sequence and arrowhead denotes predicted cut-site. Note extensive mismatch around predicted cut-site, indicating robust editing. (FIG. 2G) Major mutated APP-loci resulting from CRISPR editing, and their frequencies. (FIG. 2H) Predicted APP translational products (post-editing) for the major mutant alleles observed in deep sequencing. Note that after editing, APP is translated up-to amino acid 659 (red arrowheads; similar results were seen in HEK cells, see FIG. 6E).

FIGS. 3A-3G show the effect of APP C-terminus editing on neuronal physiology. (FIG. 3A) AAV9-sgRNA and AAV9-Cas9 expression vectors. Note that the sgRNA vector co-expresses GFP and the Cas9 is tagged to HA, for identification of transduced neurons. (FIG. 3B) Cultured hippocampal neurons were transduced with AAV9s carrying APP-sgRNA/Cas9 (or Cas9 only) and immunoblotted with the Y188 and 22C11 antibodies (in the presence of GSI). Note attenuation of CTFs by the APP-sgRNA. (FIG. 3C) Neurons were transfected (at the time of plating) with a vector expressing APP-sgRNA and Cas9. Neuritic/axon outgrowth was analyzed after 5-6 days. Neurons were transfected or infected at DIV7 with APP CRISPR, and synapse structure/function was analyzed after 14-17 days. (FIG. 3D) Top: Representative images of neurons transfected with the APP-sgRNA/Cas9 (or Cas9 alone). Bottom: Axon length and number of neurites/branches in the APP-sgRNA/Cas9 (or Cas9 alone) groups; note that there was no significant difference (mean±SEM; axon length: 30 cells for Cas9 only and 27 cells for moAPP-sgRNA from two independent experiments, $p=0.2462$; neurite number: 35 cells for Cas9 only and 31 cells for moAPP-sgRNA from two independent experiments, $p=0.2289$; branch number: 27 cells for both conditions from two independent experiments, $p=0.6008$). (FIG. 3E) Neurons were infected with AAV9 viruses carrying APP-sgRNA/Cas9 (or Cas9 only as controls), and fixed/stained with the presynaptic marker VAMP2. Note that the presynaptic density (VAMP2 puncta) was similar in both groups (quantified on right, mean±SEM of VAMP2 staining along 27 dendrites for Cas9 only and 25 dendrites for moAPP-sgRNA from two independent experiments, $p=0.3132$). (FIG. 3F) Neurons were transfected with APP-sgRNA/Cas9 (or Cas9 only as controls). Spine density in the APP-sgRNA/Cas9 (or Cas9 only) groups was also similar, quantified on right (mean±SEM of 18 dendrites for Cas9 only and 16 dendrites for moAPP-sgRNA from two independent experiments, $p=0.7456$). (FIG. 3G) Miniature excitatory postsynaptic currents (mEPSC) were recorded from neurons infected with AAV9-APP-sgRNA/Cas9 or AAV9-Cas9 alone. Top: Representative mEPSC traces in control and APP-sgRNA transduced neurons. Corresponding alignments of mEPSCs with average (white traces) are shown on right. Bottom: Cumulative histograms of mEPSC amplitude, 20-80% rise-time and inter-event interval in APP-sgRNA/Cas9 and the Cas9-only infected neurons (note no significant differences).

FIGS. 4A-4G show gene editing of APP C-terminus in vivo. (FIG. 4A) AAV9-sgRNA and AAV9-Cas9 were stereotactically co-injected into dentate gyrus of 8-week old mouse brains (bottom). Two weeks after viral delivery, brains were perfused, fixed, and immunostained with anti-GFP, anti-HA and anti-APP(Y188) antibodies. (FIG. **4**B) Co-expression of AAV9-sgRNA-GFP and AAV9-HA-Cas9 in the dentate gyrus. Note that majority of neurons are positive for both GFP and HA (~87% of the cells were positive for both; sampling from 3 brains). (FIGS. **4**C-**4**D) Coronal section of a mouse hippocampi injected on one side (marked by arrow) with the AAV viruses as described above. Note attenuated Y188 staining of neurons on the injected side, indicating APP-editing. The image of mouse hippocampus injected with Cas9 only is not shown. Fluorescence quantified in (d), mean±SEM, data from three brains. One-way ANOVA: p<0.0001. Tukey's multiple comparisons: p=0.4525 (Un-injected vs Cas9 only); p<0.0001 (Un-injected vs APP-sgRNA); p<0.0001 (Cas9 only vs APP-sgRNA). (FIG. **4**E) Intracerebroventrical injection of the AAV9 viruses into P0 pups. Note widespread delivery of gRNA into brain, as evident by GFP fluorescence. (FIG. **4**F) Brain sections from above were immunostained with the Y188 antibody. Note attenuated Y188 staining in the APP-sgRNA/Cas9 transduced sample, suggesting APP-editing. (FIG. **4**G) Western blots of the brains from (e). Note decreased expression of CTFs in the APP-sgRNA/Cas9 transduced brains; blots quantified on right (mean±SEM of three independent experiments, **p<0.01).

FIGS. **5**A-**5**E show mechanistic details of CRISPR-guided APP editing. (FIG. **5**A) APP/BACE-1 interaction—as evaluated by fluorescence complementation in cultured hippocampal neurons—was attenuated in neurons transfected with an APP C-terminus truncation mimicking the post-edited translational product (APP659:VN; quantified below, mean±SEM of 12 cells for APP(WT) and 13 cells for APP(659) from two independent experiments, p<0.0001). (FIG. **5**B) APP β-cleavage is also attenuated in cells transfected with APP659. HEK cells were co-transfected with APPWT (or APP659) tagged to VN, and BACE-1:VC; and immunoblotted with the 6E10 antibody. Note decreased β-CTFs in cells carrying the truncated APP plasmid. (FIG. **5**C) Schematic showing the CRISPR-edited C-terminus portion of APP. Note that the threonine at 668 position, and the endocytic YENPTY motif (dashed boxes) are thought to play roles in Aβ production (see text). (FIG. **5**D) APP/BACE-1 interaction—as evaluated by fluorescence complementation in cultured hippocampal neurons—was most markedly attenuated in neurons transfected with mutant YENPTY (mean±SEM of 32 cells for APP(WT), 37 cells for APP(T668A), 45 cells for APP(YENPTY) and 49 cells for APP(T668A+YENPTY) from two independent experiments). One-way ANOVA: p<0.0001. Tukey's multiple comparisons: p=0.0022 (APP vs APP^{T668A}); p<0.0001 (APP vs APP^{YENPTY}); p<0.0001 (APP vs $\text{APP}^{T668A+YENPTY}$); <0.0001 ($\text{APP}^{T668A}$ vs APP^{YENPTY}); p<0.0001 (APP^{T668A} vs $\text{APP}^{T668A+YENPTY}$); p=0.7568 ($\text{APP}^{YENPTY}$ vs $\text{APP}^{T668A+YENPTY}$). (FIG. **5**E) Strategy of APP internalization assay. Neuro 2a cells are transfected with APP:GFP or APP659:GFP. After incubation with anti N-terminal APP antibody (22C11) for 10 min, the cells were fixed and stained with secondary antibody to visualize the cell surface and internalized APP. Note the cell surface accumulation and decreased internalization of APP659 (mean±SEM of 21 cells from two independent experiments, p<0.0001).

FIGS. **6**A-**6**E show the choice of CRISPR editing site at APP C-terminus. (FIG. **6**A) Strategy to integrate APP:VN and BACE-1:VC into the H4 genome and generation of a stable cell line expressing single copies of the two proteins (see results and methods for details). (FIG. **6**B) APP and BACE-1 expression in the $\text{H4}^{single\ copy}$ cell line. Note negligible expression of endogenous proteins in native H4 cells. (FIG. **6**C) The $\text{H4}^{single\ copy}$ cell line was transduced with lentiviral vectors carrying non-targeting control-sgRNA/Cas9 or various human APP C-terminus targeting sgRNAs/Cas9 (see Table 5 for targeting sequences). The APP/BACE-1 Venus complementation was visualized by fluorescence microscopy. Note attenuation of complementation, indicating editing by the APP-sgRNAs (quantified on right, mean±SEM of three independent experiments). One-way ANOVA: p<0.0001. Tukey's multiple comparisons: p<0.0001 (control-sgRNA vs APP659-sgRNA); p<0.0001 (control-sgRNA vs APP670-sgRNA); p<0.0001 (control-sgRNA vs APP676-sgRNA); p=0.0064 (APP659-sgRNA vs APP670-sgRNA); p=0.0015 (APP659-sgRNA vs APP676-sgRNA); p=0.6207 (APP670-sgRNA vs APP676-sgRNA). (FIG. **6**D) ELISA of media from the $\text{H4}^{single\ copy}$ cell line (treated as above). Note decreased Aβ in the APP-sgRNAs treated samples (mean±SEM of three independent experiments). One-way ANOVA for Aβ 40 and 42: p<0.0001. Tukey's multiple comparisons for Aβ 40: p<0.0001 (control-sgRNA vs APP659-sgRNA; control-sgRNA vs APP670-sgRNA; control-sgRNA vs APP676-sgRNA); p=0.0331 (APP659-sgRNA vs APP670-sgRNA); p=0.0071 (APP659-sgRNA vs APP676-sgRNA); p=0.6673 (APP670-sgRNA vs APP676-sgRNA). Tukey's multiple comparisons for Aβ 42: p<0.0001 (control-sgRNA vs APP659-sgRNA; control-sgRNA vs APP670-sgRNA; control-sgRNA vs APP676-sgRNA); p=0.0068 (APP659-sgRNA vs APP670-sgRNA); p=0.0221 (APP659-sgRNA vs APP676-sgRNA); p=0.8079 (APP670-sgRNA vs APP676-sgRNA). (FIG. **6**E) HEK cells were transduced by lentiviral vectors carrying APP-sgRNAs and Cas9 (or non-targeting control-sgRNA/Cas9 as control), and APP C-terminus was sequenced. Left: Deep sequencing of APP659-sgRNA treated cells, and Sanger sequencing followed by ICE analyses for APP670-sgRNA and APP676-sgRNA treated cells. Red underlines mark the sgRNA-targeting sequences and arrowheads denote predicted cut-sites. Right: Predicted APP translational products after CRISPR/Cas9 editing in human HEK cells for the major mutant alleles observed in sequencing analyses. Red arrowheads indicate the amino acids where APP genes were translated up to after editing.

FIGS. **7**A-**7**D show evaluation of CRISPR editing by immunoblotting in mouse Neuro2a cells. (FIG. **7**A) Neuro2a cells were co-transfected with a sgRNA that knocked out the entire APP gene and Cas9 (see Table 5 for APP targeting sequence), and immunostained with APP N-terminal and C-terminal antibodies (after 5 days in culture). Note attenuation of staining for both Y188 and 22C11. (FIG. **7**B) Neuro2a cells were transfected with various APP C-terminus targeting sgRNAs (or non-targeting control-sgRNA), and immunostained with APP N-terminal and C-terminal antibodies (after 5 days in culture in the presence of GSI). Note attenuation of staining by Y188 but not 22C11, indicating selective editing of the APP C-terminus. (FIG. **7**C) Neuro2A cells were transduced by lentiviral vectors carrying APP-sgRNA and Cas9 (or non-targeting control-sgRNA/Cas9 as control) and immunoblotted with the APP antibodies CT20 and M3.2 (CT20 recognizes last 20 aa; M3.2 recognizes an extracellular domain located upstream of the CRISPR/Cas9 targeting site). A GSI was added to allow detection of accumulated APP CTF's. Note attenuated signal with CT20- but not M3.2-antibody, indicating selective editing of the APP C-terminus. (FIG. **7**D) Post-editing translational products in mouse (neuro 2a) cells. Note effective truncation of APP at aa 659.

FIGS. **8**A-**8**G show APP C-terminus editing by CRISPR/Cas9. (FIG. **8**A) HEK cells were transfected with human-specific APP-sgRNA and Cas9 (or Cas9 only), and immunostained with the Y188 antibody (after 5 days in culture). Note attenuation of staining, quantified on right (mean±SEM of 25 cells for Cas9 only and 43 cells for huAPP-sgRNA from two independent experiments, $p<0.0001$). (FIG. **8**B) HEK cells were transduced by lentiviral vectors carrying APP-sgRNA and Cas9 (or non-targeting control-sgRNA/Cas9 as control) and immunoblotted with the Y188 and 22C11 antibodies (in the presence of GSI). Note attenuation of APP-CTFs in APP-sgRNA treated cells, indicating CRISPR-editing (mean±SEM of three independent experiments, $p<0.0001$). (FIG. **8**C) HEK cells above were immunoblotted with CT20 and 2E9 antibodies (CT20 recognizes last 20 aa; 2E9 recognizes APP extracellular domain upstream of the CRISPR/Cas9 targeting site). Note attenuated signal with CT20- but not 2E9-antibody, indicating selective editing of the APP C-terminus. (FIGS. **8**D-**8**E) Human ESCs were transduced by lentiviral vectors carrying human APP-sgRNA/Cas9 (or non-targeting sgRNA/Cas9). Samples were immunostained with the Y188 antibody (d) or immunoblotted with the Y188 and 22C11 antibodies (e). Note attenuation of APP-CTFs in sgRNA-transduced group (for immunostaining, mean±SEM of 17 colonies for control-sgRNA and 20 colonies for huAPP-sgRNA from two independent experiments, $p<0.0001$; for western blotting, mean±SEM of three independent experiments, $p=0.001$ for total APP and $p<0.0001$ for CTFs). (FIG. **8**F) Media from iPSC derived neurons were immunoblotted for extracellular sAPPβ (in the absence of GSI). Note decrease in APP β-cleavage in the APP-sgRNA treated samples. (FIG. **8**G) Media from $H4^{single\text{-}copy}$ cells were immunoblotted for extracellular sAPPα with 6E10 antibody and sAPPβ (in the absence of GSI). Note enhanced APP α-cleavage and attenuated APP β-cleavage in the APP-sgRNA treated samples.

FIGS. **9**A-**9**C show gene editing by APP-sgRNA likely does not influence APP γ-cleavage. (FIG. **9**A) Strategy to evaluate γ-cleavage of post-edited APP. Neuro2a cells were transfected with either full length (FL) C99, or C99 truncated at aa 659 (to mimic the post-editing translational product; all constructs were GFP-tagged to confirm expression). γ-cleavage of the FL and 659 C99 was evaluated by western blotting (note that neuro2a cells have all components of the γ-secretase complex). (FIG. **9**B) Schematic showing expected C99-cleavage patterns. Note that upon γ-cleavage, both C99-fragments will be further truncated. However, if the 'CRISPR-mimic' (659) fragment did not undergo γ-cleavage, this truncation would not occur. (FIG. **9**C) Western blotting of the cells from (a) indicates that both C99 fragments (FL and 659) undergo γ-cleavage—as indicated by the shift upon inhibiting γ-cleavage by GSI. These data suggest that gene editing by the APP-gRNA likely does not affect APP γ-cleavage, and that the effects seen on the amyloid pathway are likely due to modulation of APP-β-cleavage.

FIGS. **10**A-**10**G show off target analyses of APP-sgRNA. (FIG. **10**A) Computationally predicted top five off-target (OT) sites in the genome, that can be potentially targeted by the mouse and human APP-sgRNAs (mismatched nucleotides in the targeting sequence are marked in red). Genomic locations corresponding to the sequences is shown on the right column (note most are in non-coding regions). (FIG. **10**B) Strategy of T7 endonuclease digestion assay to detect genome-editing events. Genomic DNA was PCR amplified with primers bracketing the modified locus. PCR products were then rehybridized, yielding three possible structures. Duplexes containing a mismatch were digested by T7 endonuclease I. DNA gel analysis was used to calculate targeting efficiency. Note digested fragments in the gel indicates cleavage. (FIG. **10**C) Gene edits at the APP locus by the APP-sgRNA, as seen by T7 endonuclease digestion. Note two digested fragments were recognized after T7 endonuclease digestion. (FIGS. **10**D-**10**E) T7 endonuclease assays of potential off-target sites (mouse and human). No digested fragments are seen, indicating that the sgRNAs do not generate detectable gene edits at these sites. (FIG. **10**F) Comparison of APLP1 and 2 sequences with APP at the sgRNA targeting site. Asterisks mark conserved nucleotide sequences, and the PAM sites are underlined. Nucleotide mis-matches are highlighted in yellow. Note extensive mismatch of the mouse and human sequences at the sgRNA targeting site. (FIG. **10**G) Left: Off-target TIDE analysis of APP family members APLP1 and 2 in mouse (neuro 2a) and human (HEK) cell lines following lentiviral integration of Cas9 using TIDE. No modifications were detected below the TIDE limit of detection (dotted line) in either of the populations, indicating that the APP-gRNA was unable to edit APLP 1/2. Right: TIDE analysis of APLP1 and 2 loci in mouse and human cell lines. Neither of the populations had significant editing at either of the two loci, and all sequences had a near perfect correlation to the model.

FIGS. **11**A-**11**C show trafficking of vesicles carrying APP(WT) or APP(659). (FIG. **11**A) Cultured hippocampal neurons were transfected with APP(WT):GFP or APP(659):GFP, and kinetics of APP particles were imaged live in axons and dendrites. (FIG. **11**B) Representative kymographs and quantification of APP kinetics in axons. Note that there was no change in frequency of transport, and only a modest reduction in run-length and velocity. Error bars, mean±SEM of 325 APP(WT):GFP and 310 APP(659):GFP vesicles in 10-12 neurons from two independent experiments. Frequency: $p=0.4635$ (APP_antero vs APP659_antero); $p=0.6650$ (APP_retro vs APP659_retro); $p=0.7420$ (APP_stat vs APP659 stat). Velocity: $P<0.0001$ (APP_antero vs APP659_antero); $p=0.9419$ (APP_retro vs APP659_retro). Run length: $p<0.0001$ (APP_antero vs APP659_antero); $p=0.2433$ (APP_retro vs APP659_retro). (FIG. **11**C) Representative kymographs and quantification of APP kinetics in dendrites. Error bars, mean±SEM of 130 APP(WT):GFP and 115 APP(659):GFP particles in 10-12 neurons from two independent experiments. Frequency: $p=0.3245$ (APP_antero vs APP659_antero); $p=0.5438$ (APP_retro vs APP659_retro); $p=0.2394$ (APP_stat vs APP659_stat). Velocity: $p=0.0120$ (APP_antero vs APP659_antero); $p=0.6248$ (APP_retro vs APP659_retro). Run length: $p=0.1352$ (APP_antero vs APP659_antero); $p=0.4284$ (APP_retro vs APP659_retro).

FIGS. **12**A-**12**C show internalization of APP-659-GG (most common post-editing translational product). (FIGS. **12**A-**12**B) Neuro2a cells were co-transfected with untagged APP-659-GG and mCherry (or untagged WT APP and mCherry as control). After incubation with anti N-terminal APP antibody (22C11) for 10 min, the cells were fixed and stained with secondary antibody to visualize surface and internalized APP (mCherry labels transfected cells). Note accumulation of APP-659-GG on the cell surface, along with decreased internalization; quantified in FIG. **12**B. Mean±SEM of 25 cells for APP(WT) and 26 cells for APP-659-GG from two independent experiments, $p<0.0001$. (FIG. **12**C) Expression levels of exogenous APP constructs. Note that WT and APP-659-GG were expressed at similar levels in the Neuro2a cells above.

FIG. **13** shows the sequence of human APP (nucleotide sequence SEQ ID NO:11, amino acid sequence SEQ ID NO:12) and mouse APP (nucleotide sequence SEQ ID NO:13, amino acid sequence SEQ ID NO:14) along with the corresponding sequences of the gRNA used in select gene editing embodiments described herein.

FIG. **14** shows the sequence of the Cas9 nuclease gene sequence.

FIG. **15** shows the sequence and vector map of an exemplary vector (SEQ ID NO:17) for APP truncation at amino acid 659. The vector includes the gRNA sequence (lowercase italics) and the Cas9 nuclease sequence.

FIG. **16** shows the sequence and vector map of an exemplary Cas9 vector (SEQ ID NO:18).

FIG. **17** shows the sequence and vector map of an exemplary APP sgRNA vector (SEQ ID NO:19).

FIG. **18** shows the sequence and vector map of an exemplary lentiviral APP sgRNA vector (SEQ ID NO:20).

INCORPORATION BY REFERENCE

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though set forth in their entirety in the present application.

DETAILED DESCRIPTION OF THE INVENTION

In General

Gene-editing methods, such as CRISPR/Cas9 guided gene-editing, hold promise as a therapeutic tool. However, few studies have applied the technology to neurodegenerative diseases. Moreover, the conventional approach of mutation-correction is limited in scope to inherited diseases which are a small fraction of neurodegenerative disease cases. The present invention introduces a strategy to edit endogenous amyloid precursor protein (APP) at the extreme C-terminus and selectively attenuate the amyloidogenic pathway—a key pathologic cascade in Alzheimer's disease (AD). In the method of the present invention, the APP N-terminus remains intact and protective α-cleavage is up-regulated.

The Examples below demonstrate that robust APP-editing is demonstrated in cell lines, human stem cells, cultured neurons, and in mouse brains. Physiologic parameters remain unaffected. Without being bound by any particular theory, the present invention works by restricting the physical interaction of APP and BACE-1, said interaction being the rate-limiting step in amyloid-β (Aβ) production. The Examples below delineate underlying mechanisms that abrogate APP/BACE-1 interaction in this setting. The invention offers an innovative 'cut and silence' gene-editing strategy that could be a new therapeutic paradigm for AD.

CRISPR/Cas9 works by inducing sequence-specific double-stranded breaks (DSBs) in DNA. After such breaks, the cell undergoes an error-prone repair process called non-homologous end joining, leading to a disruption in the translational reading frame, often resulting in frameshift mutations and premature stop codons. For the system to work, at least two components must be introduced in cells: a Cas9 nuclease and a guide RNA. Described herein are CRISPR/Cas9 constructs suitable for truncation of the APP protein and disruption of amyloid-β production.

Constructs of the Present Invention

In a first aspect, the present invention provides a construct for CRISPR mediated cleavage of the APP gene. The constructs of the present invention include a nucleotide sequence encoding a Cas9 nuclease and a guide RNA (gRNA). In some embodiments the sequence encoding the Cas9 nuclease and the gRNA are included on a single vector construct. In some embodiments the sequence encoding the Cas9 nuclease is included in a vector construct separate from a vector construct encoding for the gRNA. Additionally, the construct may include a promoter, a poly(A) tail, an optional reporter element, and an optional selection marker such as an ampicillin selection marker.

As used herein "Cas9 nuclease" refers to the RNA-guided DNA endonuclease enzyme associated with the CRISPR adaptive immunity system in *Streptococcus pyogenes* and other bacteria. The Cas9 nuclease includes two nuclease domains, a RuvC-like nuclease domain located at the amino terminus, and a HNH-like nuclease domain. In some embodiments, the sequence of the Cas9 nuclease is the sequence included in FIG. **14** (SEQ ID NO:15).

In some embodiments, the Cas nuclease is expressed under the control of a neuron specific promoter or ubiquitous promoter. The neuron specific promoter may be any neuron specific promoter known in the art (see for example, Swiech L et al., In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nature Biotechnology 2015 January; 33(1): 102-6). In some embodiments the neuron specific promoter is the human synapsin 1 (hSyn1) promoter. In some embodiments the neuron specific promoter is the mouse Mecp2 promoter (pMecp2). In some embodiments the ubiquitous promoter is the chicken β-actin promoter. In some embodiments, the ubiquitous promoter is an EFS promoter.

In one embodiment of the present invention, the construct is specific for the extreme C-terminus of the APP gene. By "APP gene" or "amyloid precursor protein", we mean to include the human APP gene as disclosed in Hendricks et al (Hendriks L et al. Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the beta-amyloid precursor protein gene. Nature Genetics 1992 June; 1(3): 218-21) and recited herein as SEQ ID NO:11. The amino acid sequence of the APP gene is recited as SEQ ID NO:12.

As used herein "extreme C-terminus," refers of a portion of the C-terminus of the APP protein which, when absent, is sufficient to disrupt the interaction between APP and BACE. The truncated APP lacking the extreme C-terminus will still include its native N-terminus, the transmembrane domain and the residual C-terminal region. Typically, the extreme C-terminus of the APP protein will mean 8 or more amino acids at the C-terminus of the APP protein. This may be accomplished by CRISPR/Cas9 mediated cleavage of the APP gene such that the expressed APP protein is truncated to a length selected from the group consisting of 659, 670, 676, or 686, relative to SEQ ID NO:12 (human) or SEQ ID NO:14 (mouse). In some embodiments, the APP gene is cleaved following a nucleotide selected from the group consisting of 1978, 2009, 2010, 2029, and 2058 relative to SEQ ID NO:11 (human) or SEQ ID NO:14 (mouse). A list of these cleavage sites is included in the table below.

As used herein "guide RNA (gRNA)" refers to the 20 nucleotide target sequence which directs Cas9 mediated cleavage within the APP gene. The gRNA will be encoded on a synthetic RNA construct which additionally includes the tracrRNA sequence. While the gRNA sequence is variable and will be specific for the cleavage site of interest, the tracrRNA is the same for all gRNA sequences used. The tracrRNA sequence is SEQ ID NO:16 The gRNA described herein are specific for the truncation of the C-terminal segment of APP. Suitable target sequences within the APP gene for design of gRNA sequences are recited below, which includes the sequence of the gRNA.

tracrRNA (SEQ ID NO:16):
5'-GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC

CGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT-3

TABLE 1

Human APP (full length 695 aa)					
sgRNA name	sgRNA sequence (5'-3')	SEQ ID NO:	Position relative to APP	Cas9 cleavage between nucleo-tides	Length of trunca-ted protein
sgRNA 1	atccattcat catggtgtgg	1	1962-1981	1978/1979	659 aa
sgRNA 2	tggacaggtg gcgctcctct	2	2007-2026	2009/2010	670 aa
sgRNA 3	ttggacaggt ggcgctcctc	3	2008-2027	2010/2011	670 aa
sgRNA 4	gtagccgttc tgctgcatct	4	2027-2046	2029/2030	676 aa
sgRNA 5	tgctcaaaga acttgtaggt	5	2056-2075	2058/2059	686 aa

TABLE 2

Mouse APP (full length 695 aa)					
sgRNA name	sgRNA sequence (5'-3')	SEQ ID NO:	Position relative to APP	Cas9 cleavage between nucleo-tides	Length of trunca-ted protein
sgRNA 1	atccatccat catggcgtgg	6	1962-1981	1978/1979	659 aa
sgRNA 2	tggagagatg gcgctcctct	7	2007-2026	2009/2010	670 aa
sgRNA 3	ttggagagat ggcgctcctc	8	2008-2027	2010/2011	670 aa
sgRNA 4	atatccgttc tgctgcatct	9	2027-2046	2029/2030	676 aa
sgRNA 5	tgctcaaaga acttgtaagt	10	2056-2075	2058/2059	686 aa

Cleavage of the APP gene will occur between the 3rd and 4th nucleotides from the PAM site associated with the target sequence in the APP gene. For the sgRNA 1, the PAM site is on the sense strand of the APP gene, the sgRNA of SEQ ID NOs:1 and 6 are complementary to the antisense strand of the APP gene, and the cleavage will occur between nucleotides 1978 and 1979 relative to SEQ ID NO:11 (human) or SEQ ID NO:14 (mouse). For sgRNA 2, 3, 4 and 5, the PAM site is on the antisense strand of the APP gene, the sgRNA of SEQ ID NOs:2-5 and 7-10 are complementary to the sense strand of the APP gene, and the cleavage site is between nucleotides 2009 and 2010 for sgRNA 2, between nucleotides 2010 and 2011 for sgRNA 3, between nucleotides 2029 and 2030 for sgRNA 4 and between nucleotides 2058 and 2059 for sgRNA 5, relative to SEQ ID NO:11 (human) or SEQ ID NO:14 (mouse).

In some embodiments, the gRNA or tracrRNA is modified by any means known in the art. Common methods for gRNA or tracrRNA modification include chemical modifications or modifications to axillary sequences appended to the RNA to increase efficiency known in the art.

In some embodiments, the gRNA is expressed under the control of an RNA Pol III promoter. Examples of RNA Pol III promoters include, but are not limited to, U6 and H1 promoters. A promoter, generally, is a region of nucleic acid that initiates transcription of a nucleic acid encoding a product. A promoter may be located upstream (e.g., 0 bp to −100 bp, −30 bp, −75 bp, or −90 bp) from the transcriptional start site of a nucleic acid encoding a product, or a transcription start site may be located within a promoter. A promoter may have a length of 100-1000 nucleotide base pairs, or 50-2000 nucleotide base pairs. In some embodiments, promoters have a length of at least 2 kilobases (e.g., 2-5 kb, 2-4 kb, or 2-3 kb).

In some embodiments, the construct comprises an optional reporter element. The reporter element may be any reporter known in the art including, but not limited to, mCherry, green fluorescent protein, and human influenza hemagglutinin (HA).

In some embodiments, the constructs are packaged in a vector suitable for delivery into a mammalian cell, including but not limited to, an adeno-associated viral (AAV) vector, a lentiviral vector, or a vector suitable for transient transfection. Suitable vector backbones are known and commercially available in the art. For example, see Deverman et al. (Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain, Nature Biotechnology, 34(2):204-209, 2016) and Chan et al. (Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous system, Nature Neuroscience, 20(8): 1172-1179, 2017) which are incorporated herein by reference in their entirety. In some embodiments, the vector is an AAV vector and the gRNA and Cas9 constructs are encoded on separate vectors. In some embodiments, the vector is a lentiviral vector and the gRNA and Cas9 constructs are encoded on a single vector. In some embodiments, the vector is a vector suitable for transient transfection and the gRNA and Cas9 constructs are encoded on a single vector. In one embodiment the vector includes the sequence of SEQ ID NO:17. In some embodiment, the gRNA and Cas9 constructs are encoded on separate AAV vectors wherein the gRNA is encoded on a vector comprising SEQ ID NO:19 and the Cas9 construct is encoded on a vector comprising SEQ ID NO:18. In some embodiments, the vector is a lentiviral vector and comprises the sequence of SEQ ID NO:20. The vectors of SEQ ID NOs:17-20 are included in FIGS. **15-18**.

In some embodiments, the vectors encoding the constructs described herein may optionally include a monoclonal antibody tag (e.g., FLAG), one or more origins of replication (e.g., fl ori), one or more terminator sequences (e.g. bGH), one or more polyadenylation tags (bGH poly (A)), and one or more inverted terminal repeats (ITR). The vector may also include one or more selectable markers, such as an antibacterial resistance marker such as an ampicillin selectable marker. A skilled artisan will be familiar with the elements and configurations necessary for vector construction to encode the constructs described herein.

Methods of the Present Invention

The constructs described herein may be formulated with a pharmaceutically acceptable carrier for administration to a patient in need thereof. A pharmaceutically acceptable carrier may be, but is not limited to, a nanoparticle cage including the one or more vectors of the present invention.

To function as therapeutic agents, the constructs described herein are delivered into neurons in the patient's brain, crossing the blood brain barrier (BBB). In one embodiment, one would attach or associate the CRISPR components with a delivery system, such as a nanoparticle delivery system. In some embodiments, the constructs are formulated using an AAV vector and are delivered intravenously. In some embodiments, the constructs are delivered intrathecally into the spinal fluid of the patient. In some embodiments, the constructs are deliver directly into the brain of the patient.

As used herein, the terms "treat" and "treating" refer to therapeutic measures, wherein the object is to slow down or alleviate (lessen) an undesired physiological change or pathological disorder resulting from Alzheimer's disease. For purposes of this invention, treating the disease, condition, or injury includes, without limitation, alleviating one or more clinical indications, reducing the severity of one or more clinical indications of Alzheimer's disease, diminishing the extent of the condition, stabilizing the subject's Alzheimer's disease (i.e., not worsening), delay or slowing, halting, or reversing Alzheimer's disease and bringing about partial or complete remission Alzheimer's disease. Treating Alzheimer's disease also includes prolonging survival by days, weeks, months, or years as compared to prognosis if treated according to standard medical practice not incorporating treatment with the constructs described herein.

Subjects in need of treatment can include those already having or diagnosed with Alzheimer's disease as well as those prone to, likely to develop, or suspected of having Alzheimer's disease, such as a subject with a genetic predisposition to or family history of Alzheimer's disease. Subjects in need to treatment may be those with a familial AD mutation or wild-type patients without a mutation. In some embodiments, a subject in need of treatment may be a subject who had been diagnosed by a positron emission tomography (PET) scan, a blood test or other means known in the art to have AD or to be predisposed to AD. Pre-treating or preventing Alzheimer's disease according to a method of the present invention includes initiating the administration of a therapeutic (e.g., the APP gRNA and Cas9 constructs described herein) at a time prior to the appearance or existence of the disease or injury, or prior to the exposure of a subject to factors known to induce Alzheimer's disease. Pre-treating the disorder is particularly applicable to subjects at risk of having or acquiring the disease injury.

As used herein, the terms "prevent" and "preventing" refer to prophylactic or preventive measures intended to inhibit undesirable physiological changes or the development of Alzheimer's disease. In exemplary embodiments, preventing Alzheimer's disease comprises initiating the administration of a therapeutic (e.g., the APP gRNA and Cas9 constructs described herein) at a time prior to the appearance or existence of Alzheimer's disease such that the disease, or its symptoms, pathological features, consequences, or adverse effects do not occur. In such cases, a method of the invention for preventing Alzheimer's disease comprises administering the APP gRNA and Cas9 constructs described herein to a subject in need thereof prior to the onset or development of Alzheimer's disease in a patient at risk for Alzheimer's disease such as a patient with a genetic risk factor or a patient with a family history of Alzheimer's disease.

As used herein, the terms "subject" or "patient" are used interchangeably and can encompass a human or mouse. As used herein, the phrase "in need thereof" indicates the state of the subject, wherein therapeutic or preventative measures are desirable. Such a state can include, but is not limited to, subjects having Alzheimer's disease or a pathological symptom or feature associated with Alzheimer's disease.

Examples

The embodiment described in this example demonstrates truncation of the C-terminus of the APP protein, attenuation of APP-β-cleavage and Aβ production, and manipulation of the amyloid pathway using CRISPR/Cas9 gene editing.

A common theme in neurodegenerative diseases is that proteins normally present in the brain (APP, tau, α-synuclein, TDP-43, etc.) acquire toxic properties—or trigger pathologic cascades—that ultimately lead to synaptic loss and neurodegeneration. Our broad idea is to rationally edit small segments of endogenous proteins known to play key roles in the progression of disease, with the ultimate goal of attenuating their pathologic activity. As endogenous proteins expectedly play physiologic roles, it is also important to conserve their normal function, as far as possible. Here we show conceptual proof of this 'selective silencing' approach for APP. APP is a single-pass transmembrane protein, cleaved by the enzymes β- and γ-secretases to ultimately generate Aβ—a neuropathologic hallmark of AD. APP cleavage by the β-secretase BACE-1 is the rate limiting step in this 'amyloidogenic' pathway. Alternatively, APP is cleaved by α-secretases—the 'non-amyloidogenic' pathway—that is thought to be neuroprotective because it precludes β-cleavage of APP (6,7); and studies have highlighted neuroprotective effects of APP-α-cleavage products in vivo (8,9).

We recently developed a Bi-molecular fluorescence complementation (BifC) assay to visualize the physical approximation of APP and BACE-1 in neurons (10). As a control for validation, we found that a C-terminal deletion also abrogated APP/BACE-1 complementation (10); in line with previous studies showing that deletions/mutations of the APP C-terminus can attenuate Aβ production (11-13). Collectively, these observations originally gave us the idea of using CRISPR/Cas9-mediated truncation of native APP to attenuate APP-β-cleavage and Aβ production in AD. Using CRISPR-tools, cell/molecular biology, live imaging, deep sequencing, electrophysiology and in vivo animal studies, here we highlight a strategy to favorably manipulate the amyloid pathway by gene editing.

Results and Discussion

CRISPR/Cas9 editing of APP C-terminus—The CRISPR/Cas9 system consists of a Cas9 nuclease enzyme that generates double-stranded breaks in DNA, and a custom-designed single guide-RNA (sgRNA) that targets the Cas9 to specific sites in the host genomic DNA. Typically, the synthetic sgRNAs are complementary to stretches of genomic DNA containing 3-nt PAM (protospacer adjacent motif) and flanking 20-nt sequences. Since subsequent repair after DNA-breaks is naturally error-prone, insertions and deletions (indels) are generated at the cut-sites, leading to disruption of the translational reading frame and effectively truncated proteins (reviewed in 14). We identified three PAM sites at the APP C-terminus that are conserved in both human and mouse, and synthesized sgRNAs targeting these regions (FIG. 1A). To compare the editing efficiency of these sgRNAs, we engineered a stable H4 neuroglioma cell line expressing single copies of APP:VN and BACE-1:VC ($APP/BACE^{single\_copy}$), where editing efficiency of a given sgRNA could be determined as a simple fluorescence on/off readout and the effect of APP truncation could be assessed by evaluating secreted Aβ (for details, see FIGS. **6**A and **6**B and methods). The APP-sgRNA predicted to cut human APP at the 659 aa. (amino acid) position was the most efficient—both in editing APP as well as in attenuating Aβ—and also led to minimal indels (FIGS. **6**C-**6**E). Accordingly, we used the APP659-sgRNA for further characterization (henceforth called 'mo-APP-sgRNA' or 'hu-APP-sgRNA' representing mouse and human specific sequences).

The TGG PAM and preceding 20-nt genomic target sequence recognized by the mo-APP-sgRNA is shown in FIG. **1**A (top right); and FIGS. **1**B-**1**F shows gene editing by this sgRNA in mouse cells. Note that upon editing, the Y188 antibody—recognizing the last 20 amino acids of APP—would not be able to identify the resultant translational product. Robust editing of endogenous APP was seen in mouse neuroblastoma cells, as determined by attenuation of immunofluorescence with the Y188 antibody (FIG. **1**B), and decreased Y188-signal in western blots (FIGS. **1**C-**1**D; FIG. **1**E shows time-course of editing). Note that the edited APP is recognized by antibodies to the N-terminus, indicating selective editing of the C-terminus by the APP-sgRNA (FIGS. **1**C and **1**E). However, the N-terminus antibody was unable to detect APP when the entire gene was deleted (FIG. **7**A). Similar results were obtained with other sgRNAs targeting APP C-terminus and other C- and N-terminus APP antibodies (FIGS. **7**B and **7**C). Genomic deep-sequencing confirmed efficient editing of mouse APP at the expected loci, APP-659 (FIG. **1**F). Post-editing translational products show that the last 36 amino acids are effectively truncated by APP-sgRNA (FIG. **7**D). Though the TGG PAM at this site is conserved in both mouse and human APP, and the upstream sgRNA-targeting sequences only differ by two nucleotides (FIG. **2**A, arrowheads); the mouse APP-sgRNA was unable to edit human APP (not shown). However, a sgRNA specific to the human APP targeting sequence robustly edited APP in HEK293 (FIGS. **8**A-**8**C), as well as in human embryonic stem cells (FIGS. **8**D-**8**E). CRISPR editing of APP did not alter the steady-state levels of holo-APP (note data throughout with multiple N-terminus antibodies in various cell lines).

Reciprocal Manipulation of the APP β/α Pathway by CRISPR/Cas9 Editing—Next, we examined APP editing in human iPSC-derived neurons. As shown in FIG. **2**B, immunostaining with the Y188 antibody was attenuated in iPSC-neurons transduced by the hu-APP-sgRNA. To examine effects of APP editing in an "AD-like setting", we also tested the hu-APP-sgRNA in a heterozygous knock-in iPSC line carrying the most common familial AD mutation (APPV717I, also called the 'London mutation'; see methods for details of iPSC line). Both cell-lysates and supernatants were examined, to look for cellular and secreted APP products (see schematic in FIG. **2**C). Immunoblotting with the Y188 antibody confirmed robust—and C-terminus selective—APP editing in both WT and APP-London iPSC lines (FIG. **2**C). Examination of supernatants revealed that interestingly, APP-editing also led to increased sAPPα in both WT and London lines (FIG. **2**D); suggesting upregulation of the neuroprotective α-cleavage pathway. ELISAs and western blot showed attenuated secretion of Aβ40/42 (FIG. **2**E) and sAPPβ (FIG. **8**F), confirming inhibition of the amyloidogenic pathway in these neurons. Genomic deep sequencing showed efficient editing of human APP by the sgRNA, with truncation of the last 36 amino acids in human embryonic stem cells (FIGS. **2**F-**2**H).

The data from iPSC-neurons suggest that the APP-sgRNA has reciprocal effects on APP β- and α-cleavage. To validate this in a more controlled setting, we tested the effects of APP editing in the H4 APP/BACE$^{single\_copy}$ cell line, where APP-cleavage is tightly regulated. In line with the data from iPSC-neurons, the hu-APP-sgRNA had reciprocal effects on APP β- and α-cleavage in APP/BACE$^{single\_copy}$ cells as well, confirming that our editing strategy has reciprocal effects on β/α cleavage (FIG. **8**G). Further experiments using an APP-C99 construct (wild-type and truncated construct mimicking the CRISPR-product, APP-659) precludes an effect of the sgRNA on APP-γ-cleavage (FIGS. **9**A-**9**C), indicating that our editing strategy is selectively affecting APP β-cleavage. Collectively, the available data strongly suggest that our gene editing strategy targeting the APP C-terminus is favorably manipulating the amyloid pathway by attenuating APP β-cleavage, while reciprocally up-regulating protective α-cleavage.

Off-target analysis and effect of APP C-terminus editing on neuronal physiology—Off-target effects of CRISPR/Cas9, due to unwanted editing of DNA-stretches resembling the targeted region, are a concern. Towards this, we asked if our mouse and human APP-sgRNA were able to edit the top five computationally predicted off-target sites (FIG. **10**A; also see Table 3). No editing was seen using T7 endonuclease assays (FIGS. **10**B-**10**E). Though APP null mice are viable, there is compensation by the two APP homologues APLP1 and 2 that undergo similar processing as APP (15,16). APLP1 and 2 were not amongst the top 50 predicted off-target sites, as their corresponding sgRNA-target sites were substantially different from APP (see sequences in FIG. **10**F). For further assurance that our sgRNA was not editing the APP homologues, we performed specific off-target TIDE (Tracking of Indels by DEcomposition) analyses (17) on cells carrying the sgRNA. As shown in FIG. **10**G, TIDE analyses showed no editing of APLP 1/2 by the sgRNA.

APP has known physiologic roles in axon growth and signaling (18). As noted above, the N-terminus of APP—thought to play roles in axon growth and differentiation—is entirely preserved in our setting. The C-terminal APP intracellular domain (AICD) has been implicated in gene transcription, though the effect appears to be both physiologic and pathologic (19,20). To examine potential deleterious effects of editing the extreme C-terminus of APP, we turned to cultured hippocampal neurons where various parameters like neurite outgrowth and synaptic structure/function can be confidently evaluated. To study pre-synapse structure and neuronal activity, we generated AAV9 viruses carrying the mo-APP-sgRNA and Cas9, tagged with GFP and HA respectively (see vector design in FIG. **3**A) that transduced almost all cultured neurons (FIG. **3**B and data not shown). In blinded analyses, we found no significant effect of the mo-APP-sgRNA on neurite outgrowth, axon-length, synaptic organization, or neuronal activity (FIGS. **3**C-**3**G). We reason that the lack of deleterious effects upon editing is likely because: 1) most of the APP molecule remains intact after editing; 2) the APP homologues APLP1/2—that undergo similar processing as APP, generate CTFs, and are known to compensate for APP function—remain unedited; and 3) APP-cleavage is not entirely blocked by our approach.

Editing of APP C-terminus in vivo and mechanistic details of APP β/α manipulation—Next we asked if the APP-sgRNA could edit endogenous APP in mouse brains. Injection of the AAV9s into mouse hippocampi (FIG. **4**A) led to efficient transduction of both sgRNA and Cas9 in dentate neurons (86.87±2.83% neurons carrying the sgRNA also had Cas9; see representative images in FIG. **4**B). Immunostaining of transduced neurons with the APP Y188 antibody showed attenuated staining, suggesting editing of endogenous APP in vivo (FIGS. **4**C and **4**D). To achieve a more widespread expression of the sgRNA and Cas9 in mouse brains—and also evaluate editing by biochemistry—we injected the viruses into the ventricles of neonatal (P0) mice and examined the brain after 2-4 weeks (FIG. **4**E). Previous studies have shown that when AAVs are injected into the ventricles of neonatal mice, there is widespread delivery of transgenes into the brain—also called somatic transgenesis (21,22). Indeed, APP Y188 immunostaining was attenuated in cortical regions (FIG. **4**F) and immunoblotting with the Y188 antibody also showed a decreased signal (FIG. **4**G); indicating that the APP-sgRNA can edit APP in vivo.

To determine the mechanism by which the APP-sgRNA manipulates the amyloid pathway, we used a "CRISPR-mimic" truncated APP construct (APP-659) that is the major post-editing translational product in both mouse and human cells (see FIG. **2**H, FIG. **6**E, and FIG. **7**D). Using our BifC assay (10), we first asked if the CRISPR-mimic APP-659 interacted with BACE-1. APP-659/BACE-1 approximation was greatly attenuated in cultured neurons (FIG. **5**A), along with a decrease in β-CTF generation (FIG. **5**B). Next we visualized axonal and dendritic transport of APP-WT and APP-659. Although there were minor changes (FIGS. **11**A-**11**C and Table 4), it seems unlikely that such small transport perturbations would lead to the dramatic attenuation of β-cleavage and Aβ-production seen in our experiments.

The CRISPR-edited segment of APP contains the residues T668 and Y682-Y687 (YENPTY motif, see FIG. **5**C; also present in APLP1/2), that have been reported to play a role in A13 production (12, 23, 24). Specifically, APP phosphorylated at T668 has been reported to colocalize with BACE-1 in endosomes (23), and the YENPTY motif is known to mediate APP internalization from the plasma membrane (25). Examining the effects of these residues in APP/BACE-1 BifC assays, we saw that the extent of APP/BACE-1 attenuation by the YENPTY mutation strongly resembled the decrease in fluorescence complementation by the APP-659 construct (FIG. **5**D). A prediction from these experiments is that endocytosis of the CRISPR-mimic APP from the cell surface should be attenuated; and indeed, this was the case in internalization assays (FIG. **5**E). Similar results were seen with an "APP-659-GG" construct that more closely resembles the most common post-editing translational product of our sgRNA (FIGS. **12**A-**12**C; also see post-editing products from human cells in FIG. **2**H and FIG. **6**E).

Collectively, the data suggest that our gene-editing approach does not have a major effect on post-Golgi trafficking of APP, but attenuates its endocytosis from cell surface, and consequently, its interaction with BACE-1 in endosomes—though we cannot exclude a direct effect of editing on APP/BACE-1 interaction. This is also consistent with previous studies showing that surface APP is internalized into endosomes, where it is cleaved by BACE-1 (26-29). Since most of the APP α-cleavage is thought to occur at the cell surface (30), this may also explain why the non-amyloidogenic pathway is enhanced by our approach.

Using CRISPR/Cas9 technology, herein we provide conceptual proof for a strategy that selectively edits the C-terminus of APP and alters the balance of APP-cleavage—attenuating β-cleavage and Aβ, while upregulating neuroprotective α-cleavage. The N-terminus of APP—known to play physiologic roles—is unaffected, along with the compensatory APP homologues APLP1/2. No deleterious effects were seen in neurophysiologic parameters. Without wishing to be bound by any particular theory, our strategy likely works by editing the terminal YENPTY motif in APP that is responsible for its internalization, subsequent APP/BACE-1 association, and initiation of the amyloidogenic pathway; while retention of APP at the plasma membrane may facilitate the upregulation of APP α-cleavage.

APP processing is regulated by α-, β-, and γ-secretases; and the various cleavage products may play physiological functions that are not fully understood (31,32). Previous studies suggest that in vivo deletion of the APP C-terminus blocks APP β-cleavage without obvious effects on neuroanatomy, behavior and neuronal activity in adult mice (13). Notably, the APP homologues APLP 1/2 also have YENPTY motifs (15,16)—that can presumably undergo endocytosis and protein-protein interactions—and are expected to compensate for the loss of the C-terminus. The precise reasoning behind enhanced α-cleavage is unclear. We propose that retention of APP at the plasma membrane might be responsible, but we cannot rule out other causes, including off-target effects, and further detailed studies may provide clarity.

Methods

Constructs, antibodies and reagents—For transient co-expression of CRISPR/Cas9 components, APP sgRNA nucleotides were synthesized and cloned into pU6-(Bbs1)_CBh-Cas9-T2A-mCherry vector at Bbs1 site. For viral transduction, a dual vector system was used to deliver CRISPR/Cas9 components using AAV9 (33). For making the AAV9 vectors, the APP sgRNA was cloned into pAAV9-U6sgRNA(SapI)_hSyn-GFP-KASH-bGH vector at Sap1 site. The CRISPR/Cas9 stable cell lines were generated by lentivirus infection as follows. The APP sgRNA was cloned into lentiCRISPR v2 vector at Bbs1 site to produce lentivirus (34). For making APP deletions and relevant constructs, the human APP659 truncation was PCR amplified and cloned at Hind3 and Sac2 sites of pVN to generate pAPP659:VN. The BBS-APP659 was PCR amplified and cloned into pBBS-APP:GFP at Hind3 and Sac2, replacing BBS-APP, to generate pBBS-APP659:GFP. The pBBS-APP^{YENFTY}:GFP was generated by site directed mutagenesis from pBBS-APP:GFP. The pAPP^{T668A}:VN and pAPP$^{T668A+YENPTY}$:VN were generated by site directed mutagenesis from pAPP:VN and pAPP^{YENPTY}:VN. Antibodies used were as follows: APP Y188 (ab32136; Abcam), APP 22C11 (MAB348; Millipore), APP 6E10 (803001; BioLegend), APP M3.2 (805701; BioLegend), APP 2E9 (MABN2295; Millipore), APP CT20 (171610; Millipore), sAPP0 (18957; IBL) BACE-1 (MAB931; R&D), GAPDH (MA5-15738, ThermoFisher), GFP (ab290, Abeam), GFP (A10262, Invitrogen), HA (901513, BioLegend), VAMP2 (104211, Synaptic Systems). Reagents were as follows: γ-secretase inhibitor BMS-299897 (Sigma), and Rho Kinase (ROCK)-inhibitor H-1152P (Calbiochem).

Cell cultures, transfections, viral production/infections, and biochemistry—HEK293 and neuro2a cells (ATCC) were maintained in DMEM with 10% FBS. Cells were transfected with Lipofectamine™ 2000 and collected 5 days after transfection for biochemical and immunostaining analysis. All the studies involving primary neuron culture were performed in accordance with University of Wisconsin guidelines. Primary hippocampal neurons were obtained from postnatal (P0-P1) CD1 mice (either sex), and transiently transfected using Lipofectamine™ 2000 or Amaxa™ 4D system (Lonza). Dissociated neurons were plated at a density of 30,000 cells/cm^2 on poly-D-lysine-coated glass-bottom culture dishes (Mattek) and maintained in Neurobasal™/B27 medium with 5% CO$_2$. For APP/BACE-1 interaction and APP transport studies, DIV 7 neurons were cultured for ~18-20 h after transfection. For spine density analysis, DIV7 neurons were transfected with Cas9, sgRNA and soluble marker, and cultured for 7 d before imaging. For testing the effect of CRISPR/Cas9 on neuronal development, neurons were electroporated with the respective constructs before plating using an Amaxa™ 4D-Nucleofector™ system with the P3 Primary Cell 4D-Nucleofector™ X kit S and program CL-133.

For western blotting, pre-synapse analyses and electrophysiology, DIV7 cultured neurons were infected with either AAV9-APP sgRNA-GFP (2.24×10^{13} Vg/ml) and AAV9-Cas9 (2.4×10" Vg/ml), or AAV9-GFP (2.58×10^{13} Vg/ml) and AAV9-Cas9 at a multiplicity of infection (MOI) of 1.5×10^{5}. Neurons were analyzed 7 days post-infection. Lentivirus was produced from HEK293FT cells as described (35). Briefly, HEK293FT cells (Life Technologies) were maintained in DMEM with 10% FBS, 0.1 mM NEAA, 1 mM sodium pyruvate and 2 mM Glutamine. Cells were transfected with lentiviral-target and helper plasmids at 80-90% confluency. 2 days after transfection, the supernatant was collected and filtered with 0.45 μm filter. For experiments with hESCs, cells were cultured on a Matrigel® substrate (BD Biosciences) and fed daily with TeSR-E8 culture media (StemCell Technologies). When the cells were around 60-70% confluent, they were infected with a 50/50 mixture of TeSR-E8 (with 1.0 μM H-1152P) and lentivirus supernatant. After 24 h, the virus was removed, and the cells were fed for 2 days (to recover). After 3 days, cells were treated with 0.33 μg/mL of puromycin for 72 h to select for virally-integrated hESCs. For HEK and neuro2a cell lines, cells were infected with the lentivirus carrying APP-sgRNA and Cas9 for 24 h. And then cells were fed for 1 day to recover. After 2 days, cells were treated with 1 μg/mL of puromycin for 72 h to select for virally-integrated cells.

Human NPCs were generated as has been described previously (36), using manual rosette selection and Matrigel® (Corning) to maintain them. Concentrated lentiviruses express control-sgRNA or APP-sgRNA were made as described previously (37), using Lenti-X™ concentrator (Clontech). The NPCs were transduced with either control-sgRNA or APP-sgRNA after Accutase® splitting and were submitted to puromycin selection the subsequent day. Polyclonal lines were expanded and treated with puromycin for 5 more days before banking. Neuronal differentiations were carried out by plating 165,000 cells/12 well-well in N2/B27 media (DMEM/F12 base) supplemented with BDNF (20 ng/mL; R&D) and laminin (1 ug/mL; Trevigen).

For biochemistry, cell lysates were prepared in PBS+ 0.15% Triton™ X-100 or RIPA supplemented with protease inhibitor cocktail, pH 7.4. After centrifuging at 12,000 g for 15 min at 4° C., supernatants were quantified and resolved by SDS-PAGE for western blot analysis. For sAPPα and sAPPβ detection, cell culture medium was collected and centrifuged at 2,000 g for 15 min at RT. The supernatants were resolved by SDS-PAGE for western blot analysis; band intensities were measured by ImageJ. Human Aβ40 and Aβ42 were detected using kits, according to the manufacturer's instructions (Thermo KHB3481 and KHB3544). Briefly, supernatants from $\mathrm{H4}^{single\ copy}$ cells or human iPSC derived neurons were collected and diluted (×5 for H4 and ×2 for iPSC-neuron). The diluted supernatants and the human A1340/42 detection antibodies were then added into well and incubated for 3 h at RT with shaking. After washing (×4), the anti-Rabbit IgG HRP solution was added and incubated for 30 min at RT. The stabilized Chromogen was added after washing (×4) and incubated for another 30 min

at RT in the dark. After addition of stop solution, absorbance at 450 nm was read using a luminescence microplate reader.

Developing a single-copy, stable APP/BACE-1 cell line—H4 tetOff FlpIn empty clone was maintained in OptiMEM® with 10% FBS, 200 μg/mL G418 and 300 μg/mL Zeocin. To generate an APP:VN/BACE-1:VC stable cell line carrying single copies of APP and BACE-1, the expressing plasmid and pOG44 plasmids were transfected with Lipofectamine™ 2000. 2 days after transfection, cells were selected with 200 μg/mL hygromycin B and 200 μg/mL G418 for 1 week. A monoclonal cell line with stable expression was selected. H4 stable cell lines were then infected with the lentivirus carrying APP-sgRNA and Cas9, as described above. After 24 h, the virus was removed, and cells were fed for 1 day to recover. After 2 days, cells were treated with 0.7 μg/mL of puromycin for 72 h to select for virally-integrated cells.

Generation of the APPLondon (V717I) knock-in iPSC line—CRIPSR/Cas9 was used to knock in the APP V717I mutation (APPLon) into a commercially available control human iPSC line IMR90 (clone 4, WiCell). sgRNAs targeting Exon17 of APP were designed using the CRISPR design tool created by Feng Zhang's lab and subcloned into the MLM3636 vector (AddGene). Efficacy of multiple sgRNAs was first assessed in HEK293 cells (Geneart™ Genomic Cleavage Detection Kit, Life Technologies). The ssDNA HDR template was designed to include a silent CRISPR blocking mutation at the PAM site of most efficacious sgRNA in addition to the APPLon mutation. sgRNA, Cas9-2A-mCherry (generously provided by Hynek Wicterle), and ssDNA HDR template were electroporated (Lonza Nucleofector™) into feeder-free IMR90 iPSCs, followed by cell sorting on mCherry signal and plating at low density on MEFs (MTI-GlobalStem). Individual clones were manually picked into a 96 well format, subsequently split into duplicate plates, one of which were used to generate gDNA as had been done previously[38]. For each clone, exon 17 of APP was amplified and initially screened by restriction digest for the presence of a de novo BclI site introduce by the APPLon mutation. Sanger sequencing was used to confirm the mutation, and successful knockin clones were expanded and banked. Potential off-target effects of CRISPR/Cas9 cleavage were analyzed by Sanger sequencing of the top 5 predicted off-target genomic locations, which demonstrated a lack of indels for multiple clones. Clone 88 was picked for future studies.

Immunofluorescence, microscopy/image analysis, APP trafficking and endocytosis assays—For immunostaining of endogenous APP or VAMP2, cells were fixed in 4% PFA/sucrose solution in PBS for 10 min at room temperature (RT), extracted in PBS containing 0.2% Triton™ X-100 for 10 min at RT, blocked for 2 h at RT in 1% bovine serum albumin and 5% FBS, and then incubated with rabbit anti-APP (1:200) or mouse anti-VAMP2 (1:1000) diluted in blocking buffer for 2 h at RT. After removal of primary antibody, cells were blocked for 30 min at RT, incubated with goat anti-rabbit (Alexa Fluor 488) or goat anti-mouse (Alexa Fluor® 594) secondary antibody at 1:1000 dilution for 1 h at RT and then mounted for imaging. z-stack images (0.339 μm z-step) were acquired using an inverted epifluorescence microscope (Eclipse Ti-E) equipped with CFI S Fluor VC 40× NA 1.30 (Nikon). An electron-multiplying charge-coupled device camera (QuantEM: 512SC; Photometrics) and LED illuminator (SPECTRA X; Lumencor) were used for all image acquisition. The system was controlled by Elements software (NIS Elements Advanced Research). z-stacks were subjected to a maximum intensity projection. For APP Y188 staining, the average intensity of single cell body (neuro2A, HEK293 and neurons) or the whole colony (hESCs) was quantified. All the images were analyzed in Metamorph® and ImageJ.

Spine density experiments were done as described previously (39). Briefly, DIV 7 neurons were transfected with desired constructs for 7 days, and secondary dendrites were selected for imaging. z-stack images were captured using a 100× objective (0.2 μm z-step) and subjected to a maximum intensity projection for analysis. For the APP/BACE-1 complementation assay, DIV 7 neurons were transfected with desired constructs for ~15-18 h and fixed. z-stack images were captured using a 40× objective (0.339 μm z-step) and subjected to a maximum intensity projection. The average intensity within cell bodies was quantified.

For trafficking studies in axons and dendrites, imaging parameters were set at 1 frame/s and total 200 frames. Kymographs were generated in MetaMorph®, and segmental tracks were traced on the kymographs using a line tool. The resultant velocity (distance/time) and run length data were obtained for each track, frequencies of particle movements were calculated by dividing the number of individual particles moving in a given direction by the total number of analyzed particles in the kymograph, and numbers of particles per minute were calculated by dividing the number of particles moving in a given direction by the total imaging time.

APP endocytosis assay was done as described previously (40). Cells expressing APP-GFP, APP659-GFP, untagged APP or untagged APP-659-GG were starved with serum-free medium for 30 min and incubated with anti-APP (22C11) in complete medium with 10 mM HEPES for 10 min. And then, cells were fixed, permeablized and immunostained for 22C11. The mean intensity of 22C11 along plasma membrane was calculated by dividing the total intensity along plasma membrane (=intensity of whole cell−intensity of cytoplasm) with area of plasma membrane (=area of whole cell−area of cytoplasm). The ratio of mean intensities between plasma membrane and cytoplasm was quantified.

Stereotactic injection of AAV9s into the mouse brain and histology—All the animal procedures were performed in accordance with University of Wisconsin guidelines. In vivo injection and immunofluorescence staining was done as described previously (41). Briefly, 1.5 μl of 1:2 AAV9 mixture of AAV9-APP sgRNA-GFP (or AAV9-GFP) and AAV9-Cas9 was injected into the dentate gyrus (−2.0, ±1.6, −1.9) of 8-week old male C57BL/6 mice (either sex). 2-weeks after surgery, the mice were sacrificed by transcardiac perfusion of saline, followed by 4% PFA. The brains were dissected, post-fixed with 4% PFA overnight, immersed in 30% sucrose until saturation, and sectioned at 40 μm. Sections were immunostained with the following antibodies: mouse anti-HA (1:1000, BioLegend, clone 16B12), chicken anti-GFP (1:1000, Invitrogen, polyclonal) and rabbit anti-APP (1:200, Abcam, clone Y188). Images were acquired using Zeiss LSM800 confocal microscope. Average intensities of APP staining in cell bodies was quantified using Metamorph®.

Intracerebroventricular injections and histology—All animal procedures were approved by the Mayo Institutional Animal Care and Use Committee and are in accordance with the NIH Guide for Care and Use of Laboratory animals. Free hand bilateral intracerebroventricular (ICV) injections were performed as previously described (42) in C57BL/6 mouse pups. On post-natal day 0, newborn pups were briefly cryoanesthetized on ice until no movement was observed. A 30-gauge needle attached to a 10 μl syringe (Hamilton) was used to pierce the skull of the pups just posterior to bregma and 2 mm lateral to the midline. The needle was held at a depth of approximately 2 millimeters, and 2 μl of a mixture of AAV9 viruses (ratio 1:2 of AAV9-APP sgRNA-GFP or AAV9-GFP+ AAV9-Cas9) were injected into each cerebral ventricle. After 5 minutes of recovery on a heat pad, the pups were returned into their home cages. Mice were sacrificed 15 days after viral injection. Animals were deeply anesthetized with sodium pentobarbital prior to transcardial perfusion with phosphate buffered saline (PBS), and the brain was removed and bisected along the midline. The left hemisphere was drop-fixed in 10% neutral buffered formalin (Fisher Scientific, Waltham, Mass.) overnight at 4° C. for histology, whereas the right hemisphere of each brain was snap-frozen and homogenized for biochemical analysis. Formalin fixed brains were embedded in paraffin wax, sectioned in a sagittal plane at 5-micron thickness, and mounted on glass slides. Tissue sections were then deparaffinized in xylene and rehydrated. Antigen retrieval was performed by steaming in distilled water for 30 min, followed by permeabilization with 0.5% Triton™-X, and blocking with 5% goat serum for 1 hour. Sagittal sections were then incubated with primary anti-GFP antibody (1:250, Ayes, chicken polyclonal) and anti-APP antibody (1:200, Abcam, clone Y188) overnight at 4° C. Sections were incubated with the secondary antibodies Alexa Fluor® 488-goat anti-chicken and Alexa Fluor®568-goat anti rabbit (1:500, Invitrogen) for 2h at room temperature. Sections were washed and briefly dipped into 0.3% Sudan Black in 70% ethanol prior to mounting.

Electrophysiology—A coverslip with cultured cells at a density of 60,000 cells/cm^2 was placed in a continuously perfused bath, viewed under IR-DIC optics and whole-cell voltage clamp recordings were performed (−70 mV, room temp.). The extracellular solution consisted of (in mM): 145 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES and 10 dextrose, adjusted to 7.3 pH with NaOH and 320 mOsm with sucrose. Whole-cell recordings were made with pipette solutions consisting of (in mM) 140 KCl, 10 EGTA, 10 HEPES, 2 Mg2ATP and 20 phosphocreatine, adjusted to pH 7.3 with KOH and 315 mOsm with sucrose. Excitatory synaptic events were isolated by adding 10 μM bicuculline to block GABA (subscript A) receptors. Miniature synaptic events were isolated by adding 100 nM tetrodotoxin to prevent action potentials. mEPSCs were detected using the template-matching algorithm in Axograph X, with a template that had 0.5 ms rise time and 5 ms decay. Statistics were computed using the Statistics Toolbox of Matlab.

T7 Endonuclease 1 Assay, Off-target, and ICE analyses—Genomic PCR was performed around each sgRNA target, and related off-target sites, following the manufacturer's instruction (using AccuPrime™ HiFi Taq using 500 ng of genomic DNA). Products were then purified using Wizard® SV Gel and PCR Clear-Up System (Promega), and quantified using a Qubit® 2.0 (Thermo Fischer). T7E1 assay was performed according to manufacturer's instructions (New England Biolabs). Briefly, 200 ng of genomic PCR was combined with 24, of NEBuffer™ 2 (New England Biolabs) and diluted to 19 μL. Products were then hybridized by denaturing at 95° C. for 5 minutes then ramped down to 85° C. at −2° C./second. This was followed by a second decrease to 25° C. at −0.1° C./second. To hybridized product, 1 μL T7E1 (M0302, New England Biolabs) was added and mixed well followed by incubation at 37° C. for 15 minutes. Reaction was stopped by adding 1.5 μL of 0.25M EDTA. Products were analyzed on a 3% agarose gel and quantified using a Gel Doc XR system (BioRad). Off-target sites were identified and scored using Benchling. The top 5 off-target sites—chosen on the basis of raw score and irrespective of being in a coding region—were identified and analyzed using T7E1 assay as previously described. For TIDE (43), PCR was performed on genomic DNA using Accuprime™ Taq HiFi (Thermo Fischer) according to manufacture specifications. Briefly, reactions were cycled at 2 min at 94° C. followed by 35 cycles of 98° C. for 30 seconds, 58° C. for 30 seconds, and 68° C. for 2 minutes 30 seconds and a final extension phase of 68° C. for 10 minutes. Products were then subjected to Sanger Sequencing and analyzed using the TIDE platform. The primers used for TIDE analyses are listed in Table 3. For analyses of indel after CRISPR editing with APP670-sgRNA and APP676-sgRNA, the edited regions of genomic DNA were PCR amplified and subjected to Sanger Sequencing. The results were analyzed using the ICE platform.

Deep Sequencing Sample Preparation and data analysis—Genomic PCR was performed using AccuPrime™ HiFi Taq (Life Technologies) following manufacturer's instructions. About 200-500 ng of genomic DNA was used for each PCR reaction. Products were then purified using AMPure® XP magnetic bead purification kit (Beckman Coulter) and quantified using a Nanodrop2000. Individual samples were pooled and run on an Illumina® HiSeq2500 High Throughput at a run length of 2×125 bp. A custom python script was developed to perform sequence analysis. For each sample, sequences with frequency of less than 100 reads were filtered from the data. Sequences in which the reads matched with primer and reverse complement subsequences classified as target sequences. These sequences were then aligned with corresponding wildtype sequence using global pairwise sequence alignment. Sequences that were misaligned through gaps or insertions around the expected cut site were classified as NHEJ events. The frequency, length, and position of matches, insertions, deletions, and mismatches were all tracked in the resulting aligned sequences.

Statistical analysis—Statistical analysis was performed and plotted using Prism software. Student's t-test (unpaired, two-tailed) was used to compare two groups. One-way ANOVA test was used to compare multiple groups, following with Tukey multiple comparison test of every pair. A P-value <0.05 was considered significant.

TABLE 3

PCR Primers used for on- and off- target genomic loci amplification

	Forward primer sequence (5'-3')	SEQ ID NO:	Reverse primer sequence (5'-3')	SEQ ID NO:
Mouse APP (659)	AGGAACGGAGTGACCTGTTTCC	21	TTCCTCCATGGTAACCACGCAT	22
Human APP (659)	TGGGGAAGCCACATGTTGTACA	23	ATGTTTTGGTGGGCCATTTGGT	24
Human APP (670; 676)	AAATTATGGGTGTTCTGCAATCTTGG	25	ACTTGTGTTACAGCACAGCTGTC	26
Mouse OT1	GCCCTCCAGAAGTATTGGCTT	27	GTCAGGGCCTTGCTCTACAAA	28
Mouse OT2	CGCAAAAACTGGCTGCGTAT	29	TGTAGGCGCACATGCAGAAG	30
Mouse OT3	CAGGTAGAGCGTGGAAACTCA	31	TGTGCGCATTAGGACCAGAT	32
Mouse OT4	CACCTGACAATGCTGTCCCA	33	AGACAAGGTCTGTCTCCTTGC	34
Mouse OT5	CCAACTCTTTGCTTAGGGGC	35	ATCGTCCCTGGTGCATTCTC	36
Human OT1	GGAAAACCAGGTAGAGGGGG	37	TCTCTGGCTCGAGGGTACAT	38
Human OT2	CTGCATGCCATGGGTAGGTA	39	CAGGCTGTTTCGGGTCCTT	40
Human OT3	AGACTCTTCTCCGATTCCAGC	41	TCCAGCACGATCTGGTAGGC	42
Human OT4	AGTGCTTTTCTTTGCCTTTGCT	43	TGCTCGGGAGGTGTTTCTAC	44
Human OT5	AACAAGGCAGCTCCTCAACT	45	GACGTCAGAATTGAGGGTGGA	46
Mouse APLP1	CCAGCGGGATGAACTGGTAAGA	47	CCCAGGTCACCTTAAGGAGCAA	48
Mouse APLP2	GAGAGAGTTGGAGGCCTTGAGG	49	AACCACAGTGACAAGTGGCTCT	50
Human APLP1	GTGAATGCGTCTGTTCCAAGGG	51	GCTGCTGGGACTATCTGGGAAT	52
Human APLP2	TTTTAGGGGCTCGACCTTCCAG	53	TGCACTAATTTCCCAGGGCTCA	54

TABLE 4

Transport parameters of WT and APP659

	% Anterograde	% Retrograde	% Stationary	Anterograde Run-length (μm)	Retrograde Run-length (μm)	Anterograde velocity (μm/sec), mean ± SEM	Retrograde velocity (μm/sec), mean ± SEM
Kinetics in axons							
APP659	53.88 ± 4.57	37.13 ± 4.36	8.97 ± 1.51	8.08 ± 0.31	6.8 ± 0.26	1.66 ± 0.03	1.52 ± 0.03
APPWT	57.84 ± 2.22	34.37 ± 4.46	10.42 ± 4.24	10.44 ± 0.42	6.35 ± 0.26	1.97 ± 0.03	1.52 ± 0.03
Kinetics in dendrites							
APP659	37.81 ± 6.45	20.55 ± 6.21	41.64 ± 8.37	7.0 ± 0.48	6.94 ± 0.81	0.76 ± 0.05	0.83 ± 0.12
APPWT	45.83 ± 4.58	24.81 ± 2.97	29.35 ± 5.63	8.12 ± 0.46	7.98 ± 0.94	0.94 ± 0.03	0.9 ± 0.07

~115 APP659:GFP and ~130 APP:GFP vesicles analyzed in dendrites;

~310 APP659:GFP and ~325 APP:GFP vesicles in axons (from 10-12 neurons from 2 separate cultures.)

TABLE 5

APP sgRNAs targeting sequences

	sgRNA targeting sequence	SEQ ID NO:
Human APP 659	ATCCATTCATCATGGTGTGG	1
Human APP 670	TGGACAGGTGGCGCTCCTCT	2
Human APP 676	GTAGCCGTTCTGCTGCATCT	4
Mouse APP 659	ATCCATCCATCATGGCGTGG	6
Mouse APP 670	TGGAGAGATGGCGCTCCTCT	7
Mouse APP 676	ATATCCGTTCTGCTGCATCT	9

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aagagtacca acttgcatga ctacggcatg ttgctgccct gcggaattga caagttccga 540

ggggtagagt ttgtgtgttg cccactggct gaagaaagtg acaatgtgga ttctgctgat 600

gcggaggagg atgactcgga tgtctggtgg ggcggagcag acacagacta tgcagatggg 660

agtgaagaca aagtagtaga agtagcagag gaggaagaag tggctgaggt ggaagaagaa 720

gaagccgatg atgacgagga cgatgaggat ggtgatgagg tagaggaaga ggctgaggaa 780

ccctacgaag aagccacaga gagaaccacc agcattgcca ccaccaccac caccaccaca 840

gagtctgtgg aagaggtggt tcgagttcct acaacagcag ccagtacccc tgatgccgtt 900

gacaagtatc tcgagacacc tggggatgag aatgaacatg cccatttcca gaaagccaaa 960

gagaggcttg aggccaagca ccgagagaga atgtcccagg tcatgagaga atgggaagag 1020

gcagaacgtc aagcaaagaa cttgcctaaa gctgataaga aggcagttat ccagcatttc 1080

caggagaaag tggaatcttt ggaacaggaa gcagccaacg agagacagca gctggtggag 1140

acacacatgg ccagagtgga agccatgctc aatgaccgcc gccgcctggc cctggagaac 1200

tacatcaccg ctctgcaggc tgttcctcct cggcctcgtc acgtgttcaa tatgctaaag 1260

aagtatgtcc gcgcagaaca gaaggacaga cagcacaccc taaagcattt cgagcatgtg 1320

cgcatggtgg atcccaagaa agccgctcag atccggtccc aggttatgac acacctccgt 1380

gtgatttatg agcgcatgaa tcagtctctc tccctgctct acaacgtgcc tgcagtggcc 1440

gaggagattc aggatgaagt tgatgagctg cttcagaaag agcaaaacta ttcagatgac 1500

gtcttggcca acatgattag tgaaccaagg atcagttacg gaaacgatgc tctcatgcca 1560

tctttgaccg aaacgaaaac caccgtggag ctccttcccg tgaatggaga gttcagcctg 1620

gacgatctcc agccgtggca ttcttttggg gctgactctg tgccagccaa cacagaaaac 1680

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gaagttgagc ctgttgatgc ccgccctgct gccgaccgag gactgaccac tcgaccaggt   1740

tctgggttga caaatatcaa gacggaggag atctctgaag tgaagatgga tgcagaattc   1800

cgacatgact caggatatga agttcatcat caaaaattgg tgttctttgc agaagatgtg   1860

ggttcaaaca aaggtgcaat cattggactc atggtgggcg gtgttgtcat agcgacagtg   1920

atcgtcatca ccttggtgat gctgaagaag aaacagtaca catccattca tcatggtgtg   1980

gtggaggttg acgccgctgt caccccagag gagcgccacc tgtccaagat gcagcagaac   2040

ggctacgaaa atccaaccta caagttcttt gagcagatgc agaactag                2088


<210> SEQ ID NO 12
<211> LENGTH: 695
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg
1               5                   10                  15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
            20                  25                  30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
        35                  40                  45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
    50                  55                  60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
65                  70                  75                  80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                85                  90                  95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
            100                 105                 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115                 120                 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165                 170                 175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                 185                 190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
        195                 200                 205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
    210                 215                 220

Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
225                 230                 235                 240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu
                245                 250                 255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile
            260                 265                 270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
    290                 295                 300
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Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305                 310                 315                 320

Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
                325                 330                 335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
            340                 345                 350

Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
        355                 360                 365

Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
    370                 375                 380

Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385                 390                 395                 400

Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe
                405                 410                 415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420                 425                 430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
        435                 440                 445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
    450                 455                 460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
465                 470                 475                 480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
                485                 490                 495

Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
            500                 505                 510

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
        515                 520                 525

Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
    530                 535                 540

Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn
545                 550                 555                 560

Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
                565                 570                 575

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
            580                 585                 590

Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
        595                 600                 605

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
    610                 615                 620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625                 630                 635                 640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
                645                 650                 655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
            660                 665                 670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
        675                 680                 685

Phe Phe Glu Gln Met Gln Asn
    690                 695

<210> SEQ ID NO 13
<211> LENGTH: 2088
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<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 13

atgctgccca gcttggcact gctcctgctg gccgcctgga cggttcgggc tctggaggta 60

cccactgatg gcaacgccgg gctgctggca gaaccccaga tcgccatgtt ctgtggtaaa 120

ctcaacatgc acatgaatgt gcagaatgga aagtgggagt cagacccgtc agggaccaaa 180

acctgcattg gcaccaagga gggcatcttg cagtactgcc aagaggtcta ccctgaactg 240

cagatcacaa acgtggtgga agccaaccag ccagtgacca tccagaactg gtgcaagcgg 300

ggccgcaagc agtgcaagac acacacccac atcgtgattc cttaccgttg cctagttggt 360

gagtttgtga gcgacgccct tctcgtgccc gacaagtgca agttcctaca ccaggagcgg 420

atggatgttt gtgagaccca tcttcactgg cacaccgtcg ccaaagagac atgcagcgag 480

aagagcacta acttgcacga ctatggcatg ctgctgccct gcggcatcga caagttccga 540

ggggtagagt ttgtatgctg cccgttggcc gaggaaagcg acagcgtgga ttctgcggat 600

gcagaggagg atgactctga tgtctggtgg ggtggagcgg acacagacta cgctgatggc 660

ggtgaagaca aagtagtaga agtcgccgaa gaggaggaag tggctgatgt tgaggaagag 720

gaagctgatg atgatgagga tgtggaggat ggggacgagg tggaggagga ggccgaggag 780

ccctacgaag aggccaccga gagaacaacc agcactgcca ccaccaccac aaccaccact 840

gagtccgtgg aggaggtggt ccgagttccc acgacagcag ccagcacccc cgacgccgtc 900

gacaagtacc tggagacacc cggggacgag aacgagcatg cccatttcca gaaagccaaa 960

gagaggctgg aagccaagca ccgagagaga atgtcccagg tcatgagaga atgggaagag 1020

gcagagcgtc aagccaagaa cttgcccaaa gctgacaaga aggccgttat ccagcatttc 1080

caggagaaag tggaatctct ggaacaggaa gcagccaatg agagacagca gcttgtagag 1140

acacacatgg ccagagttga agccatgctc aatgaccgcc gccgcctggc cctcgagaat 1200

tacatcactg cactgcaggc ggtgccccca aggcctcatc atgtgttcaa catgctgaag 1260

aagtacgtcc gtgcggagca gaaagacaga cagcacaccc taaagcattt tgaacatgtg 1320

cgcatggtgg accccaagaa agctgctcag atccggtccc aggttatgac acacctccgt 1380

gtgatctacg agcgcatgaa ccagtctctg tccctgctct acaatgtccc tgcggtggct 1440

gaggagattc aagatgaagt cgatgagctg cttcagaagg agcagaacta ctccgacgat 1500

gtcttggcca acatgatcag tgagcccaga atcagctacg gaaacgacgc tctcatgcct 1560

tcgctgacgg aaaccaagac caccgtggag ctccttcccg tgaatgggga attcagcctg 1620

gatgacctcc agccgtggca ccctttgggg gtggactctg tgccagccaa taccgaaaat 1680

gaagtcgagc ctgttgacgc ccgccccgct gctgaccgag gactgaccac tcgaccaggt 1740

tctgggctga caaacatcaa gacggaagag atctcggaag tgaagatgga tgcagaattc 1800

ggacatgatt caggatttga agtccgccat caaaaactgg tgttctttgc tgaagatgtg 1860

ggttcgaaca aaggcgccat catcggactc atggtgggcg gcgttgtcat agcaaccgtg 1920

attgtcatca ccctggtgat gttgaagaag aaacagtaca catccatcca tcatggcgtg 1980

gtggaggtcg acgccgccgt gaccccagag gagcgccatc tctccaagat gcagcagaac 2040

ggatatgaga atccaactta caagttcttt gagcaaatgc agaactaa 2088

<210> SEQ ID NO 14
<211> LENGTH: 695
<212> TYPE: PRT

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<213> ORGANISM: Mus musculus

<400> SEQUENCE: 14

Met Leu Pro Ser Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Val Arg
1               5                   10                  15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
            20                  25                  30

Gln Ile Ala Met Phe Cys Gly Lys Leu Asn Met His Met Asn Val Gln
        35                  40                  45

Asn Gly Lys Trp Glu Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Gly
    50                  55                  60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
65                  70                  75                  80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
                85                  90                  95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Thr His Ile Val
            100                 105                 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
        115                 120                 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
    130                 135                 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
145                 150                 155                 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
                165                 170                 175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
            180                 185                 190

Ser Asp Ser Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
        195                 200                 205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Gly Glu Asp Lys
    210                 215                 220

Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Asp Val Glu Glu Glu
225                 230                 235                 240

Glu Ala Asp Asp Asp Glu Asp Val Glu Asp Gly Asp Glu Val Glu Glu
                245                 250                 255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Thr
            260                 265                 270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg
        275                 280                 285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu
    290                 295                 300

Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys
305                 310                 315                 320

Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg
                325                 330                 335

Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp
            340                 345                 350

Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu
        355                 360                 365

Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala
    370                 375                 380

Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn
385                 390                 395                 400
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Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro His His Val Phe
                405                 410                 415

Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His
            420                 425                 430

Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala
        435                 440                 445

Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu
    450                 455                 460

Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala
465                 470                 475                 480

Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn
                485                 490                 495

Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser
            500                 505                 510

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr
        515                 520                 525

Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln
    530                 535                 540

Pro Trp His Pro Phe Gly Val Asp Ser Val Pro Ala Asn Thr Glu Asn
545                 550                 555                 560

Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr
                565                 570                 575

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser
            580                 585                 590

Glu Val Lys Met Asp Ala Glu Phe Gly His Asp Ser Gly Phe Glu Val
        595                 600                 605

Arg His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
    610                 615                 620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
625                 630                 635                 640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile
                645                 650                 655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg
            660                 665                 670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys
        675                 680                 685

Phe Phe Glu Gln Met Gln Asn
    690                 695


<210> SEQ ID NO 15
<211> LENGTH: 4176
<212> TYPE: DNA
<213> ORGANISM: Streptococcus pyogenes M1

<400> SEQUENCE: 15

atgggtatcc acggagtccc agcagccgac aagaagtaca gcatcggcct ggacatcggc      60

accaactctg tgggctgggc cgtgatcacc gacgagtaca aggtgcccag caagaaattc     120

aaggtgctgg gcaacaccga ccggcacagc atcaagaaga acctgatcgg agccctgctg     180

ttcgacagcg gcgaaacagc cgaggccacc cggctgaaga gaaccgccag aagaagatac     240

accagacgga agaaccggat ctgctatctg caagagatct tcagcaacga gatggccaag     300

gtggacgaca gcttcttcca cagactggaa gagtccttcc tggtggaaga ggataagaag     360

cacgagcggc accccatctt cggcaacatc gtggacgagg tggcctacca cgagaagtac     420
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cccaccatct accacctgag aaagaaactg gtggacagca ccgacaaggc cgacctgcgg    480

ctgatctatc tggccctggc ccacatgatc aagttccggg gccacttcct gatcgagggc    540

gacctgaacc ccgacaacag cgacgtggac aagctgttca tccagctggt gcagacctac    600

aaccagctgt tcgaggaaaa ccccatcaac gccagcggcg tggacgccaa ggccatcctg    660

tctgccagac tgagcaagag cagacggctg gaaaatctga tcgcccagct gcccggcgag    720

aagaagaatg gcctgttcgg aaacctgatt gccctgagcc tgggcctgac ccccaacttc    780

aagagcaact tcgacctggc cgaggatgcc aaactgcagc tgagcaagga cacctacgac    840

gacgacctgg acaacctgct ggcccagatc ggcgaccagt acgccgacct gtttctggcc    900

gccaagaacc tgtccgacgc catcctgctg agcgacatcc tgagagtgaa caccgagatc    960

accaaggccc ccctgagcgc ctctatgatc aagagatacg acgagcacca ccaggacctg   1020

accctgctga aagctctcgt gcggcagcag ctgcctgaga agtacaaaga gatttcttc   1080

gaccagagca agaacggcta cgccggctac attgacggcg gagccagcca ggaagagttc   1140

tacaagttca tcaagcccat cctggaaaag atggacggca ccgaggaact gctcgtgaag   1200

ctgaacagag aggacctgct gcggaagcag cggaccttcg acaacggcag catcccccac   1260

cagatccacc tgggagagct gcacgccatt ctgcggcggc aggaagattt ttacccattc   1320

ctgaaggaca accgggaaaa gatcgagaag atcctgacct tccgcatccc ctactacgtg   1380

ggccctctgg ccaggggaaa cagcagattc gcctggatga ccagaaagag cgaggaaacc   1440

atcacccccct ggaacttcga ggaagtggtg gacaagggcg cttccgccca gagcttcatc   1500

gagcggatga ccaacttcga taagaacctg cccaacgaga aggtgctgcc caagcacagc   1560

ctgctgtacg agtacttcac cgtgtataac gagctgacca aagtgaaata cgtgaccgag   1620

ggaatgagaa agcccgcctt cctgagcggc gagcagaaaa aggccatcgt ggacctgctg   1680

ttcaagacca accggaaagt gaccgtgaag cagctgaaag aggactactt caagaaaatc   1740

gagtgcttcg actccgtgga aatctccggc gtggaagatc ggttcaacgc ctccctgggc   1800

acataccacg atctgctgaa aattatcaag gacaaggact tcctggacaa tgaggaaaac   1860

gaggacattc tggaagatat cgtgctgacc ctgacactgt ttgaggacag agagatgatc   1920

gaggaacggc tgaaaaccta tgcccacctg ttcgacgaca aagtgatgaa gcagctgaag   1980

cggcggagat acaccggctg gggcaggctg agccggaagc tgatcaacgg catccgggac   2040

aagcagtccg gcaagacaat cctggatttc ctgaagtccg acggcttcgc caacagaaac   2100

ttcatgcagc tgatccacga cgacagcctg acctttaaag aggacatcca gaaagcccag   2160

gtgtccggcc agggcgatag cctgcacgag cacattgcca atctggccgg cagccccgcc   2220

attaagaagg gcatcctgca gacagtgaag gtggtggacg agctcgtgaa agtgatgggc   2280

cggcacaagc ccgagaacat cgtgatcgaa atggccagag agaaccagac cacccagaag   2340

ggacagaaga acagccgcga gagaatgaag cggatcgaag agggcatcaa agagctgggc   2400

agccagatcc tgaaagaaca ccccgtggaa aacacccagc tgcagaacga gaagctgtac   2460

ctgtactacc tgcagaatgg gcgggatatg tacgtggacc aggaactgga catcaaccgg   2520

ctgtccgact acgatgtgga ccatatcgtg cctcagagct ttctgaagga cgactccatc   2580

gacaacaagg tgctgaccag aagcgacaag aaccggggca agagcgacaa cgtgccctcc   2640

gaagaggtcg tgaagaagat gaagaactac tggcggcagc tgctgaacgc caagctgatt   2700

acccagagaa agttcgacaa tctgaccaag gccgagagag gcggcctgag cgaactggat   2760

aaggccggct tcatcaagag acagctggtg gaaacccggc agatcacaaa gcacgtggca   2820
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cagatcctgg actcccggat gaacactaag tacgacgaga atgacaagct gatccgggaa   2880

gtgaaagtga tcaccctgaa gtccaagctg gtgtccgatt tccggaagga tttccagttt   2940

tacaaagtgc gcgagatcaa caactaccac cacgcccacg acgcctacct gaacgccgtc   3000

gtgggaaccg ccctgatcaa aaagtaccct aagctggaaa gcgagttcgt gtacggcgac   3060

tacaaggtgt acgacgtgcg gaagatgatc gccaagagcg agcaggaaat cggcaaggct   3120

accgccaagt acttcttcta cagcaacatc atgaactttt tcaagaccga gattaccctg   3180

gccaacggcg agatccggaa gcggcctctg atcgagacaa acggcgaaac cggggagatc   3240

gtgtgggata agggccggga ttttgccacc gtgcggaaag tgctgagcat gccccaagtg   3300

aatatcgtga aaaagaccga ggtgcagaca ggcggcttca gcaaagagtc tatcctgccc   3360

aagaggaaca gcgataagct gatcgccaga aagaaggact gggaccctaa gaagtacggc   3420

ggcttcgaca gccccaccgt ggcctattct gtgctggtgg tggccaaagt ggaaaagggc   3480

aagtccaaga aactgaagag tgtgaaagag ctgctgggga tcaccatcat ggaaagaagc   3540

agcttcgaga agaatcccat cgactttctg gaagccaagg gctacaaaga agtgaaaaag   3600

gacctgatca tcaagctgcc taagtactcc ctgttcgagc tggaaaacgg ccggaagaga   3660

atgctggcct ctgccggcga actgcagaag ggaaacgaac tggccctgcc ctccaaatat   3720

gtgaacttcc tgtacctggc cagccactat gagaagctga agggctcccc cgaggataat   3780

gagcagaaac agctgtttgt ggaacagcac aagcactacc tggacgagat catcgagcag   3840

atcagcgagt tctccaagag agtgatcctg gccgacgcta atctggacaa agtgctgtcc   3900

gcctacaaca agcaccggga taagcccatc agagagcagg ccgagaatat catccacctg   3960

tttaccctga ccaatctggg agcccctgcc gccttcaagt actttgacac caccatcgac   4020

cggaagaggt acaccagcac caaagaggtg ctggacgcca ccctgatcca ccagagcatc   4080

accggcctgt acgagacacg gatcgacctg tctcagctgg gaggcgacaa aaggccggcg   4140

gccacgaaaa aggccggcca ggcaaaaaag aaaaag                             4176


<210> SEQ ID NO 16
<211> LENGTH: 80
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 16

gttttagagc tagaaatagc aagttaaaat aaggctagtc cgttatcaac ttgaaaaagt     60

ggcaccgagt cggtgctttt                                                 80


<210> SEQ ID NO 17
<211> LENGTH: 9286
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 17

ccccacgagg gcctatttcc catgattcct tcatatttgc atatacgata caaggctgtt     60

agagagataa ttggaattaa tttgactgta aacacaaaga tattagtaca aaatacgtga    120

cgtagaaagt aataatttct tgggtagttt gcagttttaa aattatgttt taaaatggac    180

tatcatatgc ttaccgtaac ttgaaagtat ttcgatttct tggctttata tatcttgtgg    240
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aaaggacgaa acaccgatcc attcatcatg gtgtgggttt tagagctaga aatagcaagt 300

taaaataagg ctagtccgtt atcaacttga aaaagtggca ccgagtcggt gctttttgt 360

tttagagcta gaaatagcaa gttaaaataa ggctagtccg tttttagcgc gtgcgccaat 420

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<210> SEQ ID NO 22
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 22

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<400> SEQUENCE: 24

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<400> SEQUENCE: 26

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<400> SEQUENCE: 27

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<400> SEQUENCE: 28

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<220> FEATURE:
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<400> SEQUENCE: 29

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 30

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<212> TYPE: DNA
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<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 31

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<212> TYPE: DNA
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<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 32

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<210> SEQ ID NO 33
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 34

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<220> FEATURE:
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<400> SEQUENCE: 35

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<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 36

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 37

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<210> SEQ ID NO 38
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<212> TYPE: DNA
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<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 38

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 39

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<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 41

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<400> SEQUENCE: 42

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<400> SEQUENCE: 43

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<400> SEQUENCE: 44

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 45

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<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 46
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<220> FEATURE:
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<400> SEQUENCE: 47

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<220> FEATURE:
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<400> SEQUENCE: 48

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<400> SEQUENCE: 49

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<220> FEATURE:
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<400> SEQUENCE: 50

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<400> SEQUENCE: 51

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<400> SEQUENCE: 52

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<400> SEQUENCE: 53

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<220> FEATURE:
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<400> SEQUENCE: 54

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<400> SEQUENCE: 55

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<400> SEQUENCE: 56

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<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 57

ctcatatccg ttctgctgca tcttggagag                                      30


<210> SEQ ID NO 58
<211> LENGTH: 303
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 58

atggatgcag aattcggaca tgattcagga tttgaagtcc gccatcaaaa actggtgttc     60

tttgctgaag atgtgggttc gaacaaaggc gccatcatcg gactcatggt gggcggcgtt    120

gtcatagcaa ccgtgattgt catcaccctg gtgatgttga agaagaaaca gtacacatcc    180

atccatcatg gcgtggtgga ggtcgacgcc gccgtgaccc cagaggagcg ccatctctcc    240

aagatgcagc agaacggata tgagaatcca acttacaagt tctttgagca aatgcagaac    300

taa                                                                  303


<210> SEQ ID NO 59
<211> LENGTH: 100
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```
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 59

Met Asp Ala Glu Phe Gly His Asp Ser Gly Phe Glu Val Arg His Gln
1               5                   10                  15

Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile
            20                  25                  30

Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile
        35                  40                  45

Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly
    50                  55                  60

Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser
65                  70                  75                  80

Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu
                85                  90                  95

Gln Met Gln Asn
            100


<210> SEQ ID NO 60
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 60

gaagaagaaa cagtacacat ccatccatca tggcgtggtg gaggtaggta aacctggagg     60

cttgtc                                                                66


<210> SEQ ID NO 61
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 61

aacagtacac atccatccat catggcgtgg tggaggtagg taaacc                    46


<210> SEQ ID NO 62
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 62

aacagtacac atccatccat catggtggag gtaggtaaac c                         41


<210> SEQ ID NO 63
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 63

aacagtacac atccatccat catggcggag gtaggtaaac c                         41


<210> SEQ ID NO 64
<211> LENGTH: 38
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 64

aacagtacac atccatccat catggaggta ggtaaacc                          38


<210> SEQ ID NO 65
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 65

aacagtacac atccatccat catggcggtg gaggtaggta aacc                   44


<210> SEQ ID NO 66
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 66

aacagtacac atccatccat catggtggtg gaggtaggta aacc                   44


<210> SEQ ID NO 67
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 67

atccatccat catggcgtgg tgg                                          23


<210> SEQ ID NO 68
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 68

atccattcat catggtgtgg tgg                                          23


<210> SEQ ID NO 69
<211> LENGTH: 66
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 69

gaagaagaaa cagtacacat ccattcatca tggtgtggtg gaggtaggta aacttgactg  60

catgtt                                                             66


<210> SEQ ID NO 70
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 70

aacagtacac atccattcat catggtgtgg tggaggtagg taaac                  45
```

```
<210> SEQ ID NO 71
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 71

aacagtacac atccattcat catggtggag gtaggtaaac                            40


<210> SEQ ID NO 72
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 72

aacagtacac atccattcat catggtggtg gaggtaggta aac                        43


<210> SEQ ID NO 73
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 73

aacagtacac atccattcat cattggtgga ggtaggtaaa c                          41


<210> SEQ ID NO 74
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 74

aacagtacac atccattcat catggtgggt ggaggtaggt aaac                       44


<210> SEQ ID NO 75
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 75

aacagtacac atccattcat ctggtggagg taggtaaac                             39


<210> SEQ ID NO 76
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 76

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp
            20                  25                  30

Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln
        35                  40                  45
```

```
<210> SEQ ID NO 77
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 77

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Gly Gly
            20                  25


<210> SEQ ID NO 78
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 78

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Gly Gly Gly
            20                  25                  30


<210> SEQ ID NO 79
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 79

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His
            20                  25


<210> SEQ ID NO 80
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 80

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly
            20                  25


<210> SEQ ID NO 81
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 81

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His Leu Val Glu Val Asp Ala Ala
            20                  25                  30
```

```
Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln
        35                  40


<210> SEQ ID NO 82
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 82

Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu
1               5                   10                  15

Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe
            20                  25                  30

Glu Gln Met Gln Asn
        35


<210> SEQ ID NO 83
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 83

aacagtacac atccattcat catggtgtgg tggaggtagg taaac                     45


<210> SEQ ID NO 84
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 84

aacagtacac atccattcat catggtggag gtaggtaaac                           40


<210> SEQ ID NO 85
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 85

aacagtacac atccattcat catggtggtg gaggtaggta aac                       43


<210> SEQ ID NO 86
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 86

aacagtacac atccattcat catggtgggt ggaggtaggt aaac                      44


<210> SEQ ID NO 87
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic
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```
<400> SEQUENCE: 87

aacagtacac atccattcat cattggtgga ggtaggtaaa c                          41


<210> SEQ ID NO 88
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 88

aacagtacac atccattcat catgtggtgg aggtaggtaa ac                         42


<210> SEQ ID NO 89
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 89

tgctgcatct tggacaggtg gcgctcctct ggggtgacag cggcg                      45


<210> SEQ ID NO 90
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (28)..(28)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 90

tgctgcatct tggacaggtg gcgctccntc tggggtgaca gcggcg                     46


<210> SEQ ID NO 91
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 91

tgctgcatct tggacaggtg gcgcttctgg ggtgacagcg gcg                        43


<210> SEQ ID NO 92
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 92

tgctgcatct tggacaggtg gctctggggt gacagcggcg                            40


<210> SEQ ID NO 93
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 93

tgctgcatct tggacaggtg gcgctctggg gtgacagcgg cg                         42
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<210> SEQ ID NO 94
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 94

tgctgcatct tggacaggtg gcgctctctg gggtgacagc ggcg                      44


<210> SEQ ID NO 95
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 95

ttggattttc gtagccgttc tgctgcatct tggacaggtg gcgct                     45


<210> SEQ ID NO 96
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (28)..(28)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 96

ttggattttc gtagccgttc tgctgcantc ttggacaggt ggcgct                    46


<210> SEQ ID NO 97
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 97

ttggattttc gtagccgttc ttggacaggt ggcgct                               36


<210> SEQ ID NO 98
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 98

ttggattttc gtagccgttc tgctgctctt ggacaggtgg cgct                      44


<210> SEQ ID NO 99
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 99

ttggattttc gtagccgttc tgcttcttgg acaggtggcg ct                        42


<210> SEQ ID NO 100
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<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 100

ttggattttc gtagccgttc ttcttggaca ggtggcgct                          39


<210> SEQ ID NO 101
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 101

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp
            20                  25                  30

Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln
        35                  40                  45


<210> SEQ ID NO 102
<211> LENGTH: 29
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 102

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Gly Gly
            20                  25


<210> SEQ ID NO 103
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 103

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Gly Gly Gly
            20                  25                  30


<210> SEQ ID NO 104
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 104

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly
            20                  25


<210> SEQ ID NO 105
```

```
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 105

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His
            20                  25


<210> SEQ ID NO 106
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 106

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Val Val Ala Val Asp Ala
            20                  25                  30

Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln
        35                  40                  45


<210> SEQ ID NO 107
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 107

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn Gly
            20                  25                  30

Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
        35                  40                  45


<210> SEQ ID NO 108
<211> LENGTH: 41
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (21)..(21)
<223> OTHER INFORMATION: X is D or E

<400> SEQUENCE: 108

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Xaa Gly Ala Pro Pro Val Gln Asp Ala Ala Glu Arg
            20                  25                  30

Leu Arg Lys Ser Asn Leu Gln Val Leu
        35                  40


<210> SEQ ID NO 109
<211> LENGTH: 41
<212> TYPE: PRT
```

```
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 109

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Ala Arg Pro Pro Val Gln Asp Ala Ala Glu Arg
            20                  25                  30

Leu Arg Lys Ser Asn Leu Gln Val Leu
        35                  40


<210> SEQ ID NO 110
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 110

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Pro Pro Val Gln Asp Ala Ala Glu Arg Leu Arg
            20                  25                  30

Lys Ser Asn Leu Gln Val Leu
        35


<210> SEQ ID NO 111
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 111

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Arg His Leu Ser Lys Met Gln Gln Asn Ser Tyr
            20                  25                  30

Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
        35                  40                  45


<210> SEQ ID NO 112
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 112

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro
            20


<210> SEQ ID NO 113
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 113
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Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys
            20                  25


<210> SEQ ID NO 114
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 114

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Asn Gly Tyr Glu Asn
            20                  25                  30

Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
        35                  40


<210> SEQ ID NO 115
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 115

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys
            20                  25


<210> SEQ ID NO 116
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 116

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Lys Gln Asn Gly Tyr
            20                  25                  30

Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
        35                  40                  45


<210> SEQ ID NO 117
<211> LENGTH: 44
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 117

Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala
1               5                   10                  15

Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Lys Asn Gly Tyr Glu
            20                  25                  30

Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
        35                  40
```

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<210> SEQ ID NO 118
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 118

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp
            20                  25                  30

Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln
        35                  40                  45


<210> SEQ ID NO 119
<211> LENGTH: 48
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 119

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Gly Gly Arg Arg Arg
            20                  25                  30

Arg Asp Pro Arg Gly Ala Pro Ser Leu Gln Asp Ala Ala Glu Arg Ile
        35                  40                  45


<210> SEQ ID NO 120
<211> LENGTH: 47
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 120

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Gly Arg Arg Arg Arg
            20                  25                  30

Asp Pro Arg Gly Ala Pro Ser Leu Gln Asp Ala Ala Glu Arg Ile
        35                  40                  45


<210> SEQ ID NO 121
<211> LENGTH: 49
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 121

Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu
1               5                   10                  15

Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Gly Gly Gly Arg Arg
            20                  25                  30

Arg Arg Asp Pro Arg Gly Ala Pro Ser Leu Gln Asp Ala Ala Glu Arg
        35                  40                  45

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<210> SEQ ID NO 122
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 122

gtccatccat catggcctgg 20

<210> SEQ ID NO 123
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 123

ttccatccat catggcttgg 20

<210> SEQ ID NO 124
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: synthetic

<400> SEQUENCE: 124

gtccctccat catggcctgg 20

<210> SEQ ID NO 125
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 125

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<212> TYPE: DNA
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<400> SEQUENCE: 127

ctcccttcat cttggtgtgg 20

<210> SEQ ID NO 128
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<212> TYPE: DNA
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atccattcat catggtgtgg tgg                                              23
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We claim:

1. A method of treating Alzheimer's disease (AD) caused by formation of amyloid plaques composed of amyloid beta (AB) peptides, wherein the method comprises the steps of

a) obtaining a gene-editing construct specific for the amyloid precursor protein (APP), wherein the gene-editing construct facilitates truncation of the APP C-terminus when combined with a Cas9 nuclease, and

b) delivering the gene-editing construct and a construct encoding the Cas9 nuclease to a patient in need of AD therapy, wherein the APP molecule is truncated and production of AB peptides is decreased in the patient's brain, wherein the truncation of the APP C-terminus occurs at an APP residue selected from the group consisting of 659, 670, 676, and 686 relative to SEQ ID NO: 12 (human) or SEQ ID NO: 14 (mouse).

2. The method of claim **1**, wherein the gene-editing construct comprises a gRNA sequence selected from the group consisting of SEQ ID Nos: 1-10.

3. The method of claim **1**, wherein the gene-editing construct and the construct encoding the Cas9 nuclease are delivered in a composition comprising an adeno-associated viral vector and a nanocarrier delivery vehicle.

4. The method of claim **3**, wherein the composition is delivered intravenously or intrathecally.

5. A method of reducing the formation of amyloid plaques in a patient's brain, wherein the plaques comprise amyloid beta (AB) peptides, the method comprises the steps of

a) obtaining a gene-editing construct specific for the amyloid precursor protein (APP), wherein the gene-editing construct facilitates truncation of the APP C-terminus when combined with a Cas9 nuclease, and

b) delivering the gene-editing construct and a construct encoding the Cas9 nuclease to a patient in need of AD therapy, wherein the APP molecule is truncated and production of AB peptides is decreased in the patient's brain, wherein the truncation of the APP C-terminus occurs at an APP residue from the group consisting of 659, 670, 676, and 686 relative to SEQ ID NO: 12 (human) or SEQ ID NO: 14 (mouse).

6. The method of claim **5**, wherein the gene-editing construct comprises a gRNA sequence selected from the group consisting of SEQ ID NOs: 1-10.

7. The method of claim **5**, wherein the gene-editing construct and the construct encoding the Cas9 nuclease are delivered in a composition comprising an adeno-associated viral vector and a nanocarrier delivery vehicle.

8. The method of claim **5**, wherein the composition is delivered intravenously or intrathecally.

9. The method of claim **1**, wherein the gene-editing construct specific for the amyloid precursor protein (APP) comprises a sequence encoding a gRNA specific to amyloid precursor protein (APP), and wherein a Cas9 nuclease/gRNA ribonucleoprotein directs cleavage of the APP gene.

10. The method of claim **5**, wherein the gene-editing construct specific for the amyloid precursor protein (APP) comprises a sequence encoding a gRNA specific to amyloid precursor protein (APP), and wherein a Cas9 nuclease/gRNA ribonucleoprotein directs cleavage of the APP gene.

* * * * *