Supplementary Information

Genetic Reduction of STEP Tyrosine Phosphatase Reverses Cognitive and Cellular Deficits in a Mouse Model of Alzheimer's Disease

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Supplementary Methods

Mouse breeding. For experiments using Tg2576 mice, heterozygous STEP mice with and without the APP transgene were crossed to produce STEP WT mice, with or without the Tg2576 transgene, and $STEP^{-/-}$ with and without the Tg2576 transgene. For experiments using 3xTg mice, we followed similar breeding procedures as described by Bryleva (1). 3xTg-AD mice were bred with $STEP^{-/-}$ mice to generate the F1 generation. The F1 mice were crossed to generate the F2 generation. F2 mice were genotyped by using PCR for PS1 and STEP and real-time PCR for APP and Tau. Two groups of F2 mice were selected for further study: 1. mice heterozygous for STEP, and homozygous for the PS1 knock-in, human TauP301L, and human APPswe transgenes, and 2. mice that were heterozygous for STEP, and that were non-transgenic for PS1 knock-in, human TauP301L, and the human APPswe transgenes. These two groups of mice were crossed to generate the 4 groups of progeny tested in this study: 1. $STEP^{-/-}$ / homozygous for PS1 knock-in, human TauP301L, and the human APPswe transgenes (double mutant mice, DM mice), 2. $STEP^{+/+}$ / homozygous for the PS1 knock-in, human TauP301L, and the human APPswe transgenes (3xTg-AD mice), 3. $STEP^{+/+}$ / non-transgenic for the PS1 knock-in, human TauP301L, and the human APPswe transgenes (WT mice), and 4. $STEP^{-/-}$ / non-transgenic for the PS1 knock-in, human TauP301L, and the human APPswe transgenes (STEP knockout mice).

Mouse genotyping. DNA from mice tails was extracted using a DNA extraction kit (Qiagen). DNA concentrations were determined by a Thermo Scientific NanoDrop™ 3300 Fluorospectrometer. To determine PS1 genotype, PCR was performed using the

following conditions: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. PCR products were digested with BstEII enzyme at 60°C for 2 hours. To determine APP and tau genotype, the same amount of total DNA was analyzed for gene copy by quantitative real-time PCR using the SYBR Green reagent on an Applied Biosystems Prism 7900 sequence detection system as described (2). Analysis was performed with ABI Prism 7900 SDS Software. The primers are as follows: APP forward: 5'–GCT TGC ACC AGT TCT GGA TGG-3', APP reverse: 5'-GAG GTA TTC AGT CAT GTG CT-3'; tau forward: 5'–GCT TGC ACC AGT TCT GGA TGG-3', tau reverse: 5'-TTC AAA GTT CAC CTG ATA GT-3'; PS1 forward: 5'-CAC ACG CAA CTC TGA CAT GCA CAG GC-3', PS1 reverse: 5'-AGG CAG GAA GAT CAC GTG TTC AAT AC-3'; β–actin forward: 5'-ACG GCC AGG TCA TCA CTA TTG-3', β–actin reverse: 5'-AGG AAG GCT GGA AAA GAG CC-3'; STEP forward: 5'-GGC AGC AGA TGC TGG TGG C-3', STEP WT reverse: 5'-CCC TAC TCT CAT TCCTCC CTT CCC-3', STEP KO reverse: 5'-GGC GCG AAG GGG CCA CC-3'.

Subcellular fractionations and immunoblot analyses. Subcellular fractionations were obtained as described (3). In brief, brain homogenates were prepared in homogenization buffer (in mM): 10 Tris-HCl, pH 7.4, 320 sucrose, 1 EDTA, 1 EGTA, 5 NaF, 1 Na_3VO_4, and protease inhibitors. Homogenates were centrifuged at 800xg for 10 min (P1); S1 was centrifuged at 9,200xg for 15 min to produce P2. P2 was resuspended in homogenization buffer containing 35.6 mM sucrose and centrifuged at 25,000xg for 20 min to produce LP1. Proteins (30-50 μg) were loaded on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (PVDF; Biorad). Membranes were blocked with

5% milk and incubated with primary antibodies overnight at 4°C followed by incubation with secondary antibody. Bands were visualized using a G:BOX with a GeneSnap image program and quantified using Image J 1.33 (NIH).

ELISA and western blotting. $A\beta_{1-42}$ concentrations were measured using a human β amyloid HS (1-42) kit (Invitrogen) according to manufacturer's instructions. In brief, hemibrains were homogenized in cold 5 M guanidine HCl / 50 mM Tris HCl, pH 8.0 solution and mixed at room temperature for 4 hrs. Homogenates were diluted (1:30) in cold Dulbecco's phosphate buffered saline, pH 7.4, with 5% BSA, 0.03% Tween-20, 1 mM PMSF, and complete protease inhibitor cocktail (Roche). Samples were centrifuged at 16,000xg for 20 min at 4°C and supernatants were used for the ELISA. Concentrations were determined by comparing to a standard curve of synthetic Aβ.

For 6E10 western blots, hemibrains were sonicated in 3 volumes of 3% SDS, centrifuged at 13,000xg for 15 min, and supernatants (500-700 μg) were diluted with 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, protease inhibitor cocktail (TNT) to reduce the SDS concentration to 0.1%, then precleared with Protein G-sepharose (Amersham). Precleared samples were mixed with 6E10 antibody (6 μl) overnight at 4°C, and incubated with Protein G-sepharose (40 μl) for one hour. The beads were washed with TNT buffer and immunoprecipitated complexes were eluted in Tricine buffer (Biorad), loaded onto 10-20% Tris-Tricine/peptide gels (Biorad). Proteins were transferred to 0.2 μm PVDF membrane and boiled for 10 min in 1X PBS and blocked with 5% non-fat milk, probed with 6E10, and processed as above.

Immunoprecipitation. The membrane fractions (250 μg) were resuspended in RIPA buffer containing 1 mM sodium orthovanadate and complete protease inhibitors. The samples were precleared with protein G-sepharose beads (GE Health Sciences) and mixed with 4 μg of mouse monoclonal phospho-tyrosine antibody (4G10; Millipore) overnight at 4ºC. The antibody-bound complex was immunoprecipitated by adding protein G-sepharose (50 μl) and incubated for 2 h at 4ºC. Beads were washed 3 times with RIPA buffer and bound complexes were eluted using 2xSDS sample buffer and processed for western blotting. The blots were probed with pSrc Y^{416} (Cell signaling) antibody. For determining total Fyn levels in the sample, the inputs (50 μg) were loaded along with the immunoprecipitation samples and probed with anti-Fyn antibody (Millipore). Total Fyn levels were normalized to GAPDH and the ratio of pSrc Y^{416} levels verses total Fyn was determined.

Immunohistochemistry. Under anesthesia (Nembutal, 50 mg/kg), mice were perfused transcardially with normal saline followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brains were removed following perfusion, immersion fixed in 4% paraformaldehyde overnight, and cryoprotected in successive incubations of 10%, 20% and 30% sucrose solutions for 24 hrs each. Forty μm horizontal sections were cut with a cryostat and stored in PBS with 0.2% sodium azide at 4˚C. Eight to 10 sections 200 μm apart were chosen for free floating immunohistochemisrty. Procedural staining methods were analogous to those previously described (4). In summary, tissue samples were blocked for endogenous peroxidase, permeabilized in PBS with 0.2% lysine, 1% Triton X-100, incubated overnight at 4°C with primary antibodies

at the optimized working dilution made up in PBS 0.1 M (pH 7.4) and 4% goat serum. The monoclonal antibody 6E10 was used for detecting Aβ and other APP cleavage products (1:10,000; Covance). Tau levels were detected using AT180 antibody, which recognizes serine 235 and threonine 231 phosphorylation residues (1:1000) and HT7, an anti-human specific tau antibody (1:3000) (Thermo Scientific). On the second day, sections were washed in PBS and incubated for 2 h with the corresponding biotinylated secondary antibody (1:1000), and immunostaining was visualized using avidin–biotin (Vectastain: Vector Laboratories), 0.05% 3.3'-diaminobenzidine and 0.3% H_2O_2. Tissue sections were mounted onto slides, dehydrated and coverslipped. Stained sections were imaged using a Zeiss AxioCam digital camera mounted on an Axioplan Zeiss microscope using the 10X objective lens (Carl Zeiss MicroImaging). Non-overlapping images of the frontal cortex and CA1, CA3, and dentate gyrus regions of the hippocampus were taken from each animal (n=4 per group). Quantification of positive staining was determined using NIH Image J software and reported as the percent area positive stain, and ANOVA statistical analysis was performed using SPSS 16.0 (IBM Company).

Object recognition task. Mice were first habituated to the task by allowing them to explore an empty white open field box (60 cm x 60 cm) for 5 min. No data were recorded during habituation. Twenty-four hours later, mice completed the sample phase in which they were placed into the open field box with two identical objects located in the right and left corners. Mice were allowed to freely explore until they had accumulated a total of 30 sec of object exploration (i.e., contact with the object with the nose and/or front paws), at which point the trial ended. The time spent with each object was recorded. To

control for differences in activity among the groups, the elapsed time to accumulate 30 sec of object exploration was recorded. Twenty-four hours later mice completed the choice phase that was conducted in an identical manner to the sample phase except that one of the objects was substituted by a novel object. Mice have an inherent tendency to explore novel objects; therefore a preference for exploring the novel object (spending more time than the chance value of 15 sec with the novel object) demonstrates intact memory for the familiar object.

Y-maze. The Y-maze apparatus consisted of three dark gray arms (42 x 4.8 x 20 cm). Each mouse was placed at the end of one arm (the designated "start arm") and allowed to freely explore the maze for 5 min. The total number of arm entries was recorded, as was the number of entries representing alternation behavior (i.e., sequential entry into all three arms). All four paws of the mouse had to enter an arm for it to count as an arm entry. The percentage of spontaneous alternation was calculated by dividing the number of alternations by the total of arm entries completed during the trial.

Open field activity. To assess locomotor activity and exploratory behavior, mice were placed in a square box (60 x 60 x 60 cm) and allowed to explore for 10 min. A video camera mounted directly above the box recorded the trials and AnyMaze software analyzed the distance traveled in 10 min and time spent in the center of the box.

Electrophysiology. Adult (6-10 months of age) mice were decapitated, and their brains rapidly removed and immersed in ice-cold artificial cerebrospinal fluid (aCSF). The

composition of the aCSF was as follows (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 26.2 $NaHCO_3$, 11 D-glucose, 1.25 NaH_2PO_4, and 2.4 $CaCl_2$. Coronal sections (400 μm) were prepared on a Vibratome 1000 Plus. Slices were allowed to recover for at least 2 hours in a submerged incubation chamber (BSC-PC, Warner Instruments) at room temperature, continuously bubbled with a mixture of 95% O_2 and 5% CO_2.

For extracellular recordings, slices were placed in a submerged recording chamber (RC-27L, Warner Instruments), continuously perfused with oxygenated aCSF at a rate of 2 ml/min at 30^0C. A bipolar tungsten microelectrode (TM33CCNON, World Precision Instruments) was placed in the Schaffer collaterals of CA3, and extracellular field EPSPs were recorded using a glass microelectrode (2-6 MΩ) filled with aCSF, placed in the stratum radiatum of CA1. For all experiments, test stimuli were given at 0.033 Hz, and the stimulus intensity was set to give baseline field EPSP slopes of less than 50% of maximal response. A stable baseline was recorded for at least 20 min before inducing long-term potentiation (LTP). LTP was induced by theta burst stimulation (10 bursts of 5 shocks at 100Hz, with an interburst interval of 200 ms) given at baseline intensity. Field potentials were recorded using an Axon Instruments 700B amplifier and a Digidata 1440A digitizer, and data were analyzed using pClamp 10 software (Molecular Devices). All experiments were conducted in a blinded fashion with respect to genotype.

2. Supplementary Figures

Supplementary Figure 1. Mice lacking STEP have increased long-term potentiation relative to WT mice. Field potentials were recorded from the CA1 region of hippocampal slices from 6-month old, littermate-matched WT mice or STEP knockout mice ($STEP^{-/-}$). A stable baseline was recorded for at least 20 min before LTP induction by theta burst stimulation (TBS). The top panels show traces before and after TBS. The slope of the EPSP relative to the pre-TBS level is plotted as a function of time in the lower panel. For 30-60 min, the WT EPSP slope is 151%±5.6% and for STEP KO, EPSP 184%±9.7% (last 30 min averaged, repeated measures ANOVA, $P<0.05$). Data are means ± S.E.M. from separate slices. For WT, 4 slices from 4 animals; for $STEP^{-/-}$, 6 slices from 4 animals.

Supplementary Figure 2. Progeny from crosses between Tg2576 and $STEP^{-/-}$ mice restore surface NR1/NR2B levels. Crosses were made between Tg2576 and $STEP^{-/-}$ mice, and NMDAR levels were analyzed in cortical synaptosomal fractions (LP1). Representative immunoblots from 9-month old mice show pNR2B, NR2B, NR1, NR2A and total Aβ. Histograms are shown in lower panels with bands normalized to GAPDH. Tg2576 mice with WT STEP levels and high Aβ levels showed significantly lower levels of surface pNR2B Y^{1472}, NR2B and NR1 compared to WT, but not NR2A (one-way ANOVA, Fisher's LSD post hoc; pNR2B Y^{1472}, NR2B: $*P<0.05$; NR1, $**P<0.01$; NR2A, $P>0.05$; n=6). $STEP^{-/-}$ had significantly higher levels of surface pNR2B Y^{1472}, NR2B and NR1 compared to WT, but not NR2A (pNR2B Y^{1472}, NR2B: $*P<0.05$; NR1, $**P<0.01$; NR2A, $P>0.05$; n=6). Tg2576/$STEP^{-/-}$ mice, STEP deletion rescued the

reduction in pNR2B Y^{1472}, NR2B and NR1 seen in Tg2576 mice (pNR2B, NR2B: $P<0.05$; NR1: $P<0.01$; n=6). The total Aβ levels in Tg2576 and Tg2576/$STEP^{-/-}$ mouse brain homogenates (250 μg) were shown using 6E10 immunoreactivity.

Supplementary Figure 3. **Loss of STEP in 3xTg-AD mice increases pERK and pFyn levels.** (A) Representative Western blot and quantitation of pFyn Y420 levels in WT, 3xTg-AD, STEP-/-, and DM mice after immunoprecipitation with phospho-Tyr antibody. pFyn Y420 levels were normalized to total Fyn levels in the input samples after normalization of total Fyn to GAPDH. (B) Representative western blot and quantitation of pERK1/2 Y204 levels. pERK1/2 Y204 levels were normalized to total ERK1/2 levels and GAPDH.

Supplementary Figure 4. Characterization of APP and pTau in WT, STEP-/-, 3xTg-AD and DM. (A). Mouse brain membrane fractions were probed with the N-terminal APP antibody (22C11, upper panel), and CTFs with C-terminal APP antibody (643-695 specific clone 2.F2.19B4, lower panel).

3. Supplementary Table

Table 1: Primary and secondary antibodies utilized in western blot and immunofluorescence.			
Antibody	**Host**	**Dilution**	**Source**
anti-GAPDH	mouse	1:5000	Santa Cruz Biotechnology
anti-pY^{1472} NR2B	rabbit	1:1000	PhosphoSolutions
anti-NR2B	rabbit	1:1000	Millipore
anti-NR1	mouse	1:1000	Millipore
anti-NR2A	rabbit	1:1000	Millipore
anti-human tau (HT7)	mouse	1:3000	Pierce
anti-p-tau (AT180)	mouse	1:1000	Thermo Scientific
anti-beta amyloid $_{(1\text{-}16)}$ (6E10)	mouse	1:1000	Signet
anti-Fyn	rabbit	1:500	Millipore
anti-ERK2	rabbit	1:5000	Santa Cruz Biotechnology
anti-pY^{204}ERK	mouse	1:500	Santa Cruz Biotechnology
anti-pY^{416}Src	rabbit	1:500	Cell signaling
anti-APP clone 22C11	mouse	1:2000	Millipore
anti-APP clone 2F219B4	mouse	1:1000	Millipore
anti-STEP clone 23E5	mouse	1:500	Santa Cruz Biotechnology
anti-rabbit IgG	donkey	1:10,000	Amersham Biosciences
anti-mouse IgG	sheep	1:10,000	Amersham Biosciences

4. Supplementary References

1. Bryleva EY, Rogers MA, Chang CC, Buen F, Harris BT, Rousselet E, Seidah NG, Oddo S, LaFerla FM, Spencer TA, Hickey WF, Chang TY ACAT1 gene ablation increases 24(S)-hydroxycholesterol content in the brain and ameliorates amyloid pathology in mice with AD. *Proc Natl Acad Sci U S A* 107:3081-3086.

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3. Kurup P, Zhang Y, Xu J, Venkitaramani DV, Haroutunian V, Greengard P, Nairn AC, Lombroso PJ (2010) Abeta-mediated NMDA receptor endocytosis in Alzheimer's disease involves ubiquitination of the tyrosine phosphatase STEP61. *J Neurosci* 30:5948-5957.

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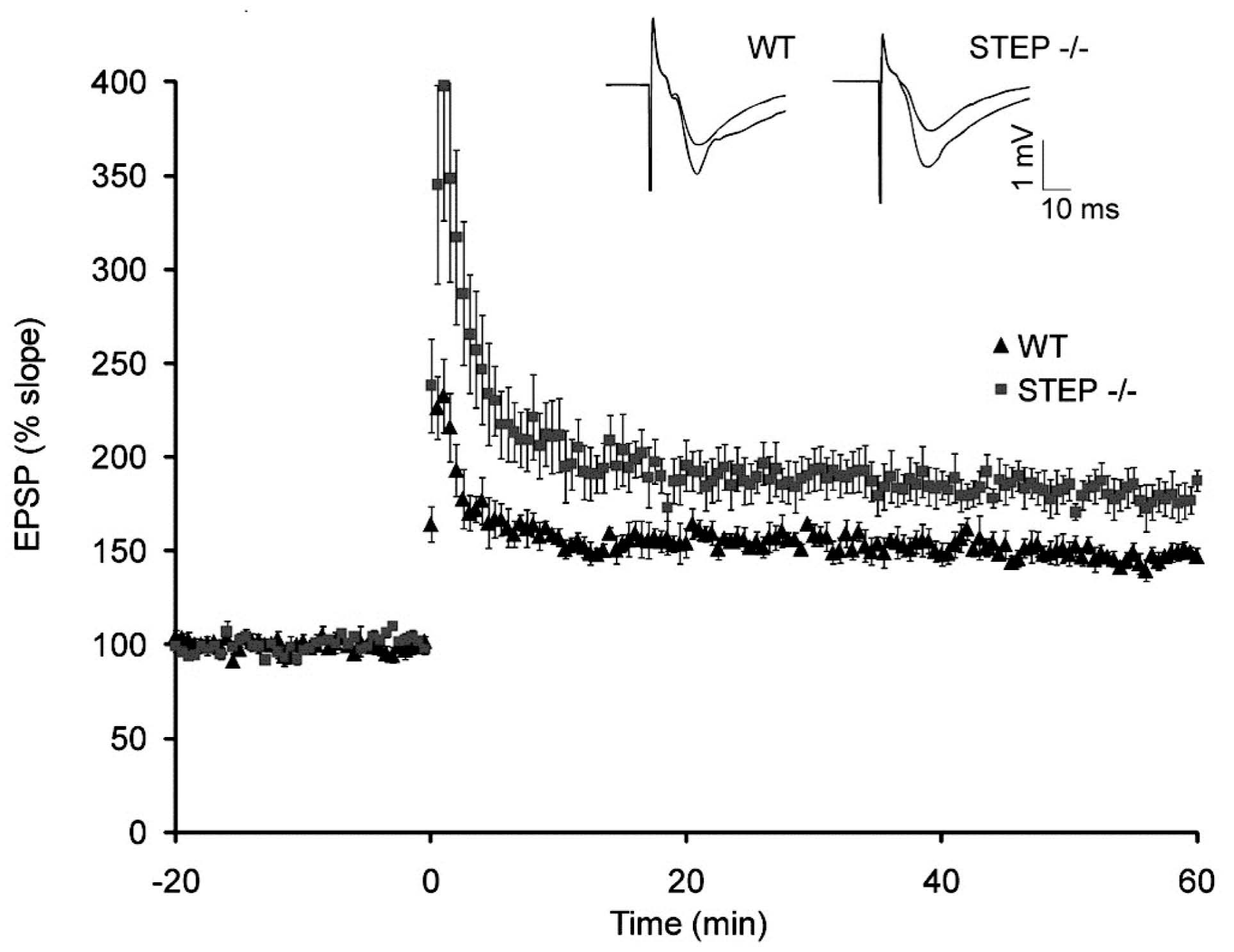
WT
STEP -/-
1 mV
10 ms
WT
STEP -/-
EPSP (% slope)
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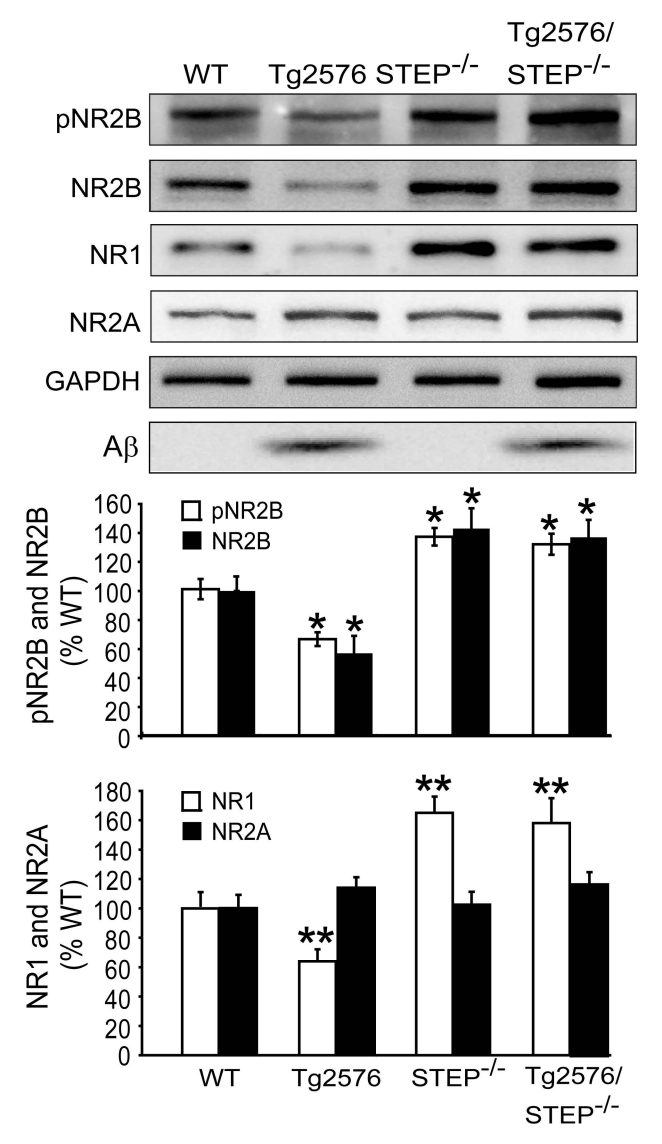
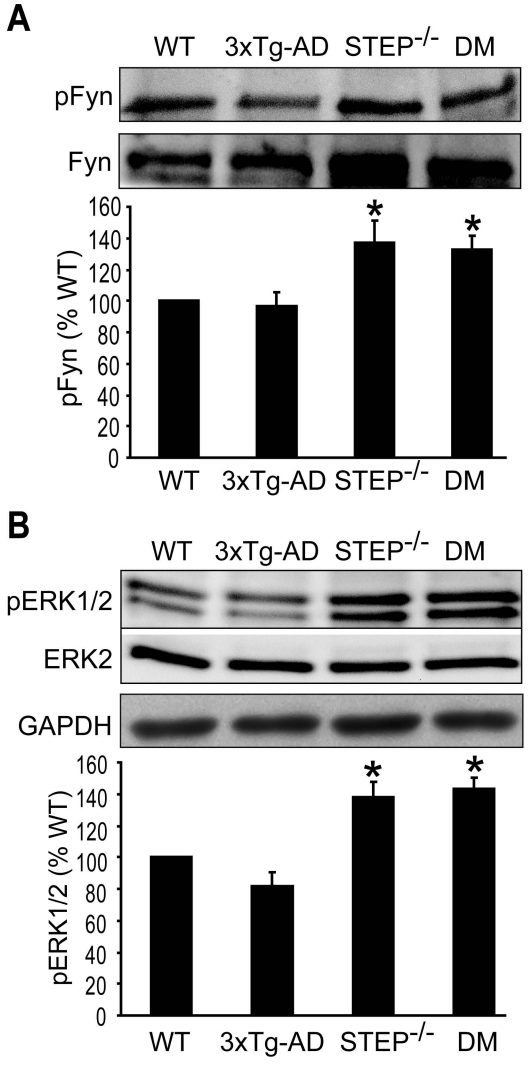
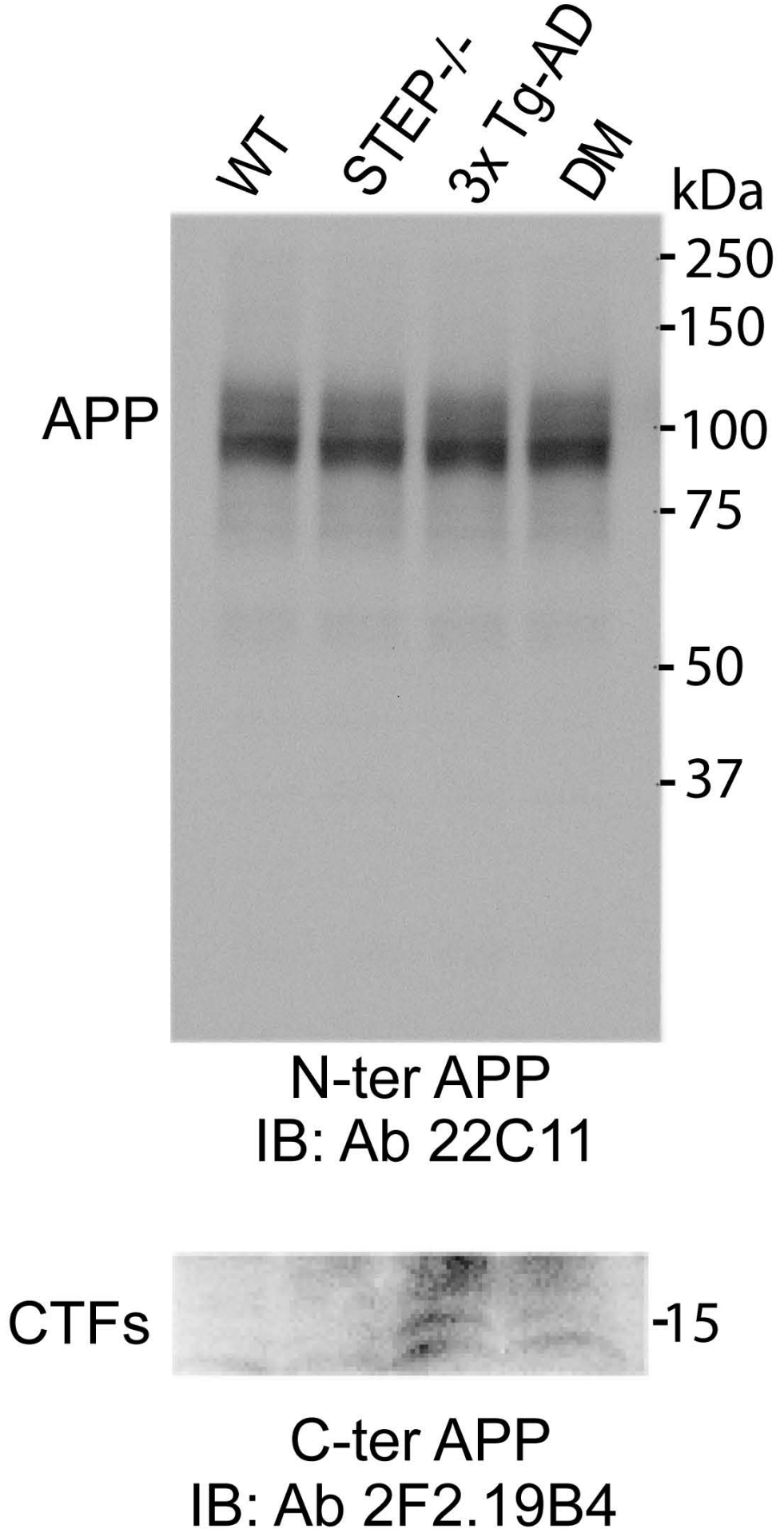
350
300
250
200
150
100
50
0
-20
0
20
40
60
Time (min)


WT
Tg2576
STEP-/-
Tg2576/
STEP-/-
pNR2B
NR2B
NR1
NR2A
GAPDH
Aβ
pNR2B and NR2B
(% WT)
pNR2B
NR2B
NR1 and NR2A
(% WT)
NR1
NR2A
WT
Tg2576
STEP-/-
Tg2576/
STEP-/-


A
WT
3xTg-AD
STEP-/-
DM
pFyn
Fyn
pFyn (% WT)
160
140
120
100
80
60
40
20
0
*
*
WT
3xTg-AD
STEP-/-
DM
B
WT
3xTg-AD
STEP-/-
DM
pERK1/2
ERK2
GAPDH
pERK1/2 (% WT)
160
140
120
100
80
60
40
20
0
*
*
WT
3xTg-AD
STEP-/-
DM


WT
STEP-/-
3x Tg-AD
DM
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150
100
75
50
37
APP
N-ter APP
IB: Ab 22C11
CTFs
15
C-ter APP
IB: Ab 2F2.19B4