Supporting Information

α_{2A} adrenergic receptor promotes amyloidogenesis through disrupting APP-SorLA interaction

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Materials and Methods

Antibodies and Chemicals

Antibodies for GAPDH and tubulin were purchased from Millipore and Developmental Studies Hybridoma Bank at The University of Iowa, respectively. APP rabbit monoclonal antibody (Y188) and golgi58 antibody were from Abcam. Aβ1-16 (6E10) antibody was from Covance. sAPPβ antibody was from Immuno Biological Lab (IBL). BACE1 and GFAP antibodies were purchased from Sigma-Aldrich. GFP monoclonal antibody was from Roche Applied Science. EEA1 and Myc-tag antibodies were purchased from Cell Signaling Technology. Rab5 and SorLA (SORL1) antibody were from Stressgen and ProteinTech, respectively. IBA-1 antibody was from Wako. AlexaFluor 488- and 594-conjugated secondary antibodies used for immunofluorescence microscopy were from Life Technologies. IRDye 680 and IRDye 800 secondary antibodies used for Western blot analysis by LI-COR Odyssey Imaging System were from LI-COR. DAB kit for detection of immunohistochemical signals was purchased from Dako EnVision+System. AlphaScreen® cAMP Assay Kit was from PerkinElmer.

Clondine and BRL44408 were purchased from Sigma-Aldrich. Idazoxan was from RBI (Research Biochemicals International). Nycodenz was from Accurate Chemical & Scientific Corp. All other chemicals were from Sigma-Aldrich or Fisher Scientific.

Animals and drug treatment.

Mice were housed in the AAALAC-accredited Animal Resources Program facility at the University of Alabama at Birmingham in accordance with procedures of the Animal Welfare Act and the 1989 amendments to the Act, and all studies followed protocols approved by the UAB Institutional Animal Care and Use Committee. All strains of mice have been backcrossed for

more than twelve generations to C57BL/6 background. Double transgenic mice carrying *APP(swe)* and *PS1(ΔE9)* transgenes, which have been integrated in the same locus (1, 2), were originally obtained from Jackson Laboratory (strain name B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J; stock number 004462). *Adra2a*$^{-/-}$ mice were crossed with *APP/PS1* mice first, and the resulting F1 *APP/PS1,Adra2a*$^{+/-}$ mice were then crossed with *Adra2a*$^{+/-}$ mice to generate *APP/PS1,Adra2a*$^{+/+}$, *APP/PS1,Adra2a*$^{+/-}$ and *APP/PS1,Adra2a*$^{-/-}$ mice. Age- and gender-matched littermate mice were compared at 6 month-old age.

APP/PS1 and non-transgenic littermates were treated with saline or clonidine (0.5mg/kg) through intraperitoneal injection twice a day starting from 4 months of age for 8 weeks. Age- and gender-matched littermates were compared. For idazoxan (3mg/kg, i.p. twice daily) treatment, male *APP/PS1* and non-transgenic littermates were injected twice a day starting from 10 weeks of age for 10 weeks. Mice were then untreated for a week to wash out drug before evaluated in behavioral assessment.

Immunohistochemistry for analysis of amyloid plaques, activated astrocytes and activated microglia.

Formalin-fixed and paraffin-embedded brain tissues were sectioned at 7μm using a microtome. Immunohistochemistry was performed as described previously (3, 4). Primary antibody 6E10 was used for detection of amyloid plaques, GFAP antibody for activated astrocytes, and IBA-1 for activated microglia. Slices immunostained with GFAP and IBA-1 antibodies were also stained with Congo red for co-detection of Aβ plaques. Multiple images (1mm^2 each) were taken and analyzed from four sections (at different septotemporal levels, but similar lateromedial positions) of each brain region (hippocampus or cortex), with an average across these sections used to generate a single data point for each animal.

Behavior assessment

Open field and elevated zero maze tests were performed as we described previously (5). Novel object recognition test (6) consists of two phases, training phase and test phase. During a training trial, two identical objects were placed and the animal was allowed to explore the identical objects for 10 min. Twenty hours later, the animal was placed back into the same box, where one of the two familiar objects was replaced with a novel one. Mice were recorded with a video camera for a 20 min testing phase. The time spent on exploring each object (touching, sniffing, and close staring) was documented. Recognition index was calculated as the percentage of time spent on exploring the novel object over the total time spent on exploring both familiar and novel objects.

Morris water maze test was performed as described previously (4, 7). During the acquisition of the spatial task, the escape latency and swim distance were recorded for 4 trials daily for 5 days. The day after the acquisition phase, a probe trial was conducted and the time spent in the target quadrant was measured in a single 60-s trial.

ELISA Measurement of Aβ levels

Brain Aβ levels in carbonate soluble and insoluble (guanidine soluble) fractions were measured as described previously (3). In brief, mouse cortex or hippocampus was homogenized first in carbonate buffer (100 mM Na_2CO_3, 50 mM NaCl, pH 11.5 plus protease inhibitors). Following centrifugation at 18,000 × g for 20 min at 4°C, the supernatant was collected as the carbonate-soluble fraction. The pellet was subjected to further homogenization for 3–4 h at room temperature in the guanidine solution (5 M guanidine HCl in 50 mM Tris-HCl, pH 8.0 plus protease inhibitors). Following centrifugation at 18,000 × g for 20 min at 4°C, the supernatant was collected as the carbonate-insoluble fraction. The levels of human Aβ40 and Aβ42 in the

soluble and insoluble fractions were measured using Aβ40- and Aβ42-specific ELISA kits (Life Technologies), respectively, following the manufacturer's protocol. The levels of mouse endogenous Aβ40 and Aβ42 in carbonate-soluble fraction were measured using specific ELISA kits (Life Technologies). To measure Aβ levels in cell culture medium, conditioned media with 48 h of vehicle or clonidine treatment was collected and diluted with the assay buffer provided in Aβ40- or Aβ42-specific ELISA kits for measurement following the manufacturer's instruction.

Cell culture

Neuro-2A (N2a) cells were cultured in DMEM/Opti-MEM (1:1, Life Technologies) supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. HEK293 cells were cultured in DMEM with 10% FBS plus penicillin, streptomycin and 2mM glutamine.

Primary culture of cortical neurons was performed as described previously (8). In brief, newborn (postnatal day 0) mouse cortices were dissected out, minced, and digested with papain for 20 min at 37°C. After dissociated by trituration, cortical neurons were plated in Neurobasal-A medium supplemented with 5% FBS, 2% B27, 2% glutamax, 0.2% gentamycin at 5×10^4 cells/well in 24-well plates. From the second day, cells were fed with Neurobasal-A medium supplemented with 2% B27, 2% glutamax, 0.2% gentamycin. Culture medium was changed every 4 days. Neurons were treated and analyzed after being cultured for 11-13 days *in vitro* (DIV). It should be noted that there is no noradrenergic input to cortical neurons in these cultures and thus the basal α_{2A}AR activity is negligible.

Measurement of secreted APP ectodomains in culture medium.

To measure secreted APP (sAPP), N2a cells stably expressing α_{2A}AR were transfected with a plasmid encoding hAPPsw. 12 hrs post transfection, cells were cultured in serum-free

medium with vehicle or clonidine (1μM). 36 hrs post treatment, culture medium was collected and concentrated with Millipore centrifugal Filter Units (Ultracel-3K) following manufacture's instruction. Cells were lysed and examined for full-length APP expression.

cAMP assays in cortical neurons.

cAMP assays were performed using AlphaScreen® Assay Kit from PerkinElmer following the manufacturer's instruction. Cortical neurons cultured on 100mm dishes for 12 DIV were washed once and collected in PBS. The pellet was resuspended with the stimulation buffer (1x HBSS, 0.1% BSA, 0.5mM IBMX, 5mM HEPES, pH7.4) at $1x10^4$ cells/μl. Cells were mixed with anti-cAMP acceptor beads and then divided into 5 groups with the following treatment: (1) vehicle; (2) 10μM foskolin; (3) 10μM foskolin and 1μM clonidine; (4) 10μM forskolin, 1μM clonidine and 10μM idazoxan; (5) 10μM forskolin, 1μM clonidine and 10μM BRL44408. 30 min post-stimulation, detection mix containing biotinylated cAMP/streptavidin donor beads in lysis buffer (0.1% BSA, 0.3% Tween20, 5mM HEPES, pH7.4) was added to cells/acceptor beads mix. After 60 min incubation, luminescence was analyzed on a Biotek Synergy2 plate reader using standard α-screen settings.

Fluorescence immunocytochemistry

Fluorescence immunocytochemistry was performed as described previously (9). Fluorescent images were taken under a confocal microscope system Nikon A1R-A1, and analyzed with imaging software NIS-Elements AR4.13.00. The degree of colocalization between two proteins was quantified by Pearson's correlation coefficient using NIS-Elements AR4.13.00 subprogram Analysis Control-Colocalization.

Subcellular fractionation

To separate organelles, a one-step subcellular fractionation method was performed using Nycodenz density gradient centrifugation (10). A working Nycodenz solution (20% w/v Nycodenz, 0.25M sucrose, 20mM Tricine, Ph7.4, 1mM EDTA and protease inhibitors) was prepared by mixing 40% w/v Nycodenz stock solution with the sucrose stock solution (1.25M sucrose, 100mM Tricine, Ph7.4 and 5mM EDTA). Then Beckman polycarbonate tubes (3.2 ml, 13x56 mm) were filled to about 90% volume with the Nycodenz working solution and allowed to freeze at -80°C overnight. The solution in tubes was thawed at room temperature several hours before use.

N2a cells stably expressing α_{2A}AR were treated with vehicle or 1 μM clonidine for 30min. Cells were washed once with ice-cold PBS and collected in the sucrose working solution (0.25 M sucrose, 20 mM Tricine, pH 7.4, 1 mM EDTA, and protease inhibitors). After being homogenized by 25g needle 10 times, samples were centrifuged for 15 min at 1,000 × *g* at 4°C to remove nuclear fractions. Then the supernatant was subjected to ultracentrifugation at 100,000 × *g* for 20 min at 4°C. The pellet (containing organelles) was resuspended in the sucrose working solution and loaded onto the thawed Nycodenz working solution in the Beckman tubes. After being centrifuged in a fix-angled rotor at 100,000 × *g* for 16 hours, 12 fractions from top to bottom were collected and subjected to Western blot analyses.

Immunoprecipitation to detect protein-protein interaction

HEK293 cells stably expressing α_{2A}AR were co-transfected with plasmids encoding myc-SorLA and hAPP695. 48h post transfection, cells were stimulated with vehicle or 1 μM clonidine for 10 min. Cells were then washed once with ice-cold PBS and lysed in immunoprecipitation (IP) buffer (10 mM Tris, pH 7.4, 150mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.3% NP-40, 10% glycerol, and protease inhibitors). APP antibody (Y188) was used to immunoisolate

APP following a procedure described previously (11). Alternatively, cell lysates were incubated with a monoclonal Myc antibody to immunoisolate myc-SorLA. To block $G_{i/o}$ subfamily of G proteins, cells were pretreated with 200ng/ml pertussis toxin (PTX) overnight before stimulation.

To examine APP-BACE1 interaction, HEK293 cells stably expressing α_{2A}AR were co-transfected with plasmids encoding BACE1-CFP, hAPP695 and myc-SorLA. 48h post transfection, cells were stimulated with vehicle or 1 μM clonidine for 10 min. Cell lysates were subjected to IP assays using an APP antibody (Y188).

For coimmunoisolation of endogenous SORLA and APP from brain lysates, adult mice were treated with saline or clonidine (1 mg/kg) through i.p. injection. 30min post-treatment, mouse brain was homogenized on ice in IP buffer described above. The detergent extract was then centrifuged at 18,000 × *g* at 4°C for 30 min, and the resulting supernatant was subjected to IP assays with an APP antibody (Y188) or a rabbit polyclonal antibody against SorLA.

Western blot quantification with LI-COR Odyssey Imaging System.

All imaging and quantification of Western blots were performed using the LI-COR Odyssey Imaging System following manufacture's instruction. Signal obtained with the digital scanner is directly proportional to the quantity of the target protein, and allows accurate quantification of both low- and high-abundance proteins in a much wider linear range (correlation coefficient $R_2 > 0.99$) than traditional films.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software. Data are expressed as mean ± SEM. To determine difference between two groups, Student's *t* test (for normally distributed data) or Mann-Whitney rank sum test (non-normally distributed data) was

performed. Two-way ANOVA was performed to determine variations in multiple groups with two variances. $p<0.05$ was considered statistically significant.

References

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Supplementary figure legends

sFig. 1. Genetic deficiency of the α_{2A}AR reduces microglial activation in *APP/PS1* mice. Representative images of IBA-1 staining for active microglia in cerebral cortex isolated from 6 month-old male mice with indicated genotypes. Scale bar, 100μm.

sFig. 2. Genetic deficiency of the α_{2A}AR reduces astrocyte activation in *APP/PS1* mice. (*A*-*C*) Representative images of GFAP staining for active astrocytes in cerebral cortex isolated from 6 month-old male mice with indicated genotypes. Scale bar, 100μm. (*D*) Quantification of relative GFAP density. Data (mean ± SEM) are expressed as the fold change to the level in *APP/PS1, Adra2a*$^{+/+}$ mice. n=7 for each genotype. *, $p<0.05$; **, $p<0.01$, compared to *APP/PS1, Adra2a*$^{+/+}$ mice.

sFig. 3. Activation of the α_{2A}AR by norepinephrine (NE) enhances production of Aβ peptides. (*A*) Cortical neurons were treated with 10μM NE (plus 1μM propranolol and 1μM prazosin) or vehicle for 48 hrs. The level of Aβ42 in culture medium was measured by ELISA. Data (mean ± SEM) are expressed as the fold change to control. n=4 for each treatment group. *, $p<0.05$, NE *vs.* control. (*B*) N2a cells expressing hAPP and α_{2A}AR were treated with 10μM NE (plus propranolol and prazosin) or vehicle for 48 hrs. The levels of Aβ40 in culture medium were measured by ELISA. Data (mean ± SEM) are expressed as the fold change to control. n=6 for each group. ***, $p<0.0001$, NE *vs.* control.

sFig. 4. Clonidine inhibits cAMP production through the α_{2A}AR and promotes Aβ generation in primary cortical neurons. (*A*) Forskolin-induced cAMP production in cortical neurons with indicated treatment. The concentration of each indicated drug was 1μM. Clon, clonidine; BR, BRL44408; ID, idazoxan. Data (mean ± SEM) are expressed as the fold change to vehicle control. n=4-6 for each treatment group. **, $p<0.01$, compared to vehicle control. (*B*) The level

of Aβ40 in culture medium of cortical neurons treated with vehicle or clonidine for 24 hrs. Data (mean ± SEM) are expressed as the fold change to control. n=4 for each treatment group. **, $p<0.01$, compared to vehicle control.

sFig. 5. Expression of endogenous APP, SorLA and BACE1 following α_{2A}AR activation. N2a cells expressing α_{2A}AR were stimulated with clonidine for indicated time durations. Total cell lysates were separated by SDS-PAGE and blotted for indicated proteins.

sFig. 6. Activation of α_{2A}AR disrupts APP-SorLA interaction. (*A*) Interaction of endogenous APP and SorLA in mouse brain. Mice were treated with saline or clonidine for 30min and lysates of cerebral cortex were subjected to IP assays using an APP antibody (Y188). (*B*) HEK293 cells expressing α_{2A}AR, Myc-SorLA and hAPP were treated with vehicle or clonidine, and cell lysates were subjected to immunoprecipitation (IP) assays using a Myc antibody.

sFig. 7. Adenosine A1R and SEMA6D cannot be co-immunoisolated with SorLA from brain lysates. Mice were treated with saline or clonidine for 30min and brain lysates were subjected to IP assays using a SorLA antibody.

sFig. 8. Activation of the α_{2A}AR enhances APP localization in recycling endosomes. N2a cells expressing the α_{2A}AR were treated with vehicle or 1μM clonidine for 15 min. (*A)* Representative images showing localization of the endogenous APP (by Y188 antibody) together with Rab11. Scale bar, 10μm. (*B*) Colocalization was quantified by Pearson's correlation coefficient. n=13-18 cells for each group. ***, $p<0.001$, clonidine *vs*. vehicle.

sFig. 9. Activation of the α_{2A}AR reduces APP localization in Golgi and enhances its localization in early endosomes in primary cortical neurons. Cortical neurons were treated with vehicle or 1μM clonidine for 15 min. Representative images showing localization of the endogenous APP (by Y188 antibody) together with Golgi58k (*A*) or EEA1 (*C*). Scale bar, 10μm. Colocalization

was quantified by Pearson's correlation coefficient (*B* and *D*). n=10-13 cells for each group. ***, $p<0.001$, compared to vehicle treated cells.

sFig. 10. The full representative Western blots with all subcellular fractions prepared from cells treated with vehicle (*A*) or clonidine (*B*). Endogenous APP, BACE1, Rab5, Golgi58k and KDEL were blotted with specific antibodies.

sFig. 11. Activation of α_{2A}AR promotes APP-BACE1 interaction in cultured cells and in mouse brain. (*A*) HEK293 cells expressing α_{2A}AR, APP and BACE1-CFP were treated with vehicle or clonidine, and cell lysates were subjected to immunoprecipitation (IP) assays using an APP antibody (Y188). (*B*) Mice were treated with saline or clonidine for 30min and lysates of cerebral cortex were subjected to IP assays using BACE1 antibody. Mature APP was co-immunoisolated with BACE1.

sFig. 12. Activation the α_{2A}AR has a negligible effect on α secretase cleavage of APP. (*A* & *B*) N2a cells exogenously expressing α_{2A}AR and hAPPsw were stimulated with clonidine (1μM) or vehicle for 36 hrs. Quantitation of C83 (*A*) and sAPPα (*B*) as a ratio over the APP-FL was measured. Representative blots are shown in Fig. 6. Data represents mean ± SEM. n=5-6 for each treatment group. (*C* & *D*) *APP/PS1* mice treated with clonidine or saline for 8 weeks. Quantitation of C83 (*C*) and sAPPα (*D*) as a ratio over the APP-FL was measured. Representative blots are shown in Fig. 6. n=5-6 for each treatment group. (*E*) N2a cells exogenously expressing α_{2A}AR and C99 were stimulated with NE (10μM plus propranolol and prazosin), clonidine (1μM) or vehicle for 36 hrs. The level of Aβ40 was measured by ELISA. n=6 for each treatment group.

sFig. 13. Idazoxan treatment reduces endogenous Aβ production in nTg mice. The levels of endogenous mouse Aβ40 (*A*) and Aβ42 (*B*) in the cortex of nTg mice treated with saline or

idazoxan for 10 weeks. Data (mean ± SEM) are expressed as the fold change to the Aβ concentration in saline-treated mice. n=10-14 in each treatment group. *, $p<0.05$; **, $p<0.01$, idazoxan *vs.* control.

sFig. 14. Open field analysis of nTg and *APP/PS1* mice treated with saline or clonidine. (*A*) Total distance traveled during the open field trial. (*B*) Time spent in the center of the open field area expressed as a percentage over total trial time.

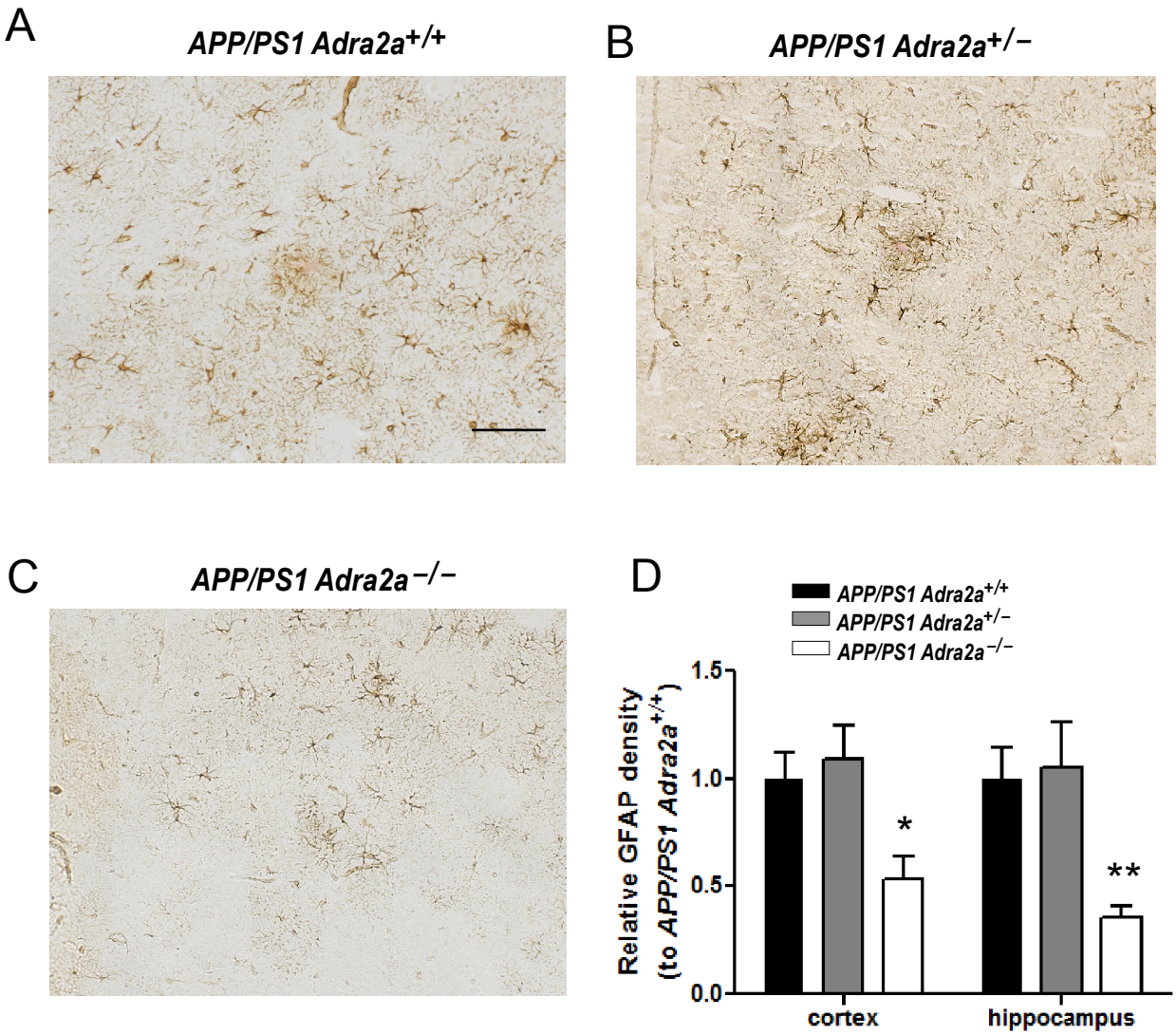
sFig. 15. Elevated zero maze analysis of nTg and *APP/PS1* mice treated with saline or clonidine. (*A*) Percent time spent in open areas of elevated zero maze during trial. (*B*) Entries into open areas of the maze.

sFig. 16. Morris water maze test of nTg and *APP/PS1* mice treated with saline or clonidine. (*A*) Travel distance in water was measured on each day of training. Data represent mean ± SEM. (*B*) Representative swim route in probe trial on the last day. (*C*) Quantification of time spent in the target quadrant in probe trial. The dashed line indicates the 25% level. n=11 for the nTg + saline and *APP/PS1* + saline; n=9 for nTg + idazoxan; n=8 for *APP/PS1* + idazoxan. *, $p<0.05$; **, $p<0.01$, saline-treated *vs.* idazoxan-treated *APP/PS1*. †, $p<0.05$; ††, $p<0.01$, saline-treated *APP/PS1 vs.* nTg.

sFig. 17. Proposed model for α_{2A}AR-mediated regulation of APP sorting and processing. Without α_{2A}AR stimulation, SorLA strongly interacts with mature APP, sorting APP from endosomes to TGN and retaining APP in TGN. As a result, amyloidogenic processing of APP is reduced. When α_{2A}AR is activated, SorLA-APP interaction is disrupted downstream of $G_{i/o}$ signaling. This leads to reduced APP distribution to TGN. Concurrently, APP colocalization and interaction with BACE1 in endosomes are enhanced. Consequently, more APP is cleaved by BACE1 and more Aβ is generated.

sFig. 1

sFig. 2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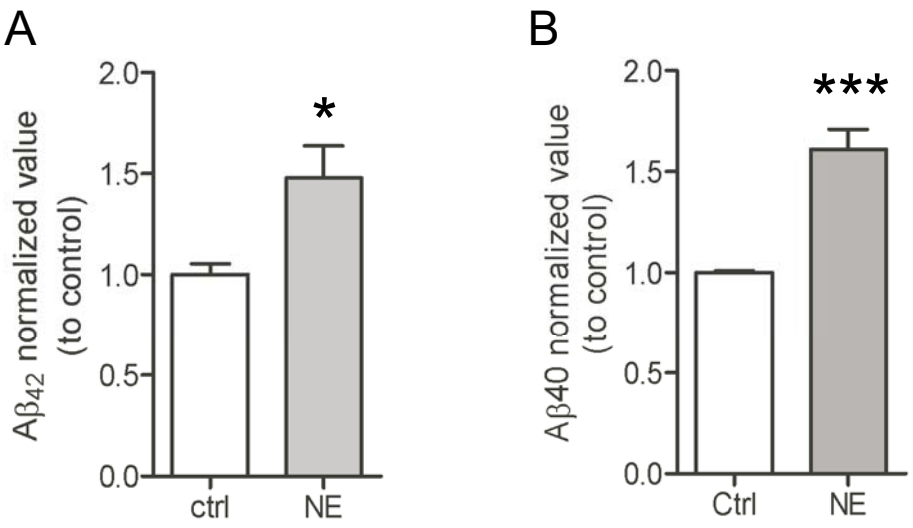
sFig. 3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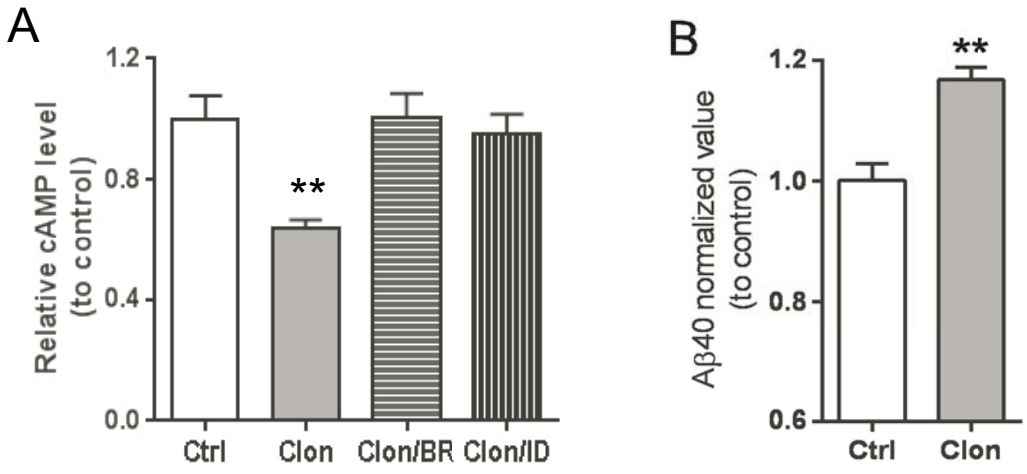
sFig. 4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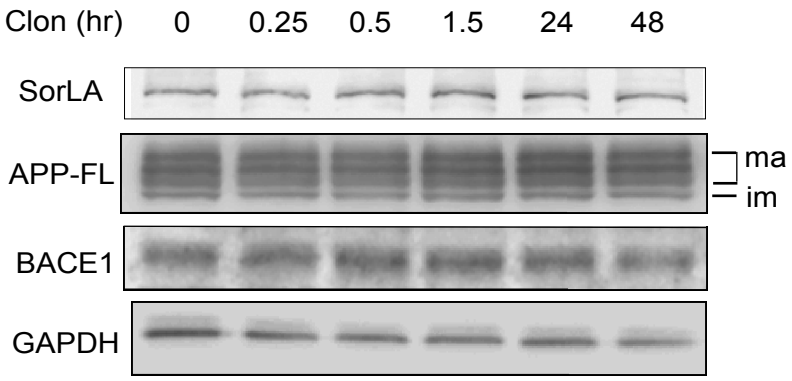
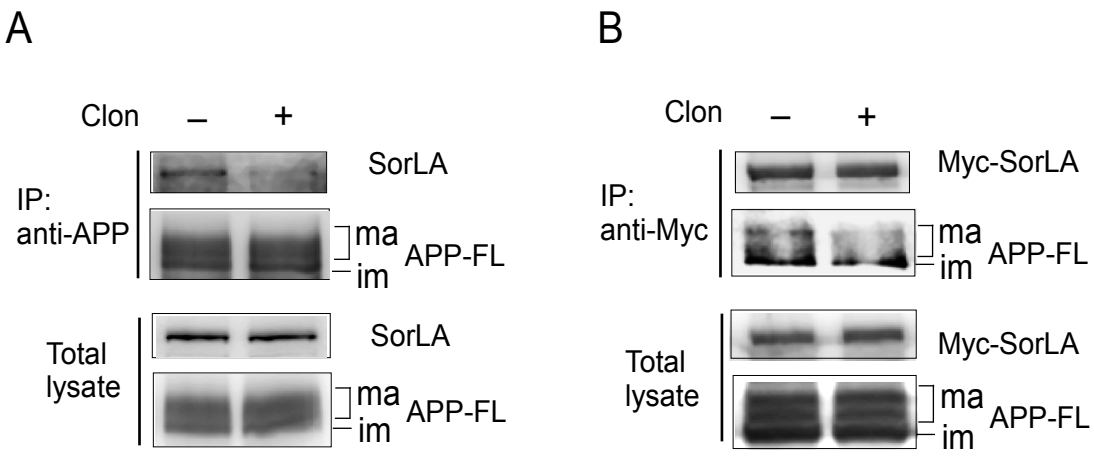
sFig. 5



sFig. 6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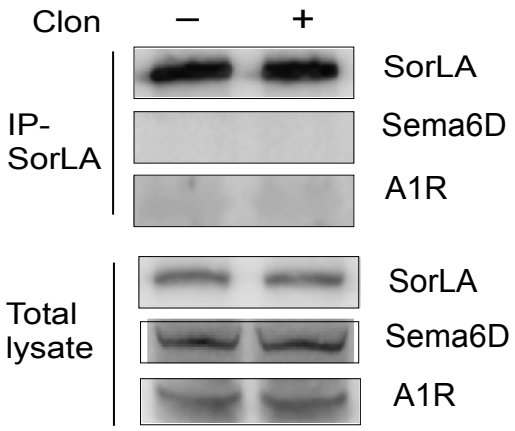
sFig. 7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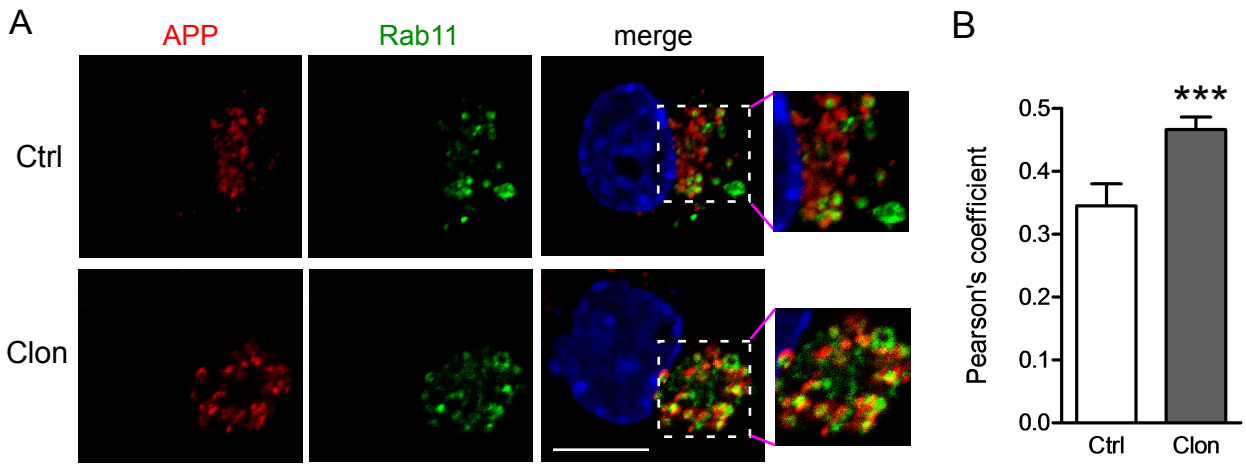
sFig. 8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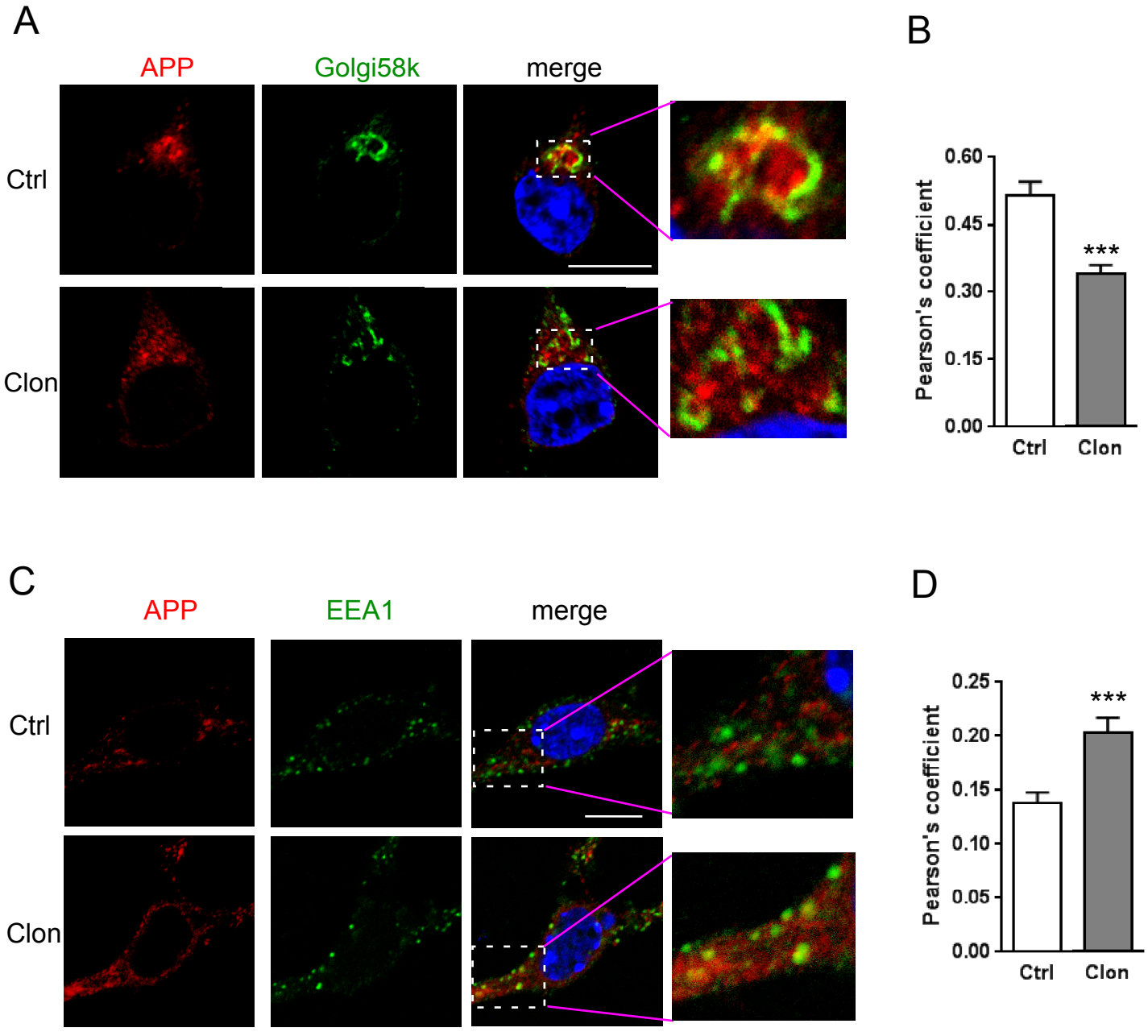
sFig. 9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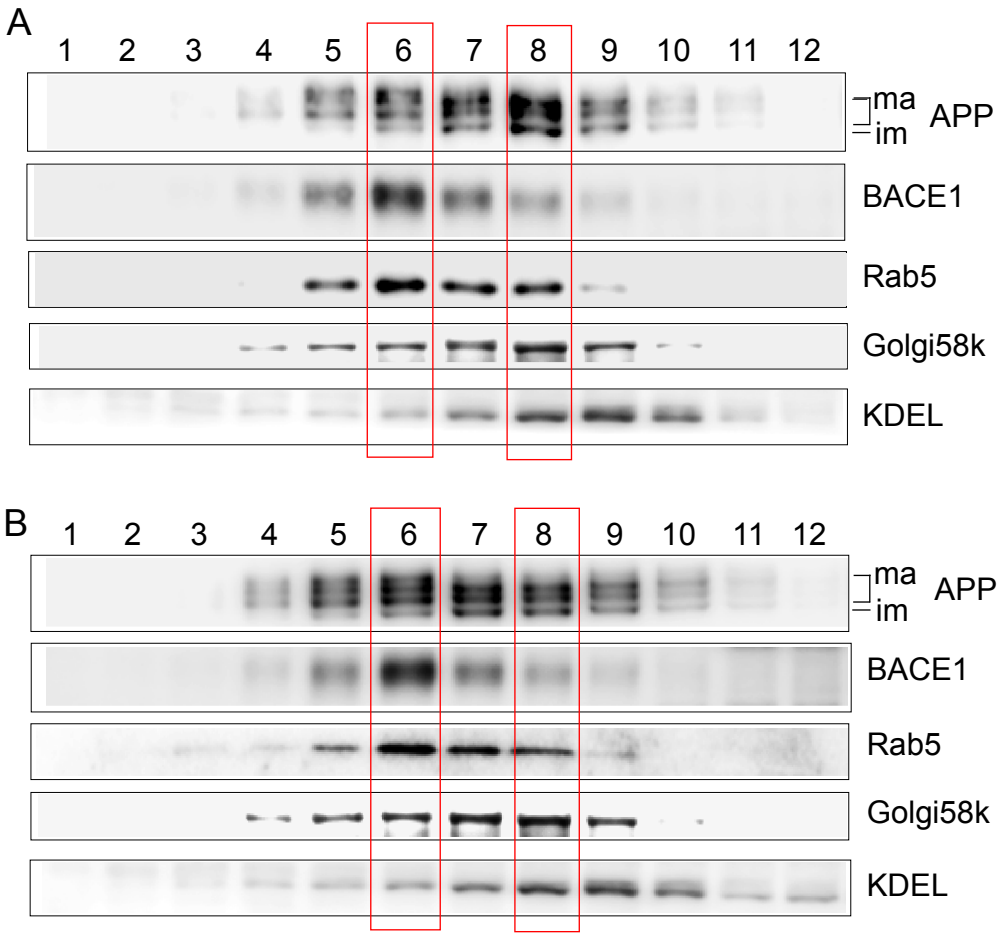
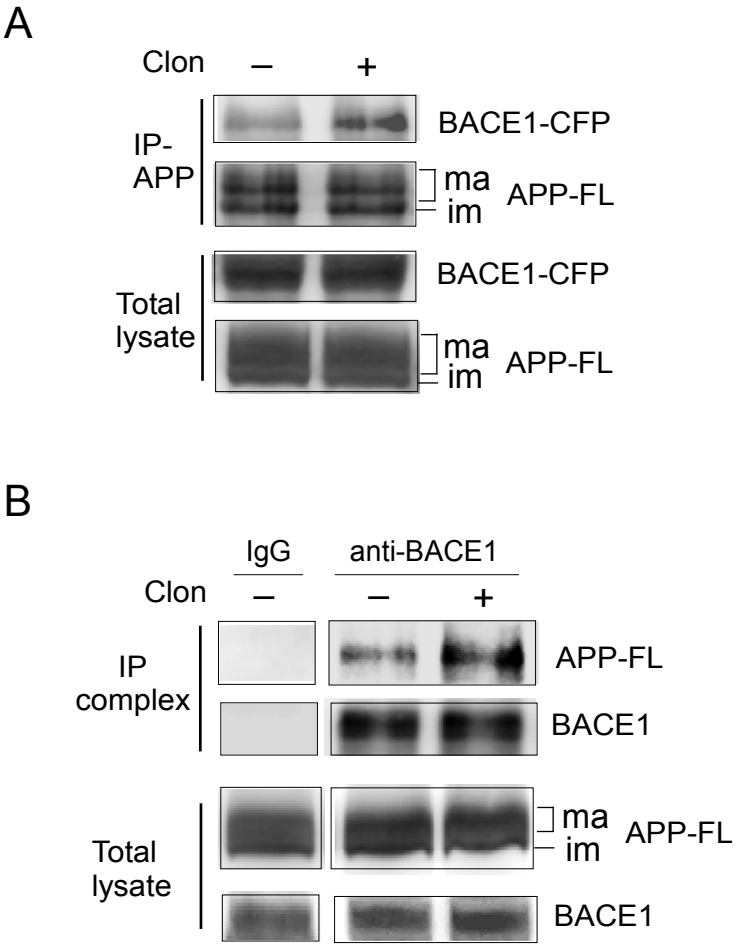
Clon

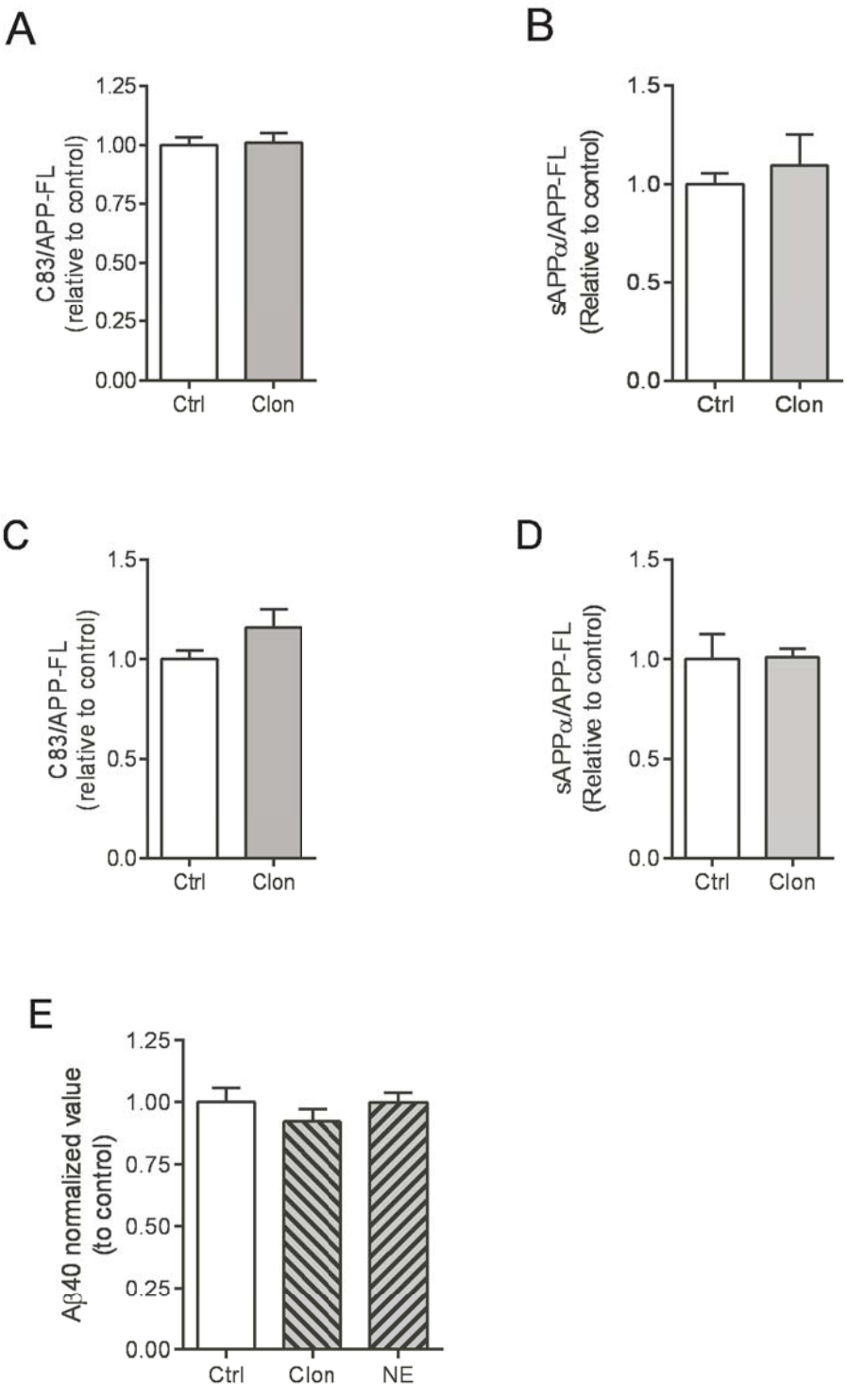
sFig. 10


A
1 2 3 4 5 6 7 8 9 10 11 12
ma
im
APP
BACE1
Rab5
Golgi58k
KDEL
B
1 2 3 4 5 6 7 8 9 10 11 12
ma
im
APP
BACE1
Rab5
Golgi58k
KDEL

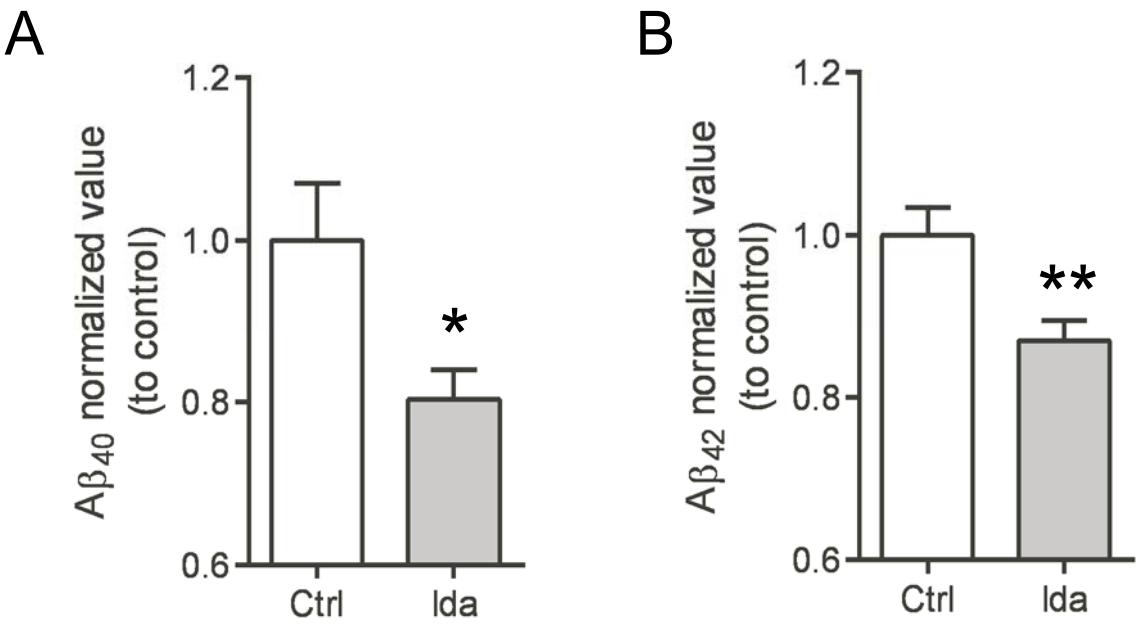
sFig. 11


A
Clon
–
+
IP-APP
BACE1-CFP
ma
im
APP-FL
Total lysate
BACE1-CFP
ma
im
APP-FL
B
IgG
anti-BACE1
Clon
–
–
+
IP complex
APP-FL
BACE1
Total lysate
ma
im
APP-FL
BACE1

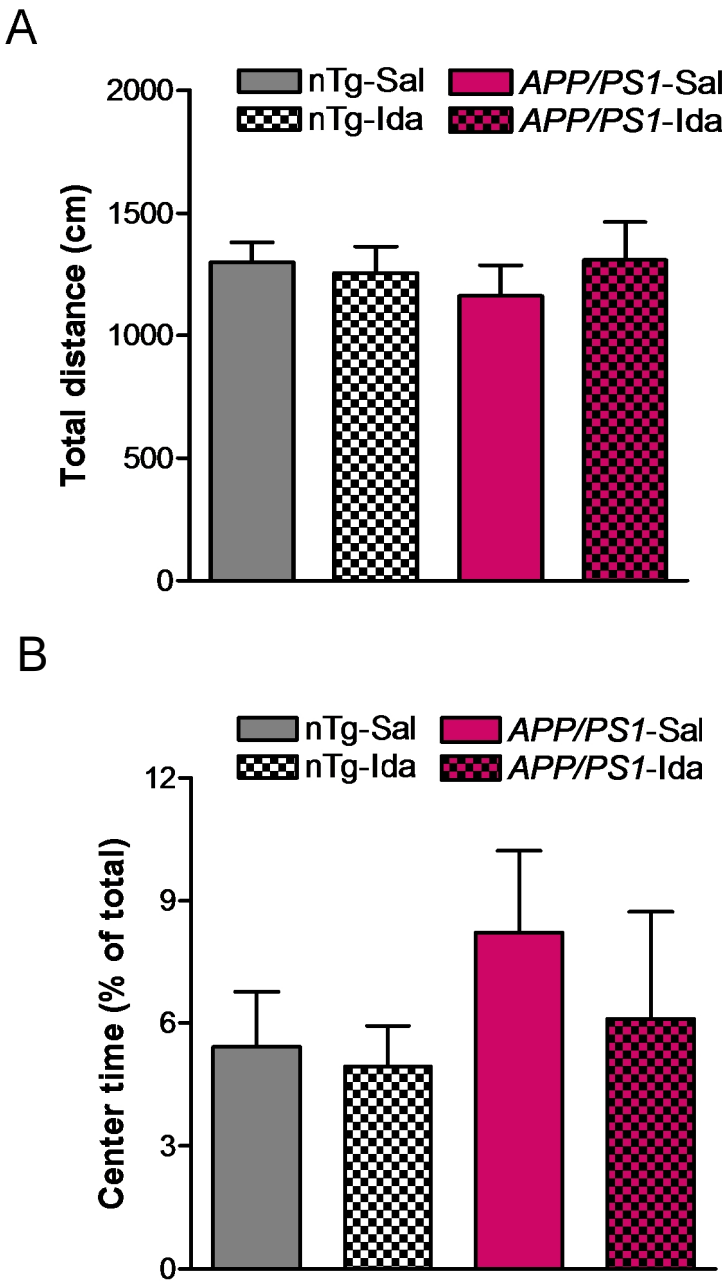
sFig. 12


A
C83/APP-FL
(relative to control)
1.25
1.00
0.75
0.50
0.25
0.00
Ctrl
Clon
B
sAPPα/APP-FL
(Relative to control)
1.5
1.0
0.5
0.0
Ctrl
Clon
C
C83/APP-FL
(relative to control)
1.5
1.0
0.5
0.0
Ctrl
Clon
D
sAPPα/APP-FL
(Relative to control)
1.5
1.0
0.5
0.0
Ctrl
Clon
E
Aβ40 normalized value
(to control)
1.25
1.00
0.75
0.50
0.25
0.00
Ctrl
Clon
NE

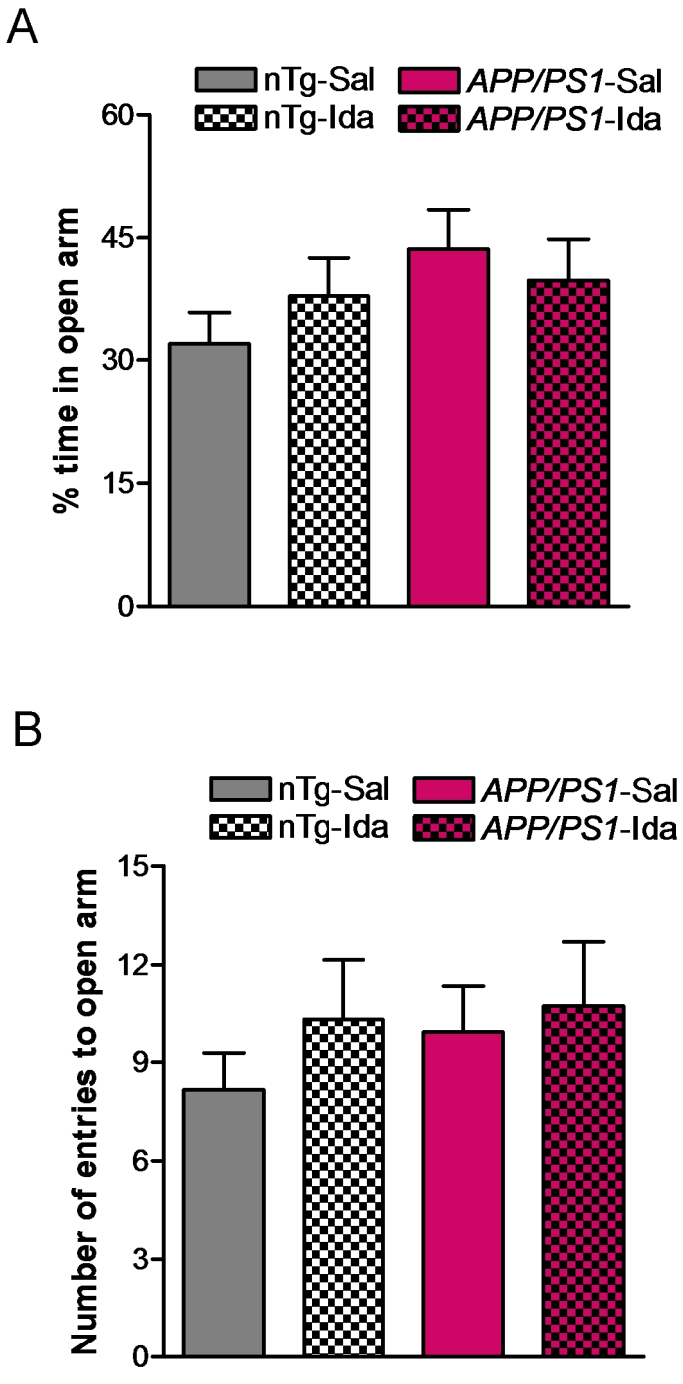
sFig. 13


A
1.2
1.0
0.8
0.6
Aβ40 normalized value
(to control)
*
Ctrl
Ida
B
1.2
1.0
0.8
0.6
Aβ42 normalized value
(to control)
**
Ctrl
Ida

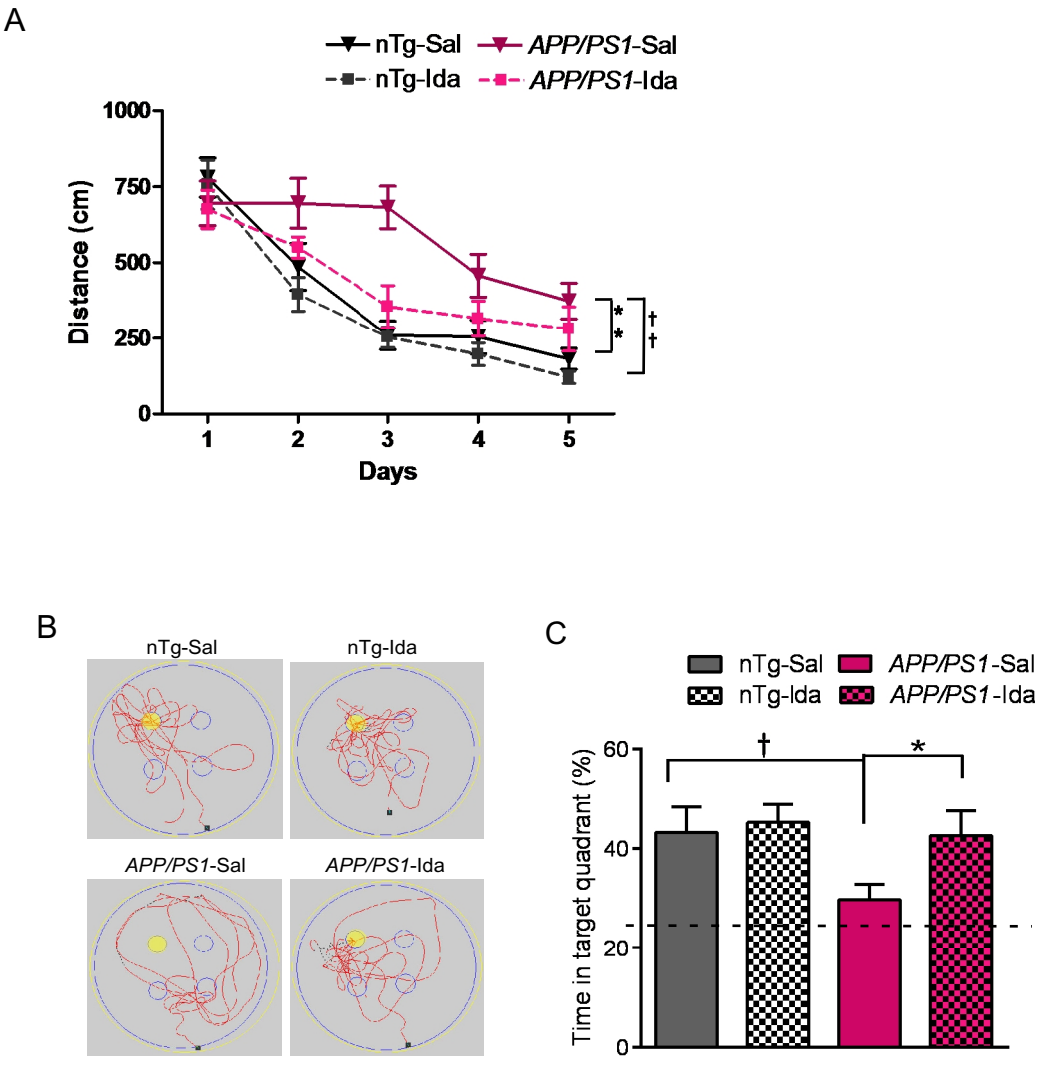
sFig. 14


A
nTg-Sal
nTg-Ida
APP/PS1-Sal
APP/PS1-Ida
Total distance (cm)
2000
1500
1000
500
0
B
nTg-Sal
nTg-Ida
APP/PS1-Sal
APP/PS1-Ida
Center time (% of total)
12
9
6
3
0

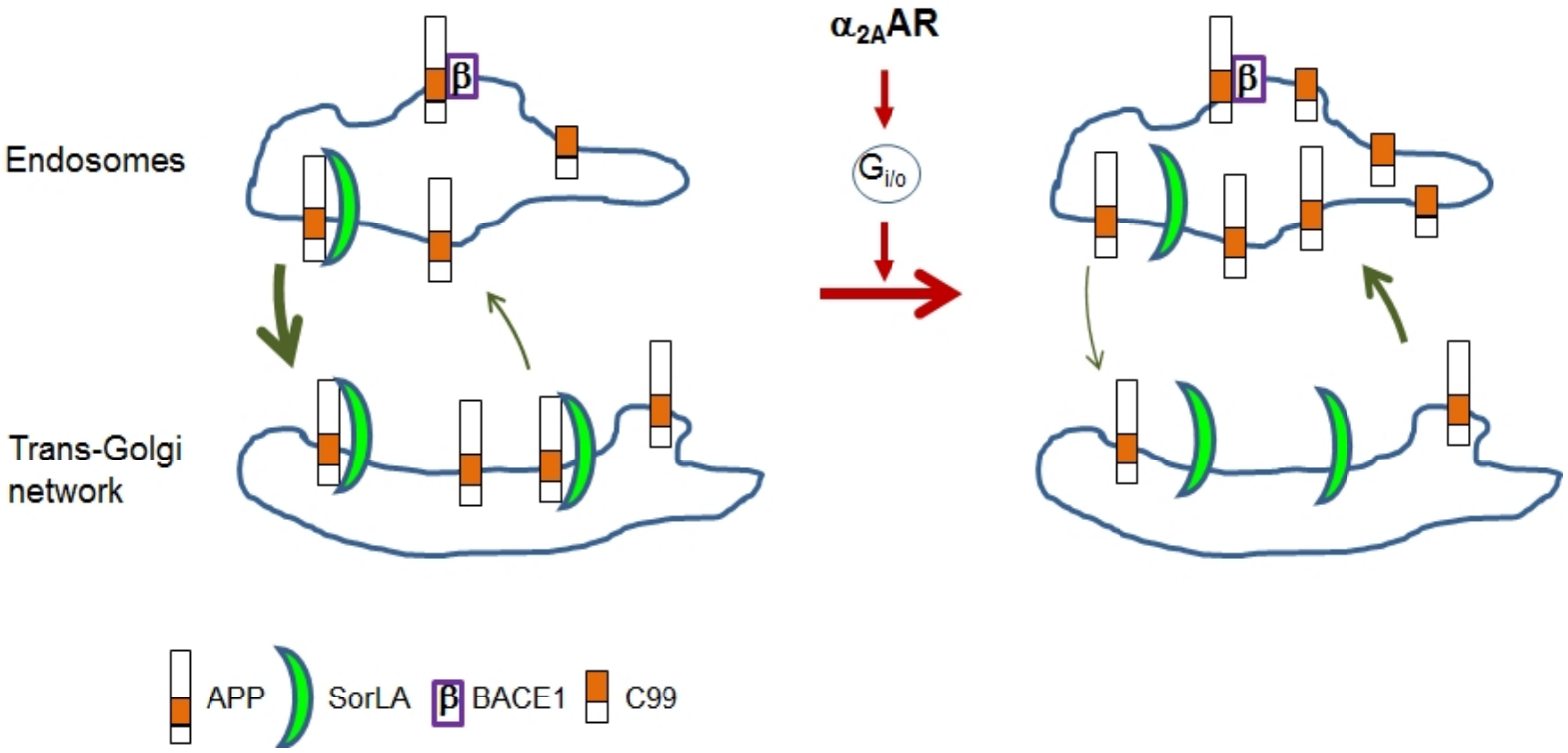
sFig. 15


A
nTg-Sal
APP/PS1-Sal
nTg-Ida
APP/PS1-Ida
% time in open arm
60
45
30
15
0
B
nTg-Sal
APP/PS1-Sal
nTg-Ida
APP/PS1-Ida
Number of entries to open arm
15
12
9
6
3
0

sFig.16


A
nTg-Sal
APP/PS1-Sal
nTg-Ida
APP/PS1-Ida
Distance (cm)
1000
750
500
250
0
1
2
3
4
5
Days
*
*
†
†
B
nTg-Sal
nTg-Ida
APP/PS1-Sal
APP/PS1-Ida
C
nTg-Sal
APP/PS1-Sal
nTg-Ida
APP/PS1-Ida
Time in target quadrant (%)
60
40
20
0
†
*

sFig. 17


Endosomes
Trans-Golgi network
β
α_{2A}AR
$G_{i/o}$
β
APP
SorLA
β
BACE1
C99