

Supplemental Data

Reduced IGF-1 Signaling Delays

Age-associated Proteotoxicity in Mice

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Supplemental figure legends:

Figure S1: The production and processing of APP is not affected by reduced IGF signaling. Quantitative RT-PCR (qPCR) revealed that reduction of IGF signaling does not significantly affect the expression level of the APP_{swe} transgene in the experimental mice. **A-C.** qPCR of human A β normalized to actin levels. **D** and **E.** β -actin controls indicate the linearity of the reaction. **F.** 12-13 month old *AD* and *AD;Igf1r^{+/-}* mouse brains contain similar quantities of APP processing enzymes and their products. Western blot analyzes of A β , APP C-terminal fragment (APP CTFs), α secretase (ADAM17) and β secretase (BACE1) quantities in brain homogenates of mice of all four genotypes. **G.** Quantification of F indicated that the total amounts of A β and of APP CTFs were higher in *AD* and *AD;Igf1r^{+/-}* mice compared to their WT and *Igf1r^{+/-}* age matched counterparts, however, no significant difference could be detected among *AD* and

AD;Igf1r+/- brain samples. **H.** No differences in the quantities of ADAM17 and of BACE1 were apparent among mice brains of all genotypes.

Figure S2: Initial behavioral analysis of *AD* mice in the context of reduced IGF signaling. **A.**

Reduced IGF signaling results in lower body weight through 16-17 month of age in both, *Igf1r+/-* and *AD;Igf1r+/-* mice compared to their *WT* and *AD* counterparts. **B and C.** Preliminary watermaze experiment indicates that the orientation impairment of *AD* and *AD;Igf1r+/-* mice is apparent at 9 months of age or later. Eight animals per genotype were trained to find a hidden platform submerged 1.5cm under opaque water in a watermaze. Average times of latency in day 2 and 3 after training are presented for *WT* and *AD* animals (**B**) and for *Igf1r+/-* and *AD;Igf1r+/-* mice (**C**). **D.** Mice of all genotypes swam at nearly identical speeds as measured by the Ethovision software (corresponding Figure 1B, C)

Figure S3: Reduced IGF signaling protects against neuronal loss due to A β expression. **A-C:**

Total neuron counts of *AD;Igf1r+/-* mice were significantly ($P < 0.05$) higher than those of *AD* animals both in cortices and hippocampus CA1 regions in young (A, 4-5 month), midlife (B, 12-13 month) and old (C, 16-17 month) ages.

Figure S4: Immuno-histochemistry using the A β antibody 6E10 indicated that A β plaques appear at similar temporal fashion in brains of *AD* and *AD;Igf1r+/-* mice. No plaques were detected in the brains of 4-5 month old mice, a few dispersed plaques observed in the brains of 8-9 month old animals and abundant plaques were seen throughout the brains of 12-13 month old animals (scale bars 200 μ m). Insets: plaques detected in *AD;Igf1r+/-* mice cortices were focal and more condensed compared to those seen in cortices of *AD* animals (corresponding Figure 4B).

Figure S5: Thioflavin-S staining and proteinase K treatment of amyloid plaques. **A.** Double labeling of A β plaques using A β antibody (82E1, red channel) and Thioflavin-S (Green channel) confirms the specificity of Thioflavin-S plaque labeling in *AD* and *AD:Igflr+/-* mice (nuclei are labeled with DAPI (Blue)). **B.** Proteinase K treatment shows higher sensitivity of plaques of *AD* animals compared to their *AD:Igflr+/-* counterparts. Plaques are stained with the A β antibody 82E1.

Figure S6: . Post embedding immuno-electron microscopy (EM) and EM quantification of animal brains used in this study. **A.** Post embedding immuno-electron microscopy indicated that A β plaques of 12-13 month old *AD:Igflr+/-* (right panel) are of higher order and density compared to those of their *AD* counterparts (left panel). Corresponding Figure 5A. **B.** No background A β gold labeling detected in cortices of WT and of *Igflr+/-* mice of all ages using A β polyclonal antibody (corresponding Figure 5A). **C.** Electron-microscope analysis of A β plaque density. Median object size and object number threshold signature for the image displayed in panels D,E and F. Shaded areas correspond to different threshold regimes and their respective segmented object size and total number of segmented objects. **D-F.** Segmented object (circled in red) for the th1 ,th2 and th3 threshold levels in C (corresponding Figure 5B). **G.** Immuno-gold label exclusion for via local threshold estimation. Shown here a representative intensity profile (red curve, bottom) measured along the pixel line pointed by the red arrow. A threshold of 0.05% is applied to the inverse of the intensity profile (gray curve, top) and the gold particle segmentation threshold is estimated by finding the first two non zeros (to the right and left moving away from the center of the scan, red dashed lines). **H.** Red squares represent pixels corresponding to the segmented gold particle. **I.** Resulting mask used for estimating median intensity. Scale bar 100nm in b-d and 10nm in e-g. Corresponding Figure 5B. **J.** The distance between each gold particle and its closest neighbor was measured in all images of *AD* and *AD:Igflr+/-* mice brains. Distance distributions in both genotypes were nearly identical,

indicating no difference in antibody accessibility among the genotypes. **K.** Brain homogenates of 4-5 month old (prior to plaque formation) *AD* and *AD;Igf1r+/-* mice seeded *in-vitro* kinetic A β aggregation assays with similar efficiencies (corresponding Figure 5C).

Figure S7: A. No significant difference observed in the non-aggregated A β_{1-40} content in brain homogenates of 4-5 month old mice as measured by ELISA assay (corresponding Figure 6A). **B.** No A β oligomers were detected in brain cytosolic fractions of 12-13 month old mice of all genotypes as measured by western blot analysis using A β antibody 6E10 (corresponding Figure 6C, D). **C-D.** Standard protein mixture was separated on the superdex 75 size exclusion column to calibrate protein sizes expected to exit the column in each fraction.

Supplemental Experimental Procedures

RNA Isolation and quantitative RT-PCR

Brains were removed from mice and flash froze. About 30mg of tissue was cut from each forebrain and transferred into 500 μ l Qiazol (Cat # 79306 Qiagen, Hilden Germany), homogenized using a syringe and needle and froze at -80°C for 4 hours. The samples were thawed, supplemented with 120 μ l chloroform and spun for 10min (18,000g). Total RNA was isolated from the supernatants using RNeasy kit (Qiagen cat # 74106). cDNA was prepared from 1 μ g RNA using Quanti Tect kit (Qiagen cat # 204341). For quantitative PCR reactions, dilutions of 10 \times were used. SybrGreen real-time qPCR experiments were performed as described in the manual using ABI Prism7900HT (Applied Biosystems). Quantification was completed using SDS2.1 software (Applied biosystems), normalizing to control levels of β -actin cDNA. A β primers: forward: CAGAATTCCGACATGACTCAGGATATGAAG, reverse: CCCACCATGAGTCCAATGATTGC. β -actin primers: forward: CTCCTCCTGAGCGCAAGTACTCTGTGT reverse: GTGCACGATGGAGGGGCCGACTCAT.

A β and AD-linked protein blotting and detection

A posterior half hemisphere (approximately 100mg) was taken from each mouse brain, supplemented with 700µl PDGF buffer (1mM HEPES, 5mM Benzamidine, 2mM β-Mercaptoethanol, 3mM EDTA, 5mM Magnesium Sulfate, 0.05% Sodium Azide, pH8.8) and phosphatase and protease inhibitor cocktails (Calbiochem, San Diego, CA cat # 524625 and 539134 respectively). The brains were sonicated and spun (5min, 5000rpm). Pellets were supplemented with 500µl PDGF buffer and sonicated again (Total). Supernatants were spun for 1h, 100,000rpm, 4°C (Beckman TL-100 desktop ultracentrifuge, rotor TL-120.2, $g_{av}=355,000g$). Ultracentrifugation supernatants were transferred to new tubes (Cytosolic) while pellets were resuspended in 300µl PDGF buffer (Particulate). BCA kit was used to measure total protein amounts. Equal total protein amounts were separated on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA cat # NP0322), transferred onto nitrocellulose membrane (Protean 0.2µm Whatman, Dassel Germany) and blotted with 6E10 Aβ antibody (SIG-39320 Covance Emeryville, CA). ECL was developed using Lumi-light plus kit (Roche, Basel Switzerland). The levels of BACE were measured using Western blot (antibody: cat # 2253, ProSci Inc. CA), ADAM17 (TACE) was detected using a monoclonal specific antibody (Cat # ab57484, Abcam, MA) and actin was blotted using Mab1501 antibody (Millipore, MA).

Immuno histochemistry (IHC)

Tissue Processing

Brains were removed and divided sagittally. The left hemibrain was post-fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4°C for 48 hr and sectioned at 40 µm with a Vibratome 2000 (Leica, Germany), while the right hemibrain was snap frozen and stored at -70°C for protein analysis.

Analysis of Neurodegeneration and Aβ Deposits

To evaluate neurodegeneration, blind-coded 40 µm thick vibratome sections were immunolabeled as previously described with monoclonal antibodies against NeuN (general neuronal marker, 1:1000, Chemicon) or GFAP (astroglial marker, 1:500, Chemicon) or synaptophysin (Millipore, MAB5258) and reacted with diaminobenzidine (DAB). The sections immunostained with anti-NeuN were analyzed with the Stereo-Investigator Software (MBF Biosciences). Images collected according to the optical disector method were analyzed as previously described (Chana et al., 2003). For the sections immunostained with anti-GFAP, tissues were imaged with an Olympus digital microscope (BX51) and images analyzed with the ImageQuant program to determine levels of corrected optical density. Three immunolabeled

sections were analyzed per mouse and the average of individual measurements was used to calculate group means. Results were expressed as the average number of GFAP+ cells per 10^5 mm^2 .

A β deposits were detected as previously described, briefly vibratome sections were incubated overnight at 4°C with the mouse monoclonal antibody 4G8 (1:600, Senetek, Napa, CA), followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Vector Laboratories). Sections were imaged with the LSCM (MRC1024, BioRad) as described previously (Mucke et al., 2000) and digital images were analyzed with the NIH Image 1.43 program to determine the percent area occupied by A β deposits. Three immunolabeled sections were analyzed per mouse and the average of individual measurements was used to calculate group means.

For analysis of the density of the A β deposits, sections were immunolabeled as previously described (Rockenstein et al., 2007) with the antibodies against A β (82E1 clone, prepared against A β 1-16) and reacted with DAB. From each case, 3 serial blind coded sections were scanned with a digital bright field photo-microscope (Olympus, BX51). From each section 4 images of the neocortex and hippocampus were obtained and analyzed for levels of optical density with the ImageQuant program. Results were averaged and expressed as mean per case. When Proteinase K (PK) treatment was applied, the sections were incubated with 10 $\mu\text{g}/\text{ml}$ PK for 8 minutes prior to immunolabeling with specific A β antibody (clone 82E1).

All sections were processed simultaneously under the same conditions and experiments were performed twice to assess reproducibility. Sections were imaged with an Olympus 60X (N.A. 1.4) objective on a digital Olympus or a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss, Germany) with an attached MRC1024 LSCM system (BioRad) (Masliah et al., 2000). To confirm the specificity of primary antibodies, control experiments were performed where sections were incubated overnight in the absence of primary antibody (deleted) or preimmune serum and primary antibody alone.

A β ELISA assay

Mouse brains were removed and cut mid sagittally. One hemisphere was homogenized in cold PBS containing complete protease inhibitor cocktail (Roche cat#1836170) using a glass tissue grinder (885482, Kontes, Vineland, NJ) to final concentration of 10% w/v. The homogenates were spun briefly to sediment debris (5000rpm, 3min, desktop centrifuge). Supernatants were transferred to new tubes and were spun again to sediment non-highly aggregated A β (rcf=10,000g, 10 min, 4°C). ELISA kits were used according to the manufacturer instructions to

measure A β ₁₋₄₀ and A β ₁₋₄₂ contents in the secondary supernatants (Cat# SIG-38940 and SIG-38942 respectively, Covance, Emeryville, CA). 9 μ l of each supernatant were used for A β ₁₋₄₀ assay and 72 μ l of each supernatant to measure A β ₁₋₄₂.

Statistical methods

Data analysis was performed using parametric linear models (one-way or two way analysis of variance) or their counterpart non-parametric substitutes for cases where the data was not normally distributed. Post hoc pair-wise contrasts (planned comparisons) were obtained by Fisher-Least Square Differences (LSD). In the current study, this need raised for the analysis of the 'probe trails' in the Morris water maze and the comparison of EM particle intensity. For this purpose, we analyzed the corresponding data using the Kruskal-Wallis non-parametric test followed by the post hoc Mann-Whitney U test. In all cases, significance was considered for p-values lower than 0.05. Normal distribution was tested using a two-sided Lilliefors' composite goodness-of-fit test with the null hypothesis that data is normally distributed. All data analysis was performed under Matlab (Mathworks Inc) by implementing the provided statistical toolbox. Neuronal counts (NeuN-based) datasets of both cortex and hippocampal areas were analyzed for each age group separately using a One-way ANOVA design. Only the hippocampal counts for the 12-13 age group was analyzed using the Kruskal-Wallis non-parametric test as it did not fulfill the Lilliefors normality test. Post-hoc comparisons were performed via Tukey-Kramer test. In all analysis, a confidence level of 95% was used, for all age groups, the degrees of freedom are the same (i.e. df = 3) and the number of cases per genotypes are presented in the following order: *WT, IgfR1+/-, AD and AD:IgfR1+/-*.

Supplemental references

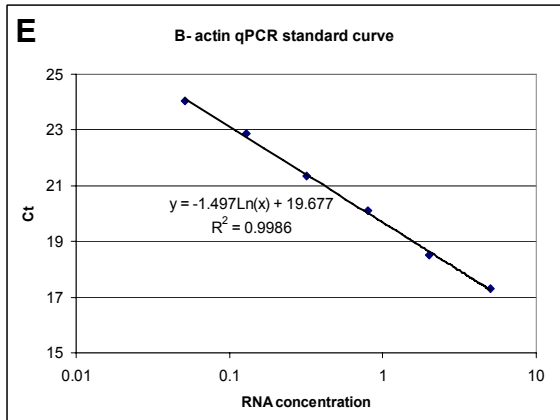
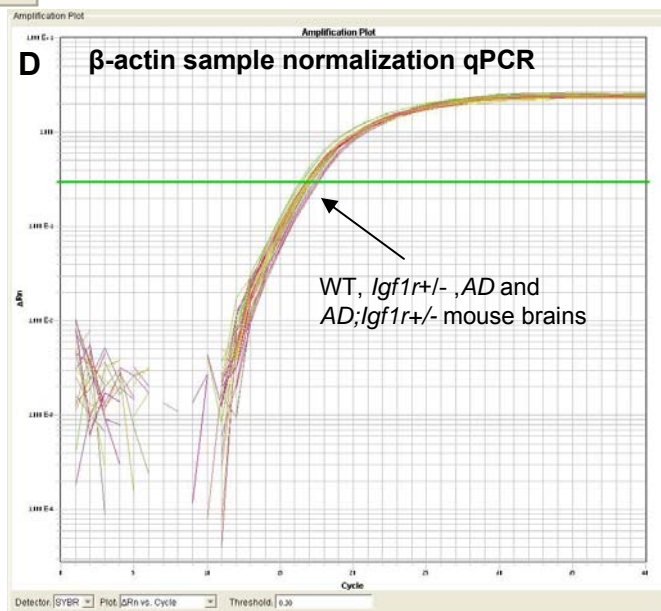
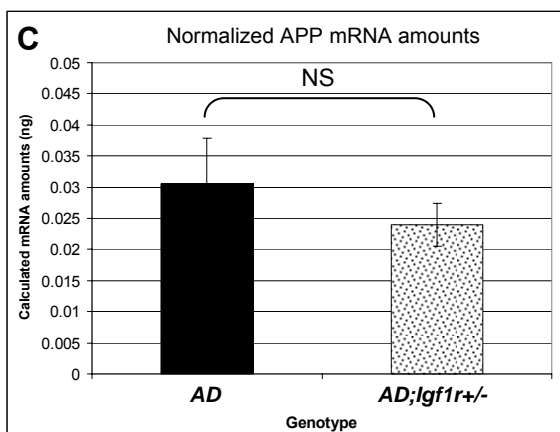
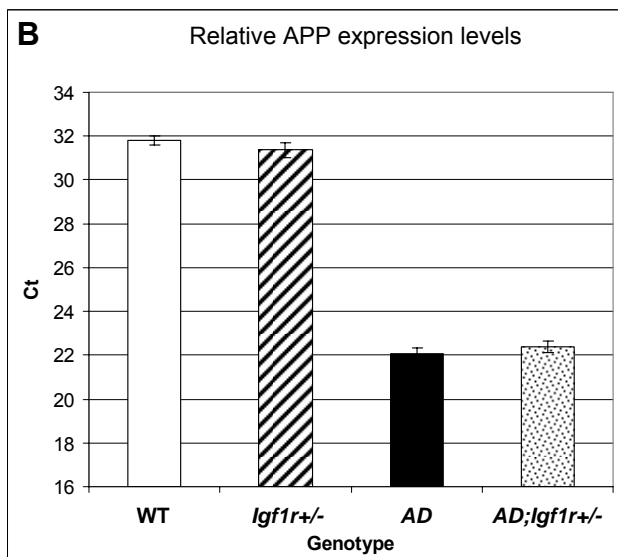
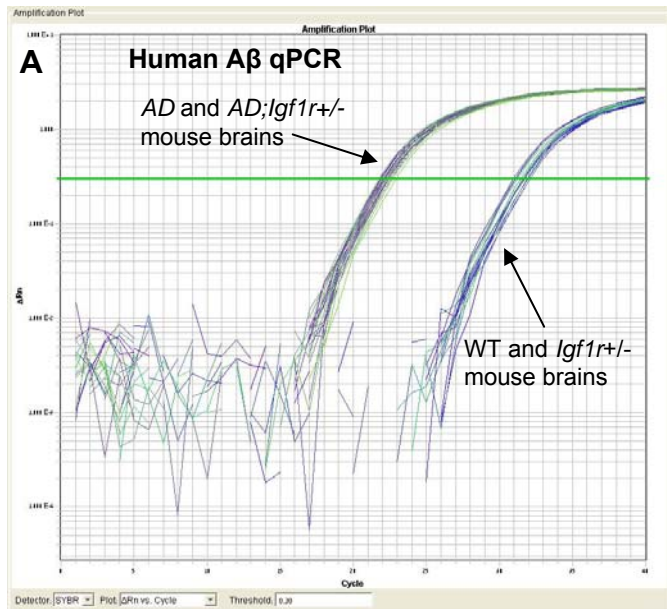
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Figure S1



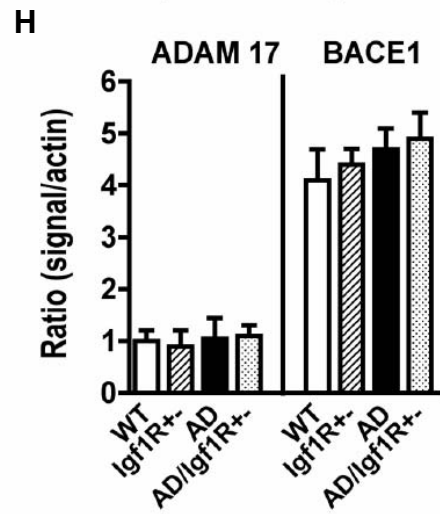
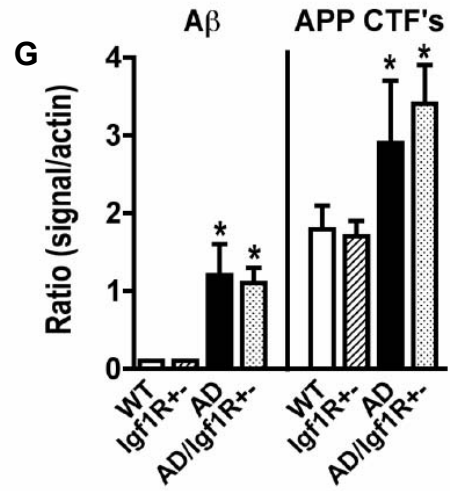
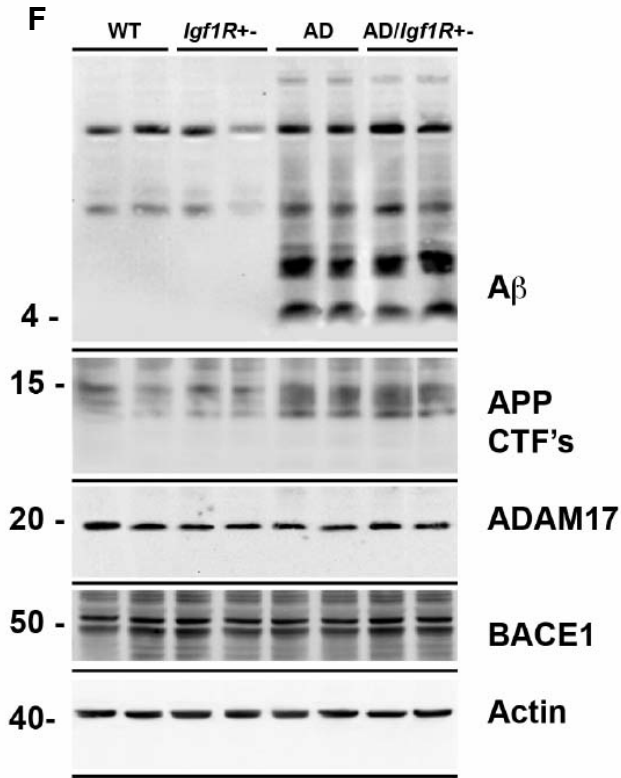


Figure S2

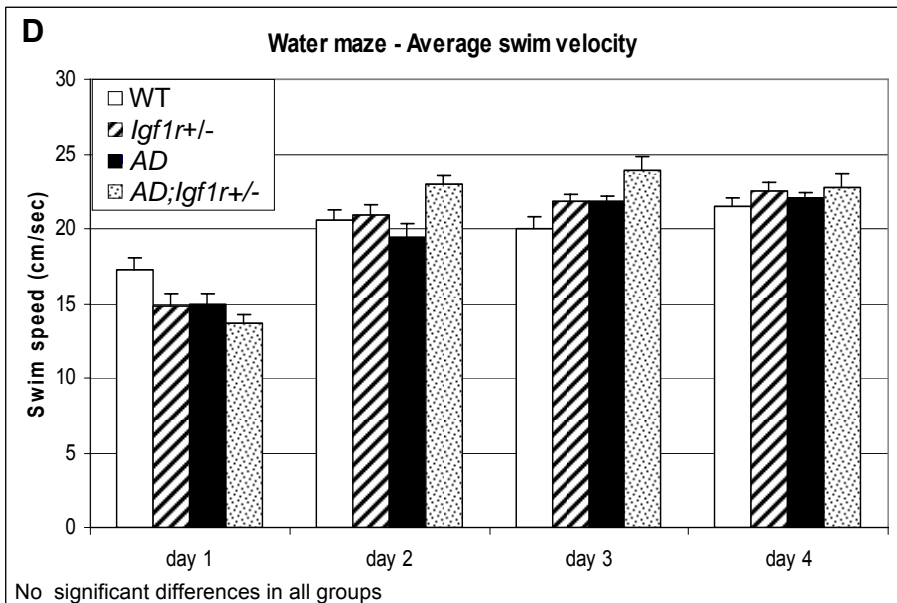
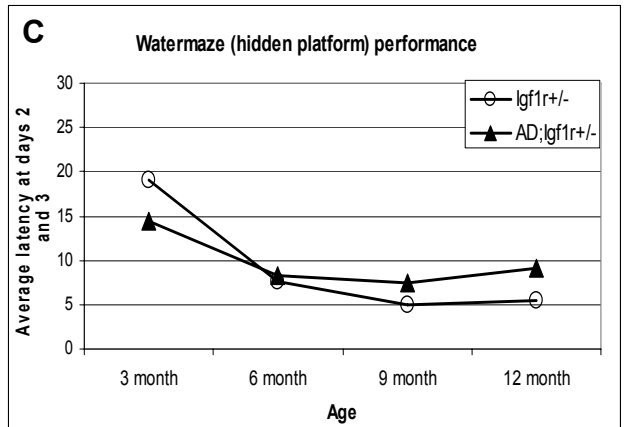
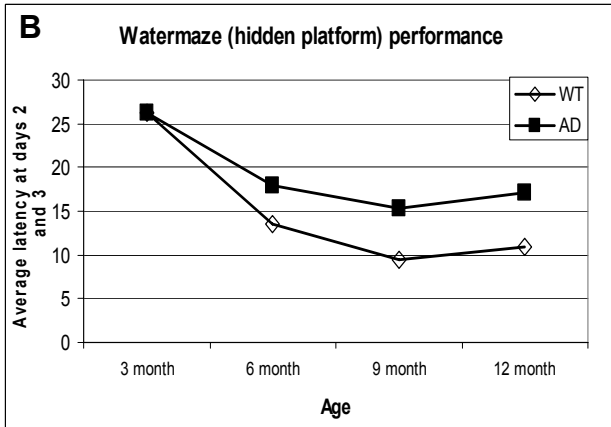
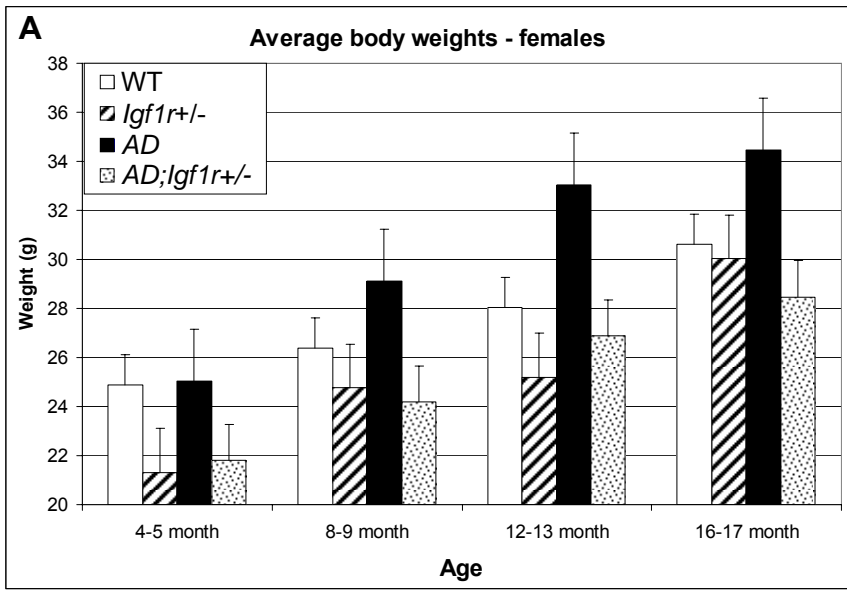
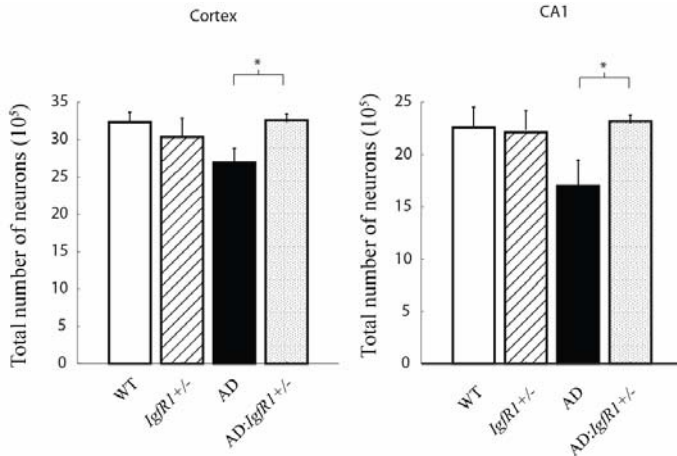


Figure S3

A Neuronal density 4-5 month



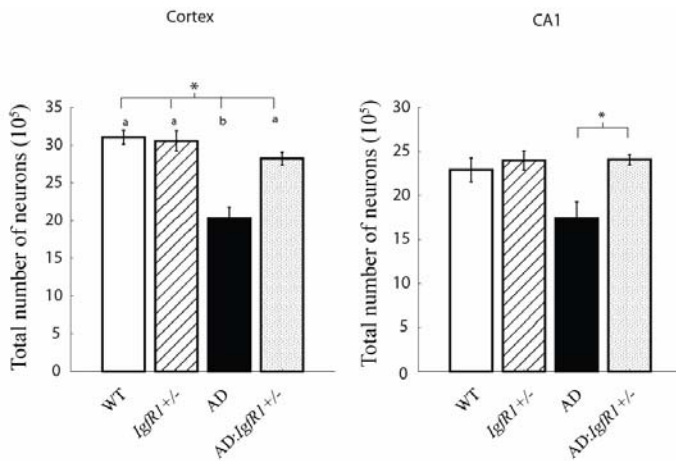
ANOVA Table

Source	SS	df	MS	F	Prob>F
Groups	74.1508	3	24.7169	4.3779	0.042151
Error	45.1663	8	5.6458		
Total	119.3171	11			

ANOVA Table

Source	SS	df	MS	F	Prob>F
Groups	89.6743	3	29.8914	4.2345	0.045556
Error	56.472	8	7.059		
Total	146.1463	11			

B Neuronal density 12-13 month



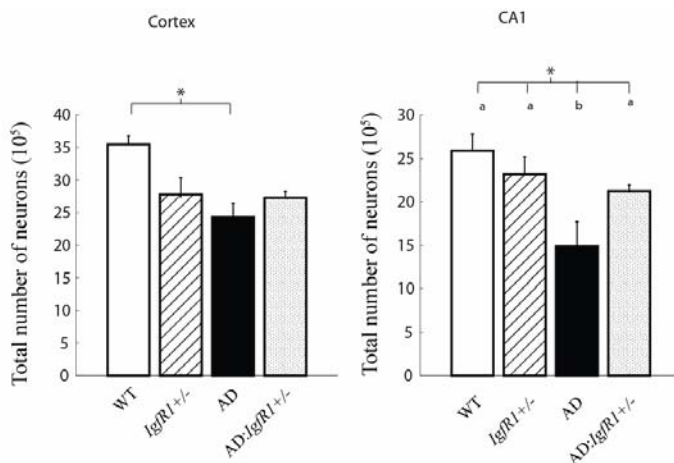
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Source	SS	df	MS	F	Prob>F
Groups	472.4325	3	157.4775	16.0297	2.5246e-05
Error	176.8337	18	9.8241		
Total	649.2662	21			

Kruskal-Wallis ANOVA Table

Source	SS	df	MS	Chi-sq	Prob>Chi-sq
Groups	394.1429	3	131.381	9.3631	0.024833
Error	489.8571	18	27.2143		
Total	884	21			

C Neuronal density 16-17 month



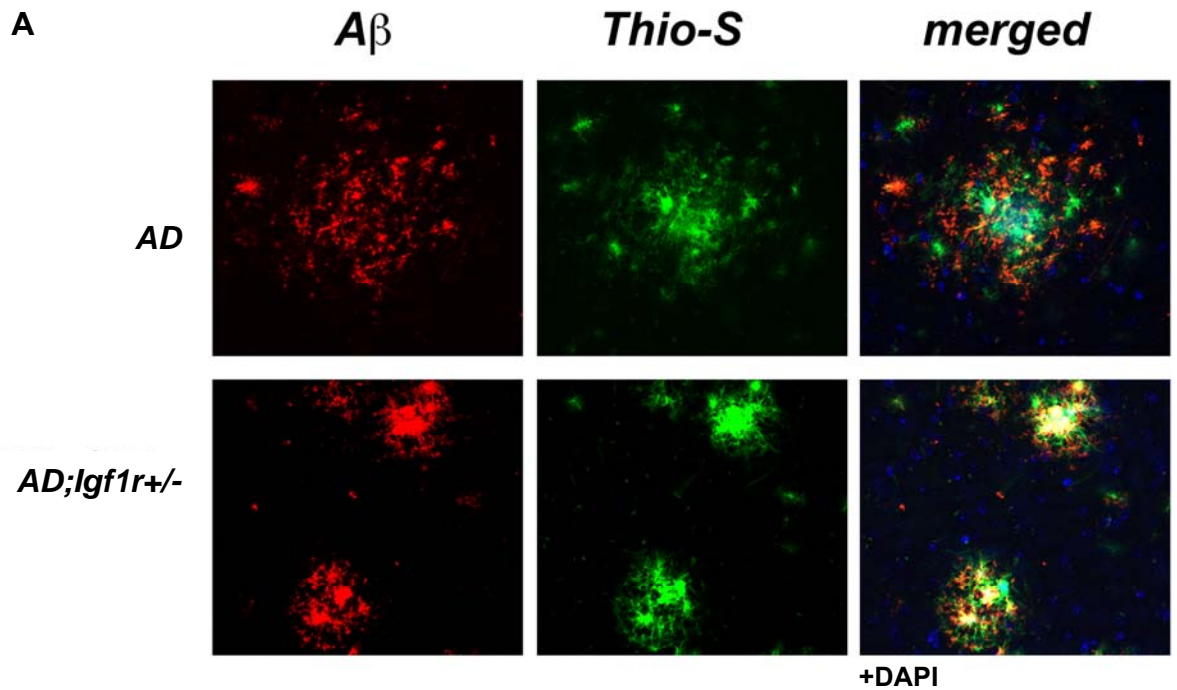
ANOVA Table

Source	SS	df	MS	F	Prob>F
Groups	154.8671	3	51.6224	4.7236	0.050714
Error	65.5719	6	10.9287		
Total	220.439	9			

ANOVA Table

Source	SS	df	MS	F	Prob>F
Groups	168.4953	3	56.1651	12.6088	0.0053165
Error	26.7267	6	4.4545		
Total	195.222	9			

Figure S5



B Effects of PK treatment upon plaque density

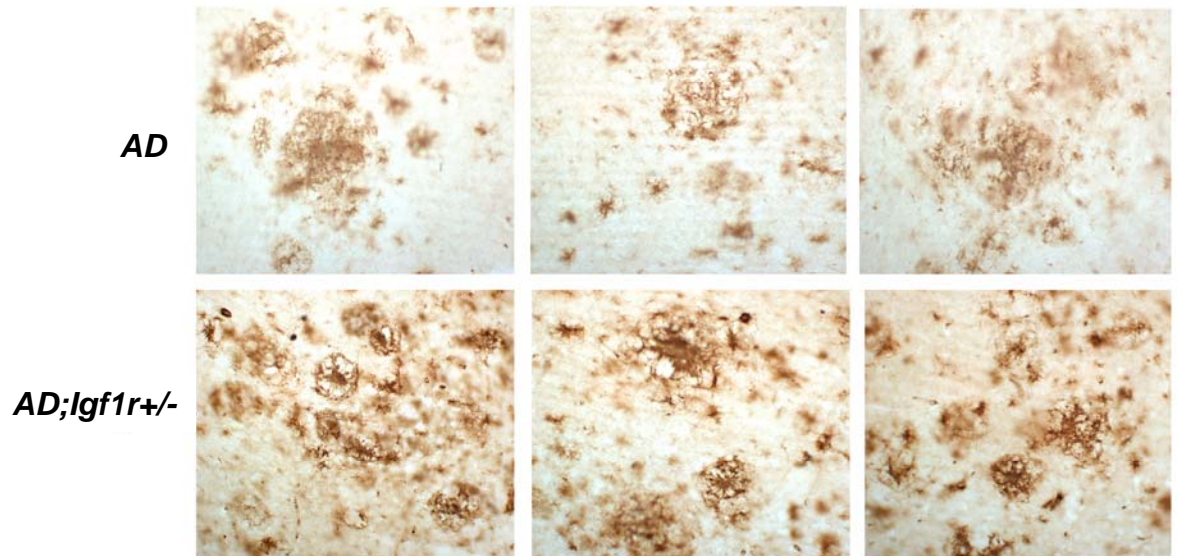
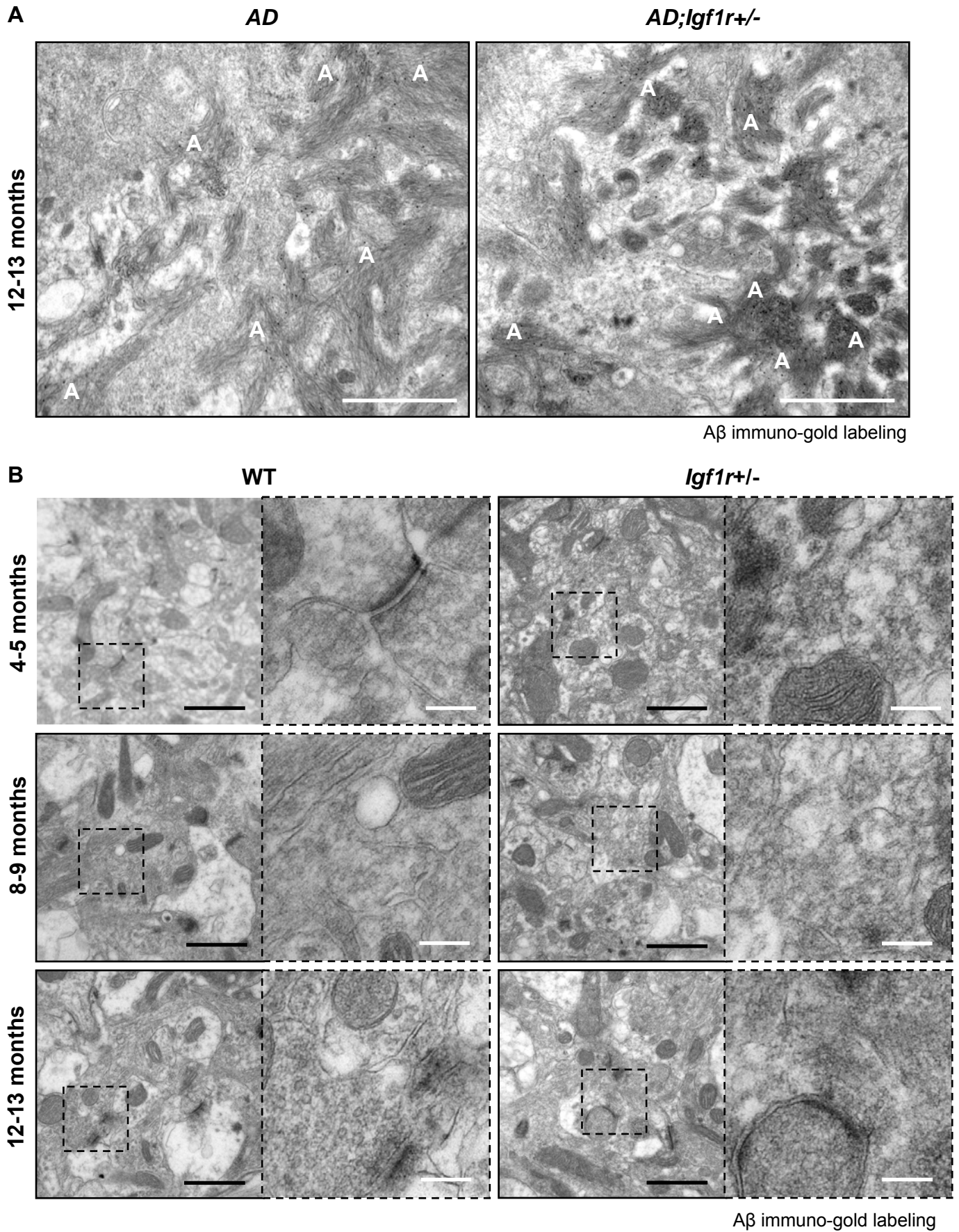


Figure S6



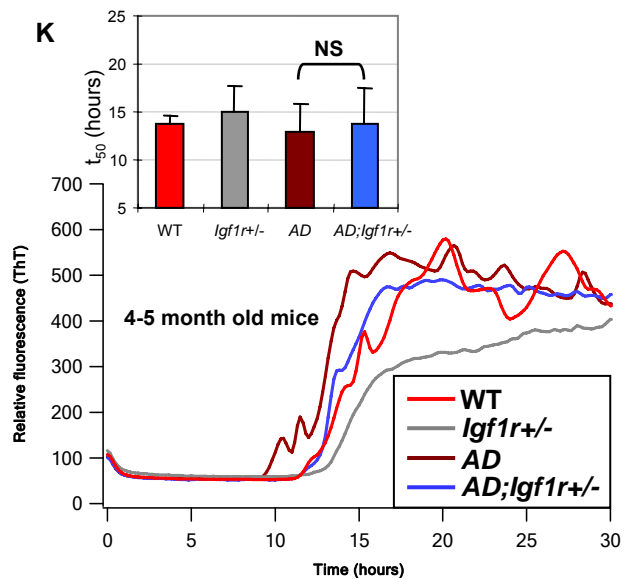
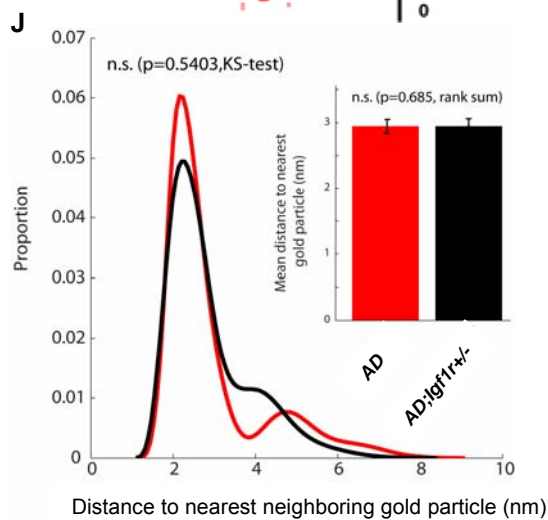
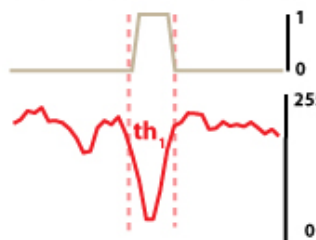
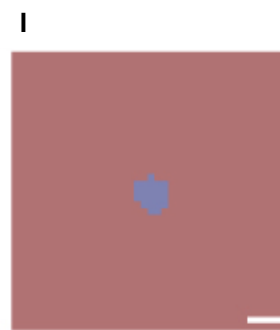
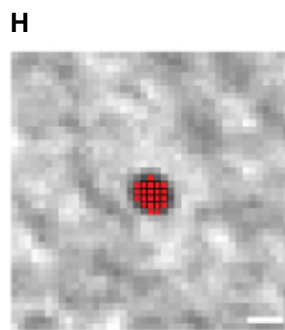
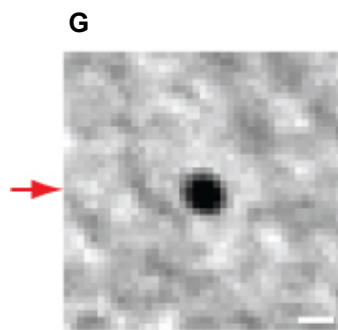
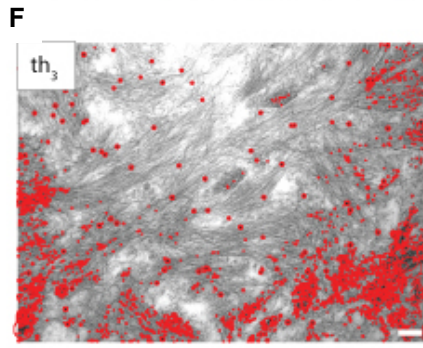
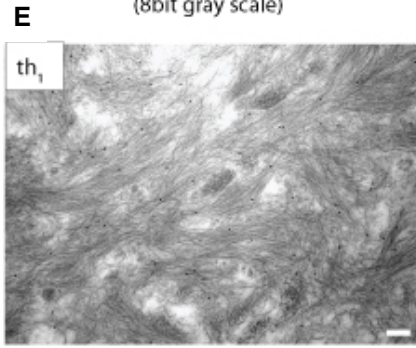
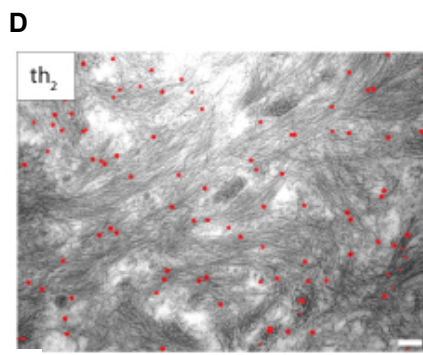
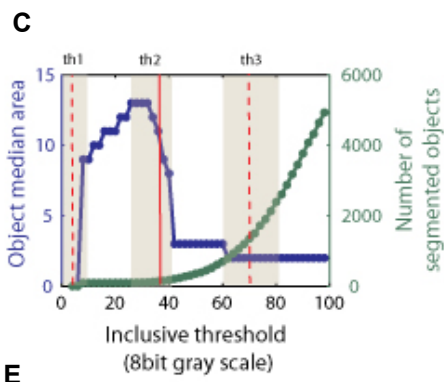


Figure S7

Relative A β_{1-40} quantities (ELISA) – 4-5 month

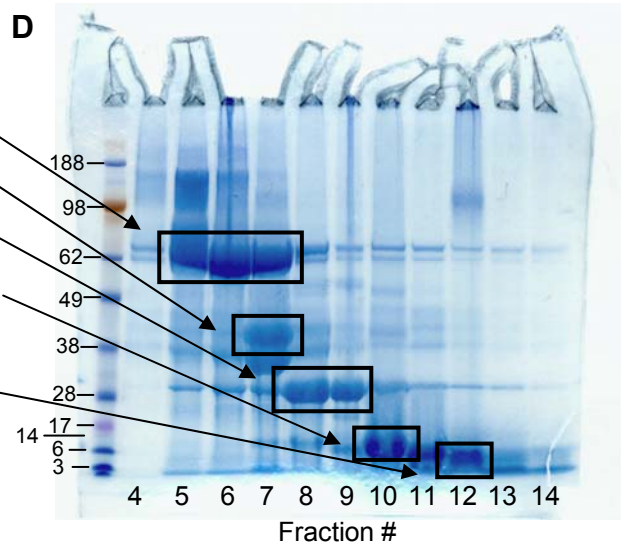
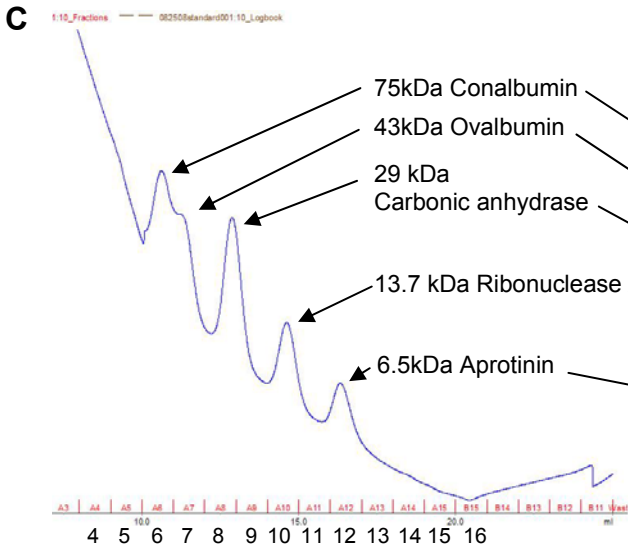
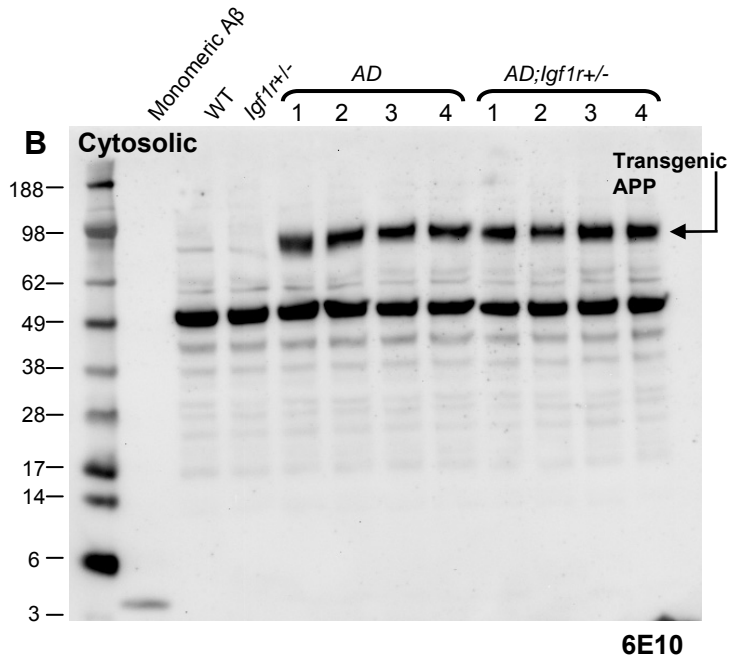
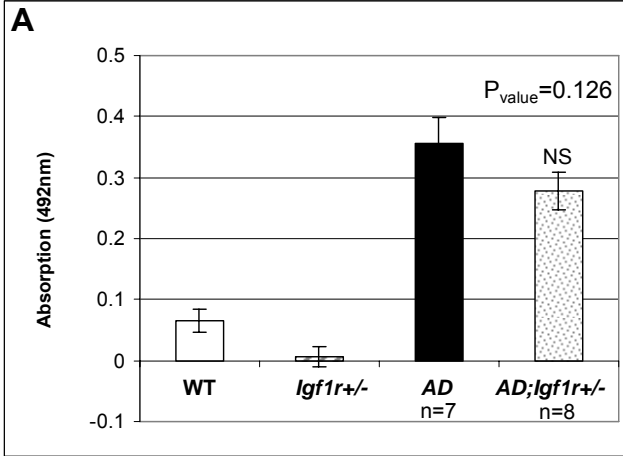
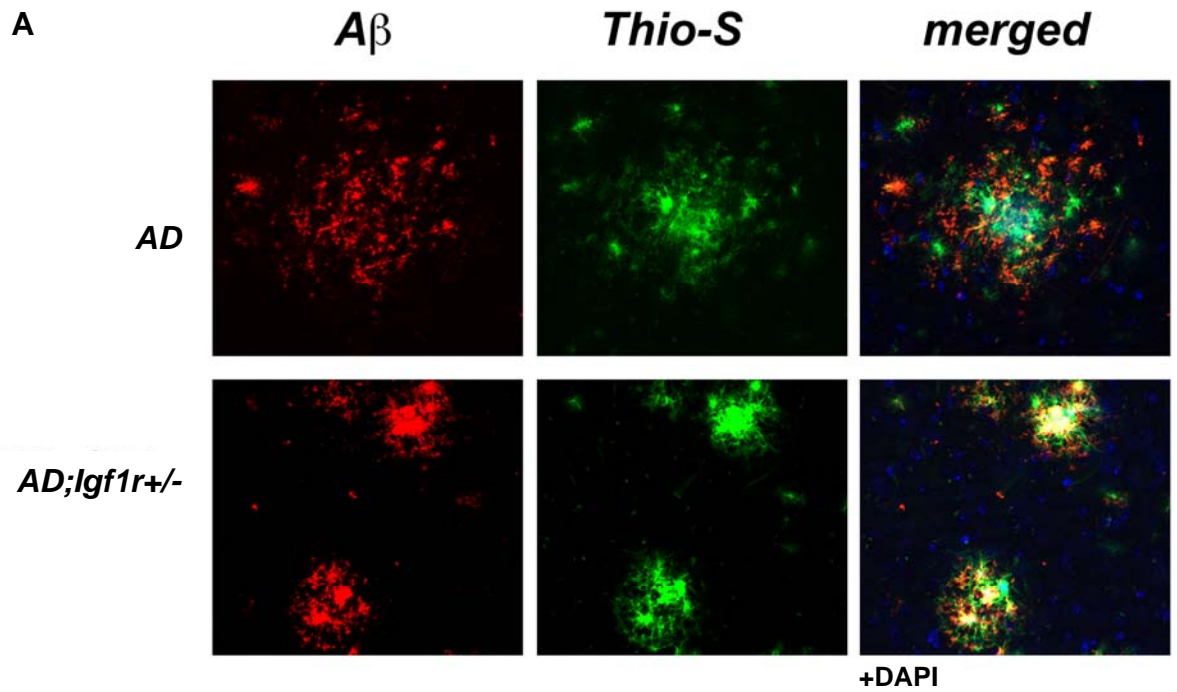


Figure S5



B Effects of PK treatment upon plaque density

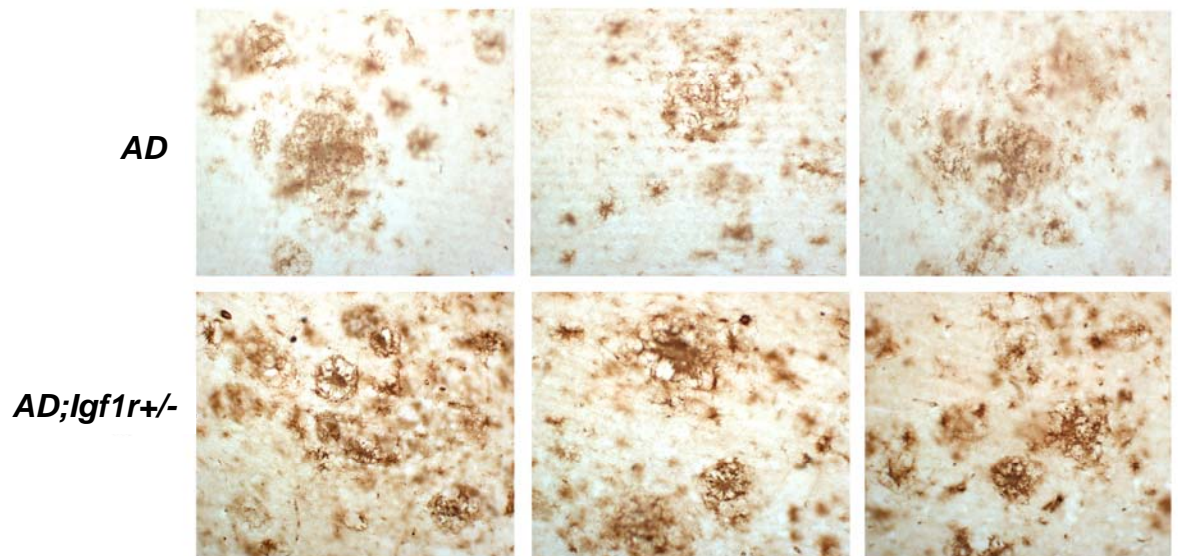


Figure S6

