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Supporting Online Material for

ApoE-Directed Therapeutics Rapidly Clear β-Amyloid and Reverse Deficits in AD Mouse Models

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Materials and Methods:

Animals

APPswe/PS1Δe9 (APP/PS1) (*27*),Tg2576 (*28*) or APPPS1-21 (*14*) mice or non-transgenic (NonTg) littermates of the appropriate genetic background and age were orally gavaged daily for 3, 7, 9, 14, 20 or 90 days with 100 mg/kg/day bexarotene or vehicle (water) .The animals were sacrificed, and one hemisphere was fixed and processed for immunohistochemistry. The hippocampus and cortex were removed from the other hemisphere and snap-frozen until subject to RNA and protein extraction as previously described (*6*). All experiments involving animals followed approved protocols by the Case Western Reserve University School of Medicine, Washington University School of Medicine, University of Pennsylvania, or the Nathan Kline Institute for Psychiatric Research's Institutional Animal Care and Use Committee.

Cell culture/Western blotting:

Primary microglia and astrocytes were prepared from P0–P3 mice and purified microglia and astrocyte cultures were obtained as previously described (*29*).For Western blot analysis 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) were used. ApoE lipidation status was monitored by native gel electrophoresis using 4-20% Tris-glycine gels (Invitrogen, Carlsbad, CA). The following primary antibodies were used: anti-human Aβ, 6E10 (Covance, Dedham, MA); anti-ApoE, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ABCA1, anti-ABCG1 (Novus Biologicals, Littleton, CO).

Quantitative Real-time PCR:

Quantification of transcripts of the Aβ degrading enzymes was performed as previously described (*30*). Primary microglia were incubated with the indicated doses of bexarotene for 24h. Cells were harvested and RNA was isolated using an RNeasy Mini Prep kit (Qiagen, Valencia, CA). RNA samples (0.5 μg) were used to synthesize cDNA using QuantiTect Reverse Transcription kit (Qiagen). cDNA was then preamplified for 14 cycles using TaqMan PreAmp Master Mix (Applied Biosystems) followed by performing real-time PCR in a 10 μl reaction for 40 cycles with the StepOne Plus Real Time PCR system (Applied Biosystems). Primers and FAM probe-sets were selected from the database of Applied Biosystems. Analysis of gene expression was performed using the comparative CT method (*31*).

Immunohistochemistry and image analysis

Post-fixed hemispheres were coronally cryostat sectioned (10 μm). For Aβ immunohistochemistry, methods were performed as previously described (*6*) with analysis of 6 sections/ mouse, obtained 1.2-1.5 mm from the midline, spaced 0.05 mm apart. Images were analyzed using Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). Plaque abundance was evaluated by 6E10 and thioflavin S staining and counterstained with DAPI and propidium Iodide, respectively. The number of thioflavin S+ plaques and 6E10 plaque area were analyzed by a blinded observer.

Intracellular Aβ clearance:

Primary mouse microglia or astrocytes were incubated with vehicle (DMSO), bexarotene, T0070907 (10 nM), or 22S-hydroxycholesterol (10μM) for 24 hours at 37°C. Cells were then treated with 2 μg/ml soluble $A\beta_{42}$ in serum-free DMEM-F12 medium for 20 hours in the presence drug. Purified human plasma apoE (rPeptide, Athens, GA) was applied at the same time as soluble $A\beta_{42}$. Aβ peptide levels were determined by ELISA following lysis of the cells in 1% SDS and normalized to total protein.

In vivo Microdialysis

In vivo microdialysis to assess brain ISF Aβ in awake APP/PS1 mice was performed as previously described (*13*) .Unilateral guide cannula and 2mm microdialysis probes (BR-2, Bioanalytical Systems) were implanted into the hippocampus. Microdialysis perfusion buffer was artificial CSF containing 4% bovine serum albumin with a constant flow rate of 1.0μl/minute. Samples were assessed for $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ by sandwich ELISA. Basal levels of ISF Aβ were defined as the mean concentration of Aβ over 6-8 hours preceding drug treatment. All ISF Aβ levels were normalized to the basal Aβ concentration for that mouse. Murine ISF Aβ40 levels in non-transgenic were assessed using a similar protocol as APP/PS1 mice except with longer sampling times of 6 hours at a 0.5μl/min flow rate. Compound E was administered intraperitoneally at 20mg/kg.

Bexarotene tissue bioavailability analysis:

Young male B6C3F1 mice (n=3) received 5 mg/kg bexarotene dissolved in DMSO via i.p. administration, with plasma and brain hemispheres collected 1 hour after dosing. Bexarotene was detected using multiple reaction monitoring of its specific collision-induced ion transitions. Samples were separated on an Aquity BEH C18 column and peak areas were plotted against concentration and a 1/x weighted linear regression curve was used to quantify tissue bexarotene levels.

Behavioral Analysis:

Olfactory Assay: Tg2576 mice (12-14 mo) were orally gavaged for 3 or 9 days with 100 mg/kg/day bexarotene. Mice were screened for olfactory deficits using the odor cross-habituation test (*17*). Odors (*n*=4; heptanone, isoamyl acetate, limonene, and ethyl valerate; Sigma Aldrich, St. Louis,MO) were diluted 1×10^{-3} in mineral oil and applied to a cotton-applicator stick enclosed in a piece of plastic tubing. Odors were delivered for 4 successive trials (1 block), 20sec each, separated by 30sec inter-trial intervals, by inserting the odor stick into a port on the side of the animal's home cage. Testing took place during the light phase of the animals' (12:12) light:dark cycle. The duration of time spent investigating, defined as snout-oriented sniffing within 1cm of the odor presentation port, was recorded across all trials by a single observer blinded to genotypes. Mice were tested in a counter-balanced order.

Contextual Fear Conditioning: Freezing behavior was monitored by automated tracking system (Coulbourn Instruments, USA). In the training phase, mice were individually placed in the shock chamber to explore the environment freely for 2 min. Mice were exposed to the conditioned stimulus (CS: an 85dB sound at 2800Hz) for 30sec. After 2 seconds, the unconditioned stimulus (US: 0.56mA) was delivered. After the CS/US pairing, the mice were kept in the chamber for another 30sec to measure the immediate freezing response. This process was

repeated 4 times. Retention tests were performed 24 hours later. Each mouse was returned to the same shock chamber for 2.5 min for contextual freezing measurement in the absence of tone and the percent of time frozen and number of freezes measured.

Morris Water Maze: The spatial version of the water maze task was used to examine cognitive decline and rescue by experimental treatment in all mouse groups following a modified version of previously published methodology (*32*). Briefly, mice were tested at the end of pharmacological treatment. Animals were trained in a black circular pool (120 cm), in a well-lighted room replete of visual cues. Pool water was whitened with non-toxic white dye and temperature was maintained at 23°C. A clear escape platform (10.5 cm in diameter) was located approximately 0.5cm beneath the water level placed in the center of four quadrants (N, S, E, W) of the pool in the same location relative to room visual cues. Animals were tested beginning in different quadrants to control for location bias. Animals were tested for 8 trials per day, subdivided into 2 blocks of 4 trials, over 6 days. Prior to the beginning of testing, the animals were allowed to swim freely in the pool for 30 seconds and then allowed to sit on the escape platform for 30 more seconds. On day 1 of testing, the platform was located in quadrant 4 (northwest quadrant (NW)) for all 8 trials. The animals were placed in the water from one of the 4 start positions at the edge of each quadrant and allowed to swim for 60 seconds. If the animal did not find the platform during the allocated task, it was guided towards it where it remained for 15 seconds and then immediately placed back into the water from the next start position for the next trial. The exact procedure was followed for four trials (one from each start position), at which point the mouse was dried and placed back into its home-cage (warmed with a heating pad) for 30-40 minutes until the start of the next trial block. Swim time, path length, and swim speed were recorded using a tracking system and software (Ethovision, Noldus Information Technology, Wageningen, The Netherlands). On day 5, the platform was placed in the same position as day 1 for the 7 trials and removed on the 8th trial for a probe trial to test for spatial strategy and retention. During this trial, the platform was removed and the animals were allowed to swim for 60 seconds without the possibility to escape; percent time spent in the quadrant where the platform was previously located was measured. On day 6, 24hrs after the last training session, a second probe trial was repeated as described above, followed by visual acuity testing. For visual testing, the platform was made visible by bringing it above the water surface and clearly marked flag; all extra-maze cues were removed completely. Eight trials in 2 4-trial blocks were administered with the platform moving to a different quadrant for each trial. Animals with escape latencies higher than 30 seconds after 8 trials were considered visually impaired and excluded from the analyses.

Nest construction assay: Nest construction with paper towel material was observed throughout the course of treatment with vehicle or bexarotene as previously described (*15*).

Electrophysiology:

Piriform cortex (PCX) local field potentials (LFPs) were recorded from awake head-fixed mice (12-14mo Tg2576 or non-transgenic) treated daily with either vehicle (water) or bexarotene. Mice were anesthetized and stainless steel wire electrodes (A-M Systems, Inc) implanted in the anterior portion of the PCX and over the contralateral neocortex (the reference electrode). The electrodes were then connected to a head-stage for chronic recordings. The animal was then allowed to recover on a heating blanket for 24 hours and treated daily for 2 days with Carprofen (Rimadyl, Pfizer Animal Care, 5mg/kg S.C.). Following 3 days of recovery from surgery, mice were positioned in the restraint device located within an enclosure box outfitted

with an exhaust fan. Mice were head-fixed daily for no more than 1 hour at a time. During fixation a Teflon odor port was positioned ~5mm from the tip of the nose for odor delivery via an automated air-dilution olfactometer. Four odors were presented, in a counterbalanced order, several times each for analysis of odor-evoked LFP activity. LFPs were recorded over 4 days (1 baseline and 3 during treatment) and the data from treatment day 3 analyzed (e.g., Fig. 3 and Fig. S4). LFP data were recorded along with odor presentation events using CED's Spike 2 (Cambridge Electro Inc., UK). Data were processed with Fast-Fourier transform analysis (~2Hz bin resolution) of LFP activity before and during the odor (2 sec epochs).

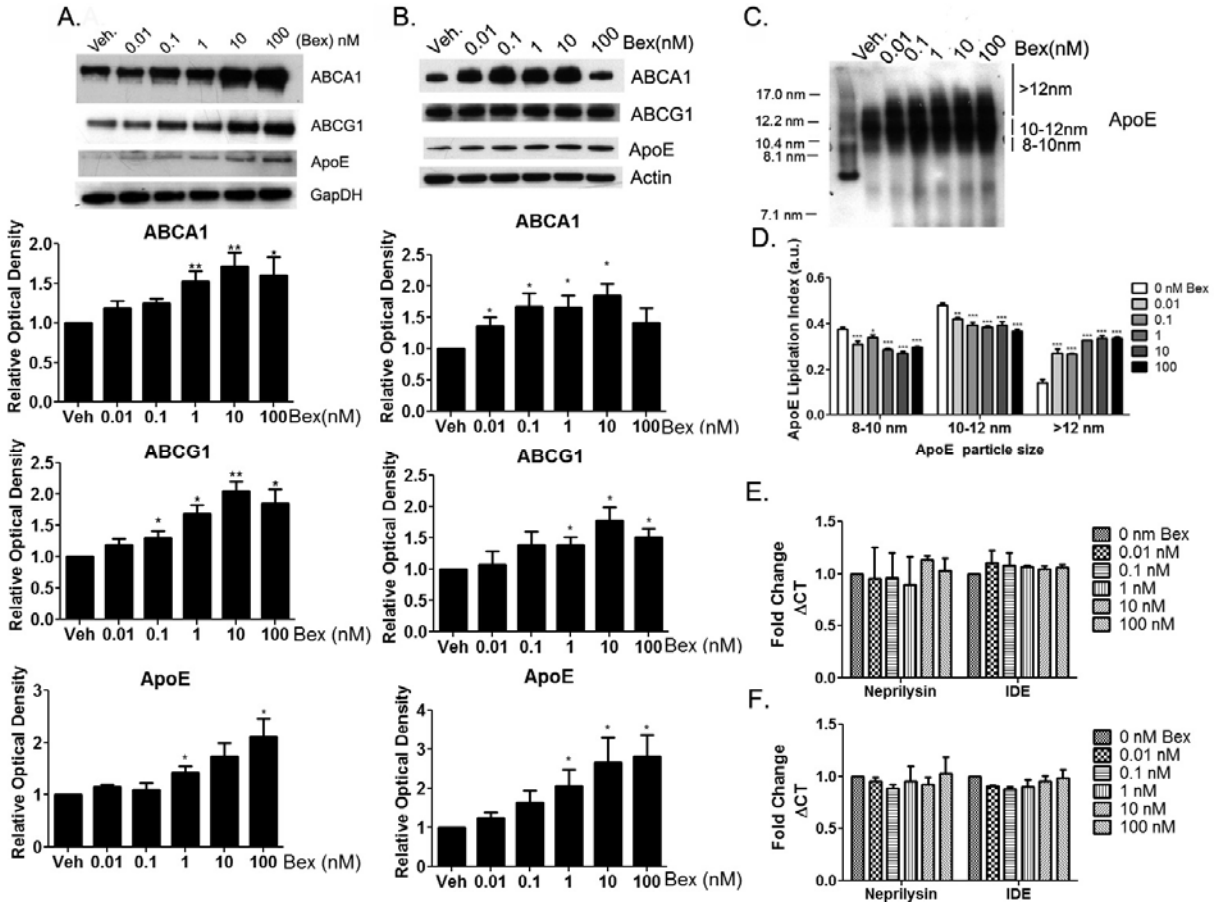

Figure S1: Bexarotene stimulates the expression of LXR target genes. Primary microglia (A) and astrocytes (B) were treated for 24 hours with the indicated doses of bexarotene or vehicle (DMSO) and gene expression measured by Western analysis (Wilcoxon signed rank test, mean ±SEM, *p<0.05, **p<0.001, n≥4). Secreted ApoE HDL particles were analyzed by native gel electrophoresis (C), and the levels of the indicated particle sizes quantified (D) Primary microglia (E) and astrocytes (F) were treated for 18 hours with the indicated doses of bexarotene or vehicle (DMSO) and mRNA expression was quantified. (Student's t test mean ±SEM *p <0.05, **p<0.01, relative to vehicle treated control, n≥3).

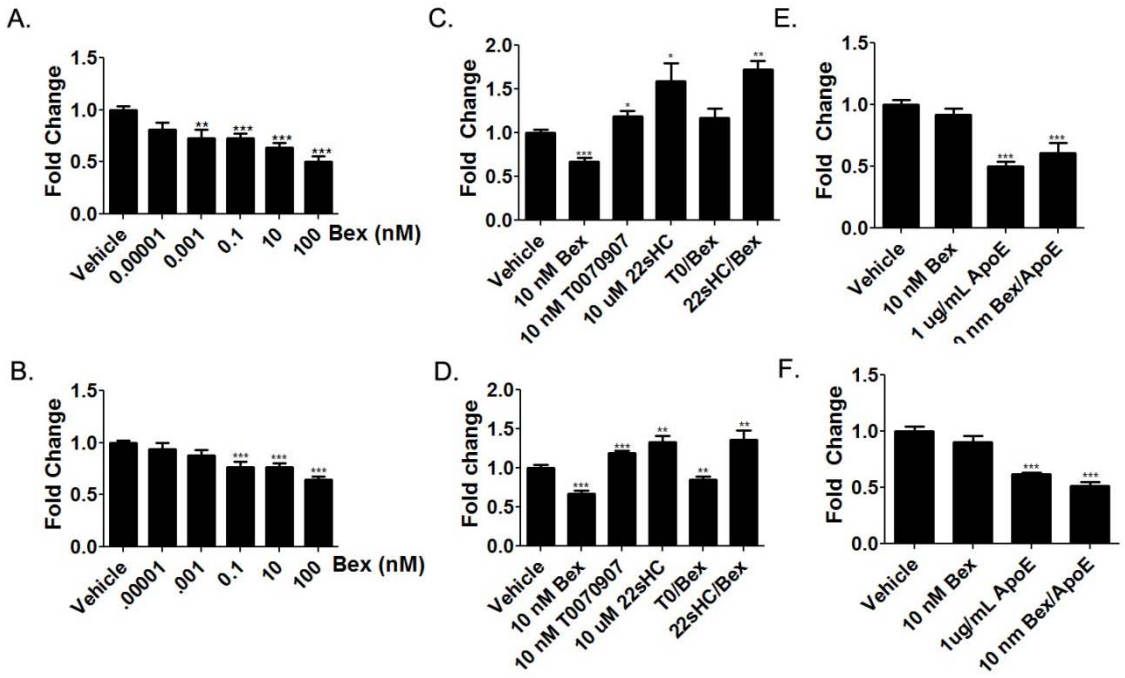

Figure S2: Bexarotene stimulates the ApoE-dependent intracellular clearance of Aβ through the actions of LXR and PPARγ. Primary microglia (A) and astrocytes (B) or *apoE* knockout microglia (E) or astrocytes (F) were treated for 24 hours with bexarotene or vehicle (DMSO) then soluble $A\beta_{42}$ was added for an additional 18 hours. Primary microglia (C) and astrocytes (D) were pretreated with competitive antagonists to LXR (22-S hydroxycholesterol) (22sHC) and PPARγ (T0070907) (T0) for 2 hours. Remaining intracellular Aβ was measured by ELISA (Student's t test; mean ±SEM, *$p<0.05$, **$p<0.01$, ***$p< 0.001$, $n\geq3$) and fold change is reported relative to vehicle-treated controls.

A.

Bexarotene	Brain uM	Plasma uM	B/P
1	2.38	2.49	0.955
2	1.98	1.97	1.001
3	2.50	2.48	1.008
AVG	2.28	2.31	0.988
SD	0.27	0.29	0.029
CV	12	13	2.96

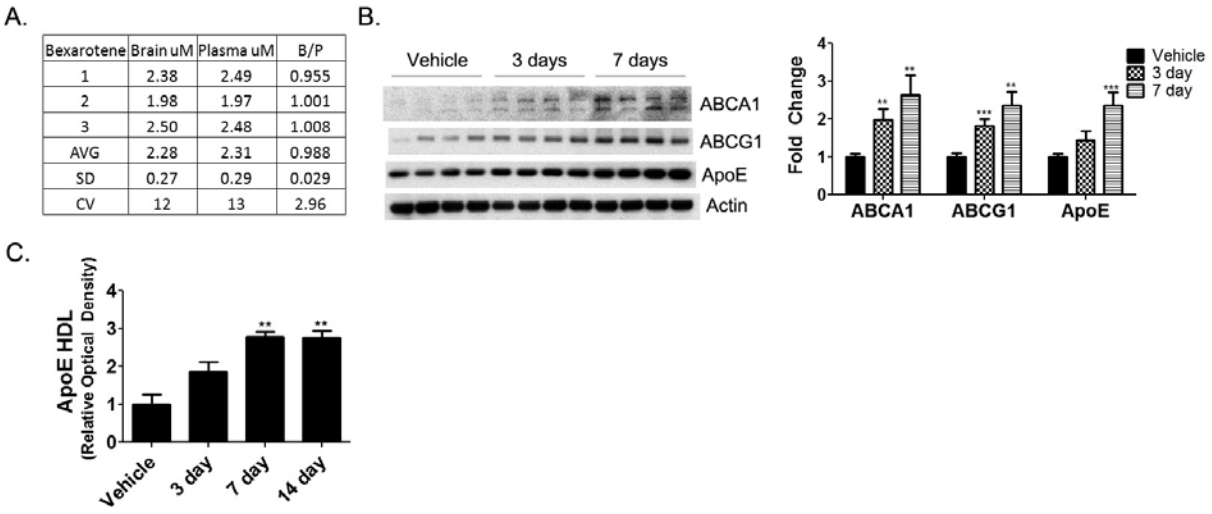

Figure S3: Bexarotene stimulates the expression of LXR target genes in vivo. C57Bl/6 mice were injected intraperitoneally with bexarotene (5 mg/kg) and 1 hour later, brain and plasma bexarotene levels were measured (A). APP/PS1 mice (6 mo, n≥6/group) were gavaged with 100 mg/kg/day bexarotene for 3 or 7 days or vehicle (water) for 7 days. Hippocampus and cortex homogenates were analyzed for the expression of ABCA1, ABCG1 and apoE by Western analysis and quantified (B). Secreted HDL particles from cortex and hippocampus homogenates from animals treated for 3, 7, or 14 days with bexarotene or water (vehicle) were analyzed by native electrophoresis and quantified (C). (Student's t test; mean ±SEM *$p<0.05$, **$p<0.01$, ***$p<0.001$) and fold change is reported relative to vehicle treated controls.

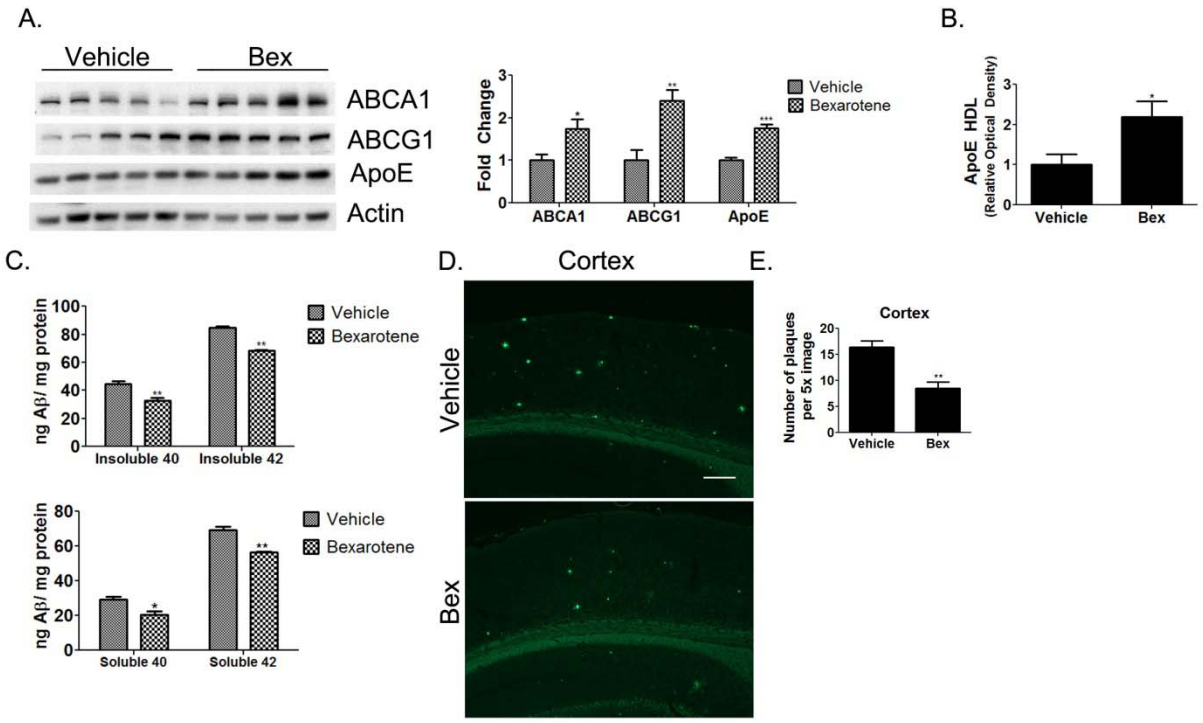

Figure S4. Short-term treatment in 11 month old APP/PS1 mice with bexarotene stimulates clearance of Aβ. APP/PS1 mice (11 mo) were orally gavaged for 7 days with bexarotene (100 mg/kg/day) or vehicle (water). Hippocampus and cortex homogenates were analyzed by Western analysis for the indicated proteins and quantified (A and B). Soluble and insoluble $A\beta_{40}$ or $A\beta_{42}$ levels were measured by ELISA (C). Representative images of sections from the cortex (D) of vehicle- and Bex-treated mice stained with thioflavin S and plaque levels quantified (E); n=5 animals/group (Student's t test. mean±SEM, *p<0.05, **p<0.01, ***p<0.001; Scale bar 100 μm).

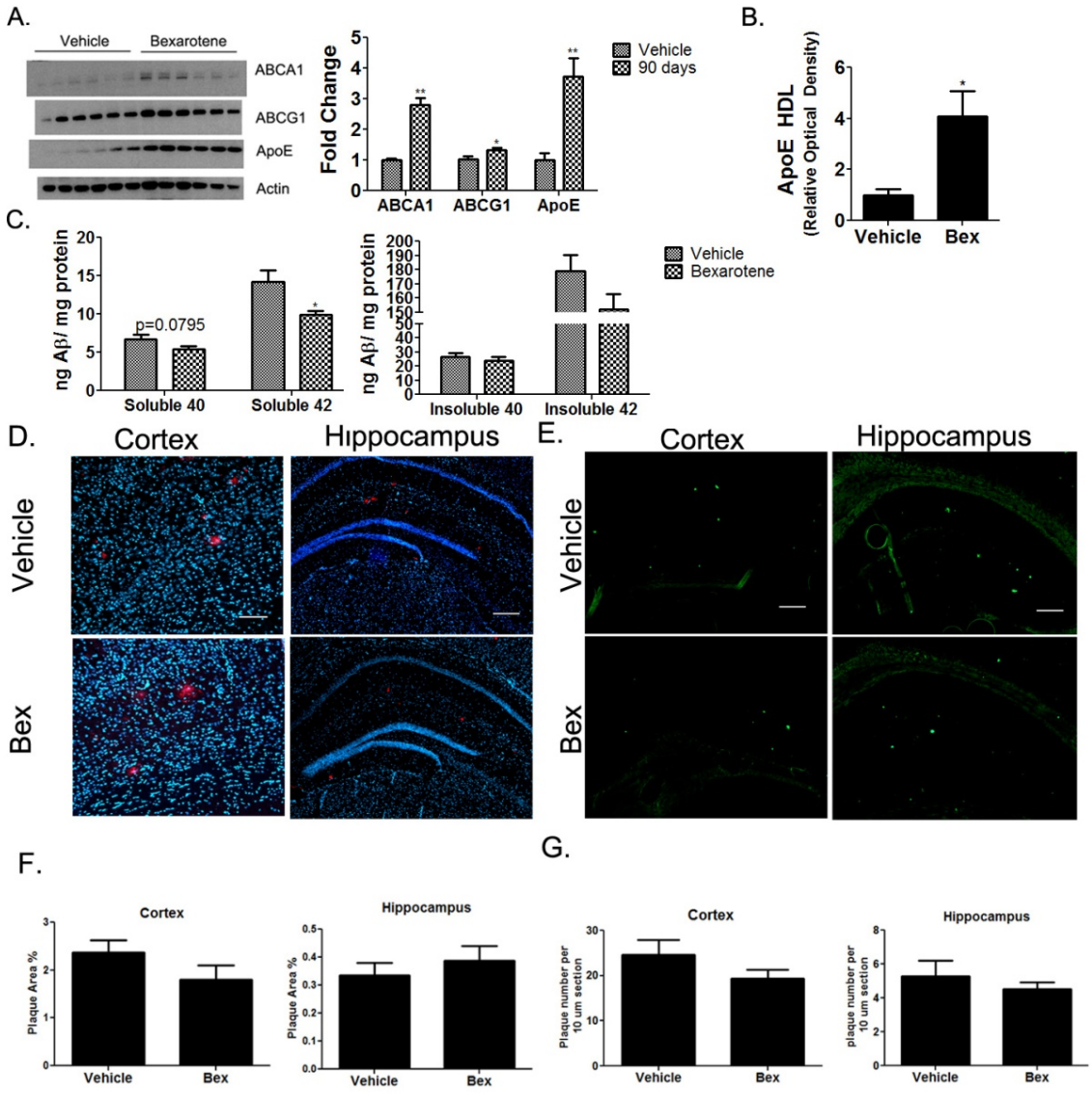

Figure S5. Chronic bexarotene treatment reduces levels of soluble Aβ in APP/PS1 mice. APP/PS1 mice (9 mo) were orally gavaged for 90 days with bexarotene (bex) (100 mg/kg/day) or vehicle (water) (behavioral analysis from same cohort: Figure 2C,F). Hippocampus and cortex homogenates were analyzed by Western analysis for the indicated proteins and quantified (A,B). Soluble and insoluble $A\beta_{40}$ or $A\beta_{42}$ levels were measured by ELISA (C). Images of the cortex and hippocampus (D and E) of vehicle- and bexarotene-treated mice stained with the anti-Aβ antibody, 6E10 (D) or thioflavin S (E) are shown and plaque levels were quantified (F,G); n= 10 animals/group (Student's t test; mean ±SEM *$p<0.05$, ***$p<0.001$ Scale bar (D) cortex 100 μm, hippocampus 200 μm, (E) all 200 μm).

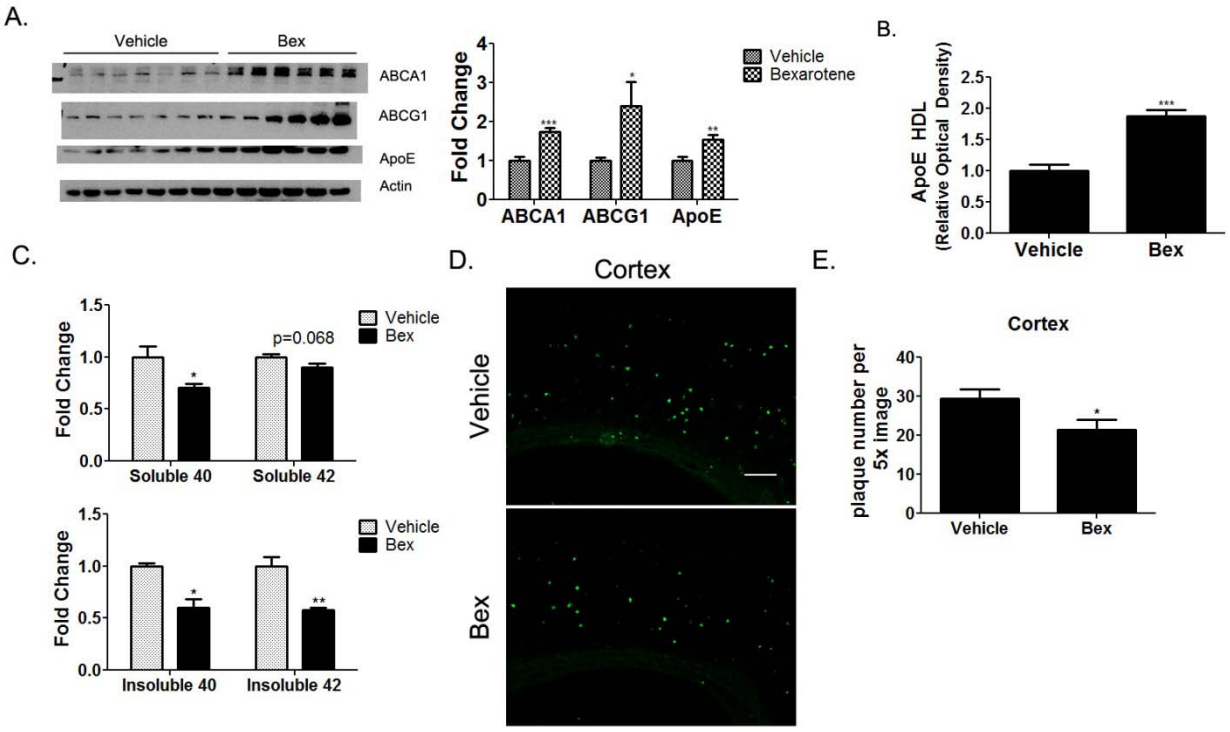

Figure S6. Bexarotene treatment of an aggressive amyloidogenic mouse model stimulates the clearance of Aβ. APPPS1-21 mice (7-8 mo) were gavaged daily for 20 days with bexarotene (Bex) (100 mg/kg/day) or vehicle (water). Hippocampus and cortex homogenates were analyzed for the indicated proteins by Western analysis and quantified (A and B). Soluble and insoluble $A\beta_{40}$ or $A\beta_{42}$ levels were measured by ELISA (C). Representative images of the cortex (D) of vehicle- and bexarotene- treated mice stained with Thioflavin S are shown and plaque levels quantified (E); n≥6 animals/group (Student's t test; mean ±SEM, *p<0.05,**p<0.01, ***p<0.001) Scale bar (D) 100 µm.

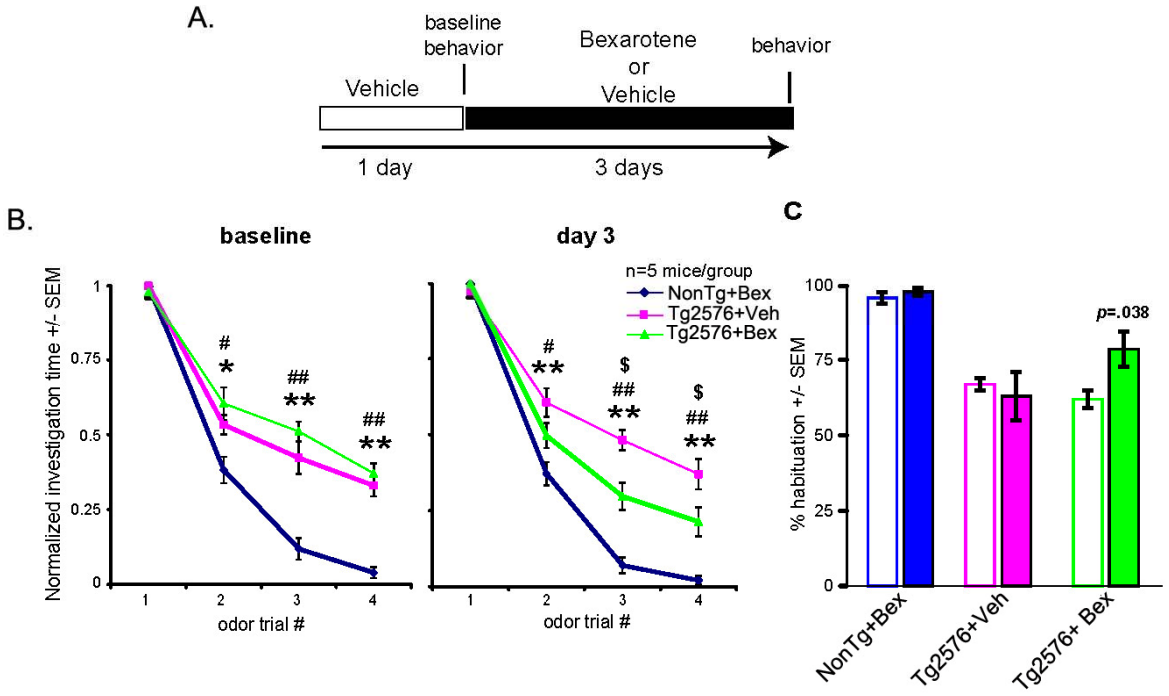

Figure S7: Improvement of odor habituation behavior in 12-14mo old Tg2576 mice treated with bexarotene for 3 days. Mice (implanted with chronic head-caps for electrophysiology) were orally gavaged vehicle (veh, water) and used for baseline olfactory behavior measures ('baseline') (A). Subsequently, mice were orally gavaged once daily with either bexarotene (Bex) or vehicle for 3 days and again tested. Line graphs showing normalized odor habituation data across 4 successive odor presentations on baseline and day 3 (B). *p<.05, **p<.001, NonTg+Bex vs. Tg2576+veh. #p<.05, ##p<.001, NonTg+Bex vs. Tg2576+Bex. $^{\$}$p<.05, Tg2576+veh vs. Tg2576+Bex. % habituation values by trial #4 (C). Open bars=baseline, closed bars=day 3. All statistics are 2-tailed *t*-tests, mean ±SEM. These data are from same mice used for electrophysiology (Fig. 4).

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