Supplementary Material

Methods

Animals: We used WT and heterogeneous TgF344-AD male and female rats. TgF344-AD rats, which are on a Fischer 344 background, coexpress human APP 695 with mutations (K595N, M596L) and PS1 with a deletion of exon 9 driven on a mouse prion promoter. A male TgF344-AD breeder was generously provided by Dr. Robert Cohen, and bred with Fischer 344 females from Charles River. All animal procedures were performed in accordance with the University of Iowa Office of Animal Resources and Institutional Animal Care and Use Committee (IACUC). Animals were maintained in temperature-controlled conditions (70-72 °F) on a 12-h light/dark cycle (6:00 a.m. to 6:00 p.m.) and provided normal chow (Envigo 7913) and water ad libitum. Animals were housed in Specific Pathogen Free (SPF) facility at the University of Iowa in standard caging (Thoren brand filtered caging) with standard cellunest bedding (Shepherd Paper Products). To eliminate the confounding factors of reduced animal interaction or environmental enrichment on neurogenesis (47, 48), animals were housed two or three to a cage throughout the duration of the experiment. In certain instances, aging animals required standard veterinary care. All animal care procedures deemed necessary were performed by a member of the University of Iowa's Office of Animal Resources veterinary staff according to IACUC regulations. In instances where surgical procedures fell within the time frame for behavior testing, analysis was conducted at least two weeks after the necessary procedures were performed, in order to eliminate any potential confounding factors of surgery and anesthetic on behavior.

(-)-P7C3-243 treatment and plasma pharmacokinetic analysis: Six-month old male and female WT and TgF344-AD rats were randomly placed into treatment groups and cohoused for the duration of the experiment. Intraperitoneal injections of vehicle (2.5% DMSO, 10% kolliphore, and 5% dextrose, pH 7.4) or 10 mg/kg/d (-)-P7C3-S243 in vehicle made fresh daily were administered every day for 9 months (15-month cohort) or 15 months (24-month cohort). Injections were alternated between sides every other day to reduce irritation at the abdominal injection site. A third cohort of animals was used to determine plasma concentration levels and thus was treated with (-)-P7C3-S243 for two months to achieve steady state blood levels. Four rats were injected once daily IP with 3 mg/kg/d (-)-P7C3-S243 for a total of 60 days. Blood samples were collected from the tail vein in ACD anticoagulant at the indicated times after each daily injection but on separate days and were processed immediately for plasma. Plasma was stored at -80 °C until analysis. All samples were collected no earlier than 14 days after the first injection. The methods used to evaluate levels of (-)-P7C3-S243 in rat plasma were identical to those described for mouse plasma by Dutca, et al. (21) except blank rat plasma (BioreclamationIVT, New York) was used as the matrix for construction of standard curves.

Open field: Locomotor activity was measured using an open field test. All open field tests were performed between 12:00-3:00 p.m. The open field apparatus (University of Iowa Medical Shop) was a black, open topped 50 × 50 × 30 cm Plexiglas box. Open field boxes were cleaned before the first run of the day, between subjects, and after the

last run of the day using 70% ethanol. Rats were brought to the experimental testing room in their home cage on the day of each testing session and remained in the experimental room for 30 minutes to habituate. Each rat was placed in the center of the open field box and allowed to freely explore for 10 minutes. Anymaze video tracking software (Stoelting Co.) was used to measure the distance traveled and the proportion of the time spent in the center of the apparatus by the rat, and analysis was conducted blind to treatment group.

Barnes maze: The Barnes maze test was conducted to analyze learning and memory. All Barnes maze tests were performed between 12:00-6:00 p.m. Rats were brought to the experimental testing room in their home cage on the day of each testing session and remained in the experimental room for 30 minutes prior to testing to habituate. The Barnes maze apparatus was a white, circular surface, 120 cm in diameter, and was raised to a height of 40 cm. The maze consisted of 20 holes, 10 cm in diameter, equally spaced around the border of the surface with an escape cup placed under one hole (University of Iowa Medical Shop). Four, high contrast images placed equal distance around the apparatus, 10 cm from the top of the table were used as spatial cues. In order to motivate the test animals to learn the location of the escape hole, which was associated with a spatial cue, the surface was brightly lit. Each test animal was subjected to four days of training comprised of four trials per day, and the time spent to find the escape location and the number of errors (nose pokes in incorrect holes) committed prior to finding the escape cup were recorded for each trial and averaged for each day. The trial ended when the rat entered the escape cup, or after 80 seconds.

The Barnes maze apparatus was cleaned with 70% ethanol before the first run of the day, between subjects, and after the last trial of the day. On day five, a probe trial was conducted, during which time the escape cup was removed and the animal's memory of the escape location based upon spatial cues was analyzed. Anymaze video tracking software (Stoelting Co.) was used to measure the percent time spent in the target area and the percent time spent in the target quadrant. The ratio of the number of nose pokes in the target hole to the number of nose pokes in incorrect holes was manually scored. Nose pokes were defined as head deflections into a hole, but successive pokes into the same hole were not counted as multiple nose pokes. An area extending 5 cm from the escape hole in all directions was used as the target area for measurements.

Reversal Barnes maze: Reversal testing was used to further assess learning and memory. All reversal Barnes maze tests were performed between 12:00-6:00 p.m. Following initial Barnes maze analysis, rats were given two days of rest and then tested for learning and memory, as well as plasticity. Accordingly, the escape hole was rotated 180° and associated with a new spatial cue. Again, each animal was given time to explore the Barnes maze platform freely for 80 seconds or until they located the new escape location. Four trials were conducted each day for three days, and the latency to find the escape and the errors committed prior to finding the correct escape location were recorded to test the animal's capacity to learn the escape location. On day four, the escape cup was removed to test the animal's memory, and the probe assay was scored as previously described above.

Morris water maze: The Morris water maze test was conducted to analyze learning and memory in 24-month old rats. All Morris water maze tests were performed between 12:00-6:00 p.m. Rats were brought to the experimental testing room in their home cage on the day of each testing session and remained in the experimental room for 30 minutes prior to testing to habituate. The Morris water maze was a silver, circular water tank, measuring 167 cm in diameter filled with 24-25 °C water to approximately 60 cm. A clear, plastic platform is placed submerged below the surface of the water in association with one of four high contrast spatial cues located around the maze. Each test animal was subjected to four days of training comprised of four trials per day, and the time spent to find the platform was recorded for each trial and averaged for each day. On day five, a probe trial was conducted, during which time the platform was removed and the animal's memory of the escape location based upon spatial cues was analyzed. Anymaze video tracking software (Stoelting Co.) was used to measure the latency to cross the previous platform location, as well as the number of times the animal crossed the previous platform location area.

Reversal Morris water maze: Reversal testing was used to further assess learning, memory, and plasticity. All reversal Morris water maze tests were performed between 12:00-6:00 p.m. Accordingly, the escape platform was rotated 180° and associated with a new spatial cue. Again, each animal was placed in the water tank until they found the location of the platform or for 120 seconds. Four trials were conducted each day for three days, and latency to find the platform was recorded to test the animal's capacity to

learn the escape location. On day four, the platform was removed to test the animal's memory and the probe assay was scored as previously described above.

Porsolt forced swim test: The Porsolt forced swim test (FST) was used to assess depression-like phenotypes. All FSTs were performed between 8:00-11:00 a.m. immediately prior to euthanasia. At the 24-month time point, one week elapsed between Morris water maze testing and FST to reduce confounding factors of repeated water interaction. Rats were brought to the experimental testing room in their home cage on the day of each testing session and remained in the experimental room for 30 minutes prior to testing to habituate. Cylindrical swim tanks measuring 20 cm in diameter and 45 cm tall was filled with 24-25 °C water to 30 cm. Rats were placed in the tank for 5 minutes and the time spent immobile, defined as no detectable movement for ≥ 2 seconds, was scored using Anymaze software to analyze videos recorded from above.

Reagents: (-)-P7C3-S243 was synthesized in one large batch by TCG Lifesciences. BrdU for injections was obtained from Sigma and an antibody against 5-BrdU was obtained from Sigma and used at 1:1000. An antibody against APP (APP C-terminal) was obtained from ThermoFisher Scientific and was used at dilutions of 1:1,000 for western blot (WB). Antibodies against abnormally phosphorylated tau (clone tau-ps199/202) were obtained from Sigma and used at a concentration of 1:1,000 for immunohistochemistry (IHC) and 1:4,000 for WB respectively. An antibody for neuronal marker NeuN (1:1,000 for WB) was obtained from Cell Signaling. An antibody against Iba1 (1:5,000 for IHC) was obtained from Wako Ltd. GFAP antibody was obtained from

Abcam and used at 1:5000 for IHC. β-Actin (1:1,000) was obtained from Santa Cruz, and secondary antibodies against mouse and rabbit (1:5,000-1:10,000 for WB) were obtained from Cell Signaling. Cresyl Violet acetate (Sigma) was used at 0.2% and prepared fresh daily.

Tissue preparation: Transcardiac perfusions with ice-cold phosphate-buffered saline were performed under deep anesthesia, and rat brains were quickly collected and separated into hemispheres. The left hemisphere was fixed in 4% paraformaldehyde (PFA) prior to routine processing and paraffin embedding for histochemical analyses. The right hemisphere was snap frozen in liquid nitrogen and stored at -80 °C until further processing. Upon processing, brains were homogenized in 2 mL of ice-cold lysis buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethylsulfonyl fluoride, phosphatase inhibitors, and protease inhibitors (Halt ThermoScientific) for biochemical analysis. Briefly, brains were mechanically dissociated using a tissue tearor homogenizer for 10 pulses. Samples were allowed to stand for 15 minutes at 4 °C and then separated into 3 equal aliquots and stored at −80 °C until further processing. One aliquot was used for tau biochemical analysis, the second aliquot was used for biochemical analysis for Aβ, and the third aliquot was used for protein analysis.

Immunohistochemistry and microscopy: Ten µm para-median sagittal sections were sliced at 30 µm intervals using a microtome and mounted on glass slides. Sections were routinely dewaxed, hydrated in a graded series of ethanol, and placed in a steamer for 25 minutes in Citra+ buffer (R&D) for antigen retrieval. Endogenous peroxidase was

quenched with 0.6% H_2O_2 for 15 minutes. Sections were blocked in normal horse serum for 20 minutes, hybridized with various primary antibodies for 1 hour, and then incubated with appropriate ImmPRESS secondary kits (Vector) for 30 minutes. Sections were developed with ImmPACT Nova Red or DAB (Vector) and counterstained with hematoxylin. Routine dehydration in a graded series of ethanols and xylene was preformed prior to coverslipping (Surgipath MM24 Leica). Immunohistochemical stained slides were scanned using an Aperio ImageScope and analyzed using Aperio Digital Slide Studio software (Aperio Group LLC, Sausalito, CA, USA). For amyloid burden, sections were directly stained with Congo Red (Sigma) according to standard practice and imaged using Cy5 fluorescent excitation. Fluorescent images were obtained using a Zeiss AxioImager.M2 microscope and quantified using contrast intensity methods in ImageJ.

Morphological analysis and whole brain neuronal estimates: For morphological analysis, para-median sagittal 10 µm sections, mounted 3 per slide, spaced 150 µm apart, were stained with hematoxylin and eosin using routine protocols. Again, sections were imaged using an Aperio ImageScope and analyzed using Aperio Digital Slide Studio software (Aperio Group LLC, Sausalito, CA, USA). Representative sections were subsequently assessed by a neuropathologist. Whole number neuronal estimates were completed using the Aperio ImageScope and analyzed using Aperio Digital Slide Studio software (Aperio Group LLC, Sausalito, CA, USA). Para-median sagittal 10 µm sections, mounted 3 per slide, spaced 150 µm apart were stained with 0.2% cresyl violet acetate (Sigma) for 15 minutes at 50 °C or NeuN according to

immunohistochemical procedures provided above. Anatomical regions of interest were defined according to the Paxinos and Watson (2005) rat brain atlas, and included the cerebral cortex, hippocampus, CA1, CA2, CA3, and dentate gyrus.

Biochemical analysis: Biochemical analysis of Aβ peptides was conducted according to a two-step extraction method (49). Briefly, the Aβ aliquot was centrifuged at 10,000 g for 15 minutes at 4 °C. The supernatant (soluble fraction) was centrifuged at 16,000 g for 20 minutes at 4 °C and the resulting supernatant was diluted 1:10 in cell lysis buffer for further analysis. The pellet from the initial centrifugation was rehomogenized in 4x (tissue/volume) 5 M guanidine HCl (insoluble fraction) and then diluted 1:12,500 for further analysis. Protein levels were normalized by BCA protein assay (Pierce Biotechnology). Aβ species were separately quantified in detergent soluble and -insoluble (5 M guanidine-HCl-extracted) fractions using $A\beta_{1\text{-}40,\ 42}$ ELISA kits (Wako Ltd.) in accordance with the manufacturer's instructions. For tau biochemical analysis, established isolation techniques were used (50). Briefly, the tau aliquot was centrifuged at 27,000 g for 20 minutes at 4 °C and the supernatant was set aside. Pellets were rehomogenized in 3x (tissue/volume) buffer supplemented with 10% sucrose and salts and then centrifuged at 27,000 g for 20 minutes at 4 °C. The supernatant was combined with the first supernatant and the pellet was saved for western blot analysis (crude pellet). To isolate sarkosyl insoluble tau, the combined supernatants were incubated for 1 hour at 37 °C in 1% sarkosyl and 1% β-mercaptoethanol. The samples were centrifuged at 150,000 g for 35 minutes at room temperature. The resulting pellet (sarkosyl insoluble P3) and supernatant (sarkosyl soluble) were analyzed via western

blotting. BCA analysis was preformed to determine protein levels for normalization of sarkosyl soluble and insoluble samples prior to electrophoresis, and 20 µg of sample was added per sample. Alternatively, crude pellet tau was obtained by re-homogenization of pellets with Tris-buffered saline (TBS) (pH 7.4), followed by centrifugation at 1,000 g for 5 min at 4 °C, and 5 µl of pure sample was loaded for electrophoresis. Aliquots of protein were electrophoretically separated using 4-20% tris-glycine gels. Electrophoresed proteins were then transferred to PVDF membranes, blocked in TBS containing 5% (w/v) bovine serum (Sigma), and subsequently hybridized with various primary antibodies. Membranes were then incubated with the appropriate HRP-conjugated secondary antibody prior to development with chemiluminescent substrates. ImageJ software was used for densitometric analysis of blots.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Behavioral experiment time course at 15 and 24 months of age conducted on two independent cohorts of animals. *Porsolt forced swim testing was conducted on day 15 of the behavior paradigm in the 15-month old cohort and was tested on day 21 for the 24-month old cohort. An extra week of rest was allotted for the 24-month old cohort, in order to reduce the confounding factor of previous introductions to water with Morris water maze testing.

Supplemental Figure 2. Visual differences noted in the same sample 90 μm apart. (A) NeuN and (B) cresyl violet staining techniques were used to quantify neuronal cell counts in various brain regions. Note an increase in cell numbers in the (A) NeuN staining, considered attributable to characteristic false positive staining in this model from engulfment of neuronal debris by microglia. Note the diffuse staining the CA1-CA3 region of the hippocampus with (B) cresyl violet staining, which complicated cell counts in these regions, as well as the dentate gyrus.

Sup figure 1

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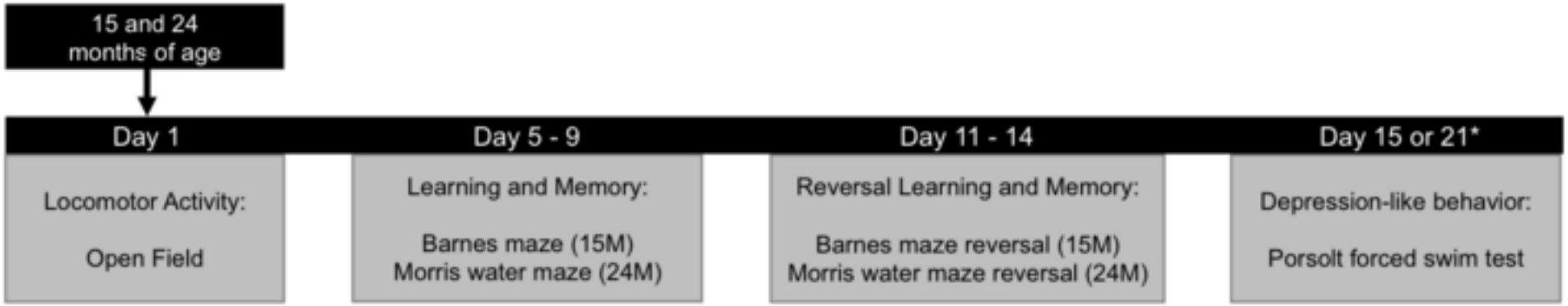

Sup figure 2

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