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### **Cognitive and disease-modifying effects of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 inhibition in male Tg2576 mice, a model of Alzheimer's disease**

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1 **Cognitive and disease-modifying effects of 11 $\beta$ -hydroxysteroid dehydrogenase type 1**  
2 **inhibition in male Tg2576 mice, a model of Alzheimer's disease**

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9  
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31 SPW have consulted for pharmaceutical companies developing selective 11 $\beta$ -HSD1 inhibitors.

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43 **Abstract**

44 Chronic exposure to elevated levels of glucocorticoids has been linked to age-related cognitive  
45 decline and may play a role in Alzheimer's disease. In the brain, 11 $\beta$ -hydroxysteroid dehydrogenase  
46 type 1 (11 $\beta$ -HSD1) amplifies intracellular glucocorticoid levels. We show that short term treatment of  
47 aged, cognitively impaired C57BL/6 mice with the potent and selective 11 $\beta$ -HSD1 inhibitor UE2316  
48 improves memory, including following intracerebroventricular drug administration to the CNS alone.  
49 In the Tg2576 mouse model of Alzheimer's disease, UE2316 treatment of mice aged 14 months for 4  
50 weeks also decreased the number of beta amyloid (A $\beta$ ) plaques in the cerebral cortex, associated with  
51 a selective increase in local insulin-degrading enzyme (involved in A $\beta$  breakdown and known to be  
52 glucocorticoid-regulated). Chronic treatment of young Tg2576 mice with UE2316 for up to 13  
53 months prevented cognitive decline, but did not prevent A $\beta$  plaque formation. We conclude that  
54 reducing glucocorticoid regeneration in the brain improves cognition independently of reduced A $\beta$   
55 plaque pathology, and that 11 $\beta$ -HSD1 inhibitors have potential as cognitive enhancers in age-  
56 associated memory impairment and Alzheimer's dementia.

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## 66 **Introduction**

67 Glucocorticoids have long been recognised to impact on cognitive function, especially with aging (1-  
68 3). Older individuals who exhibit learning and memory impairments have elevated glucocorticoid  
69 levels that parallel both cognitive deficits and shrinkage of the hippocampus, a key locus for memory  
70 formation. The hippocampus expresses a high density of corticosteroid receptors, both the lower  
71 affinity glucocorticoid receptor (GR) and the higher affinity mineralocorticoid receptor (MR), and  
72 these receptors are also abundant in other neocortical regions associated with cognition (4). Elevated  
73 glucocorticoid concentrations in vitro and in vivo promote biochemical, electrophysiological and  
74 structural changes in hippocampal neurons, which associate with poorer memory formation (5, 6).  
75 Manipulations which maintain low glucocorticoid levels from birth (neonatal programming) or mid-  
76 life (adrenalectomy and low dose steroid replacement) prevent the emergence of cognitive deficits  
77 with age (7).

78 Some patients with dementia, including those with Alzheimer's disease (AD), have elevated  
79 circulating cortisol levels, which may contribute to AD pathogenesis (8, 9). It has been postulated that  
80 excess glucocorticoids increase levels of amyloid precursor protein (APP) and APP cleaving enzyme  
81 (BACE) leading to increased amyloid A $\beta$  formation, reduced A $\beta$  degradation via attenuation of  
82 insulin degrading enzyme (IDE) and increased tau expression (10). Other relevant glucocorticoid  
83 actions include hyperglycemia/insulin resistance, angiopathic and anti-angiogenic actions, increased  
84 excitatory (NMDA) neurotransmission and post-synaptic calcium signaling promoting neurotoxicity,  
85 metabolic endangerment of neurons and deleterious alterations in neuroimmune function (11).

86 Glucocorticoid action via intracellular MR and GR is determined not only by circulating steroid levels  
87 but also by target tissue concentrations, modulated by intracellular metabolism by the isozymes of  
88 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) (12). The adult forebrain expresses 11 $\beta$ -HSD type 1,  
89 which catalyses conversion of inert 11-keto corticosteroids (cortisone, 11-dehydrocorticosterone) to  
90 active cortisol and corticosterone. 11 $\beta$ -HSD1 levels are increased in the aging rodent hippocampus  
91 and cortex and correlate with cognitive decline (13). Transgenic mice modestly overexpressing 11 $\beta$ -

92 HSD1 in the forebrain show premature memory decline with aging, while 11 $\beta$ -HSD1 null mice on  
93 two distinct genetic backgrounds, and even heterozygous null mice (with 50% less enzyme) resist  
94 cognitive decline with aging in a variety of tests (14). This protection associates with loss of the age-  
95 associated rise in intrahippocampal corticosterone levels but without changing plasma corticosterone  
96 levels (13).

97 Treatment of already aged mice with selective 11 $\beta$ -HSD1 inhibitors improves spatial memory  
98 performance. Effects are rapid, occurring within hours to days (15-17). Moreover, in small  
99 randomized placebo-controlled trials, the non-selective 11 $\beta$ -HSD inhibitor carbenoxolone improved  
100 memory in healthy aging men and in patients with type 2 diabetes (18). Whilst 11 $\beta$ -HSD1 inhibition  
101 improves glucose homeostasis and other metabolic parameters in obesity, metabolic changes were not  
102 correlated with cognitive effects in aged rodents or humans. These results support examination of  
103 selective 11 $\beta$ -HSD1 inhibitors in the treatment of age-related cognitive impairments.

104 Here we examined a crucial issue, whether selective 11 $\beta$ -HSD1 inhibition alters cognition and  
105 pathology in AD. We used a murine AD model, the well-characterized Tg2576 mouse which bears a  
106 mutated human APP gene. We generated and used UE2316, a novel and selective inhibitor of both  
107 human and rodent 11 $\beta$ -HSD1 with a low nanomolar IC<sub>50</sub> value and high penetration into the brain  
108 (19,20).

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## 110 **Materials and Methods**

### 111 **UE2316**

112 UE2316 ([4-(2-chlorophenyl-4-fluoro-1-piperidinyl)[5-(1H-pyrazol-4-yl)-3-thienyl]-methanone) was  
113 synthesized by High Force Ltd, UK according to methods previously described (21). In vitro  
114 screening of UE2316 potency in HEK293 cells stably transfected with hsd11b1 (22) showed a greater  
115 median inhibitory concentration (IC<sub>50</sub>) than our previously reported compound UE1961 (15, 20).

116 Inhibition of 11 $\beta$ -HSD1 activity in tissue extracts was quantified as previously described (22). Liver

117 brain and white adipose tissues were collected and snap frozen on dry ice. Frozen tissue (50-80mg)  
118 was homogenized in 700µl of chilled Krebs Buffer and a cleared homogenate prepared by  
119 centrifugation at 3500rpm for 5 minutes. The protein concentration of this homogenate was  
120 determined by Bradford assay. For the assay, 25µl of 10mM NADPH was added to 250µg of the  
121 homogenate in a final volume of 200µl chilled Krebs buffer and incubated at 37°C for 20 minutes.  
122 <sup>3</sup>H-cortisone (25µl of 200nM) was then added and the assay incubated for a further 15 minutes prior  
123 to termination by rapid freezing on dry ice. <sup>3</sup>H-cortisone to <sup>3</sup>H-cortisol conversion was determined in  
124 50µl of the defrosted reaction by capturing liberated <sup>3</sup>H-cortisol on anti-cortisol (HyTest Ltd)-coated  
125 scintillation proximity assay beads (protein A-coated YSi, GE Healthcare). The percentage inhibition  
126 was determined by measuring the conversion of <sup>3</sup>H-cortisone to <sup>3</sup>H-cortisol relative to that in tissue  
127 from vehicle treated mice.

## 128 **Animals**

129 All in vivo experiments were performed under a project license issued under the UK Scientific  
130 Procedures (Animals) Act, 1986, and with local ethical committee approval. Male C57Bl/6 mice were  
131 obtained from Harlan (UK). Male mice were chosen to eliminate the potential effects of gonadal  
132 hormonal fluctuations observed in females. Animals were group-housed under controlled lighting (on  
133 07.00-19.00h) and temperature (22°C), with access to food and water ad libitum. Experimental  
134 procedures are summarised in Table 1.

135 For measurement of pharmacodynamic inhibition following oral administration, oral gavages with  
136 vehicle (38% PEG, 2% DMSO, 60% saline; Sigma, Poole, UK) or UE2316 dissolved in vehicle were  
137 performed in the morning in animals aged 8-10 weeks (n=3 per dose). Animals were sacrificed post  
138 dosing (1, 4 and 6 hours) and the tissues retained for analysis of 11β-HSD1 inhibition.

139 For assessment of pharmacodynamic inhibition following subcutaneous administration, C57Bl/6 mice  
140 (8-10 months, Harlan UK) (n=3 per group) were treated with either vehicle (50:50 DMSO: PEG,  
141 Sigma) or 10mg/kg/day UE2316 in vehicle via subcutaneously implanted Alzet osmotic minipumps

142 (model 2004, Charles River, Margate, UK) for 14 days. Animals were sacrificed at this stage and the  
143 tissues retained for analysis of 11 $\beta$ -HSD1 inhibition.

144 To show that the effects of UE2316 were not merely due to any peripheral metabolic actions of 11 $\beta$ -  
145 HSD1 inhibition, the agent was administered intracerebroventricularly to aged male C57Bl/6 mice (24  
146 months, obtained from an in-house stock) were treated with either vehicle (artificial CSF; Alzet;  
147 Charles River) (n=9) or 100ng/h UE2316 in artificial CSF (n=8) administered via  
148 intracerebroventricular (icv) infusion, for 9 days, as previously described (23).

149 For assessment of the effects of UE2316 on cognition in aging animals, aged male C57Bl/6 mice (22  
150 months, Harlan UK) were treated with either vehicle (n=6), 5mg/kg/day UE2316 (n=8) or  
151 15mg/kg/day UE2316 (n=8) in vehicle (50:50 DMSO: PEG, Sigma) by subcutaneously implanted  
152 Alzet osmotic minipumps (model 2004, Charles River, Margate, UK) for 23 days, with body weights  
153 monitored at the start and end of the treatment.

154 For the assessment of the effects of UE2316 in a model of AD, male Tg2576 (Hsiao et al., 1996) and  
155 age-matched genetic control (BL6;SIL) littermates were obtained from Taconic Europe (Ry,  
156 Denmark). Animals were singly housed due to aggressive behavior. In the short term treatment study,  
157 14 month old mice of each genotype (n=10 per group) were allocated at random to receive either  
158 UE2316 (10mg/kg/day) or vehicle (50:50 DMSO: PEG, Sigma) via 2 Alzet osmotic minipumps  
159 (model 2004) implanted subcutaneously to provide sufficient volume of drug or vehicle for 29 days.  
160 Food intake and body weight were monitored weekly throughout. For the long term study in which  
161 UE2316 was administered by incorporation in the diet, 6-7 month old Tg2576 male mice that were  
162 screened for eye color, coat color and rd1 homozygosity for the Pde6b<sup>rd1</sup> retinal degeneration  
163 mutation by Taconic were fed with control diet (RM1) (n=16) or with RM1 containing 175 ppm  
164 UE2316 (for a calculated dosage of 30 mg/kg/day) (n= 32) (Special Diet Services, Broxburn, UK) for  
165 up to 57 weeks. Food intake and body weights were monitored weekly throughout the experiment and  
166 drug dosages were calculated based on average daily food intake. Each cohort of mice underwent  
167 repeated longitudinal cognitive testing.



168 **Behavior**

169 Mice were acclimatized to the behavior room for at least one hour before all procedures in order to  
170 minimize stress. All behavioral testing was conducted during the day between 9am and 12pm.

171 **Memory in passive avoidance**

172 Passive avoidance was assessed over 2 days (for aging studies in C57Bl/6 mice on days 13 and 14  
173 after the start of treatment, for the short term UE2316 study in Tg2576 mice on days 27 and 28 and at  
174 weeks 15 and 41 in the long term UE2316 Tg2576 study) in a step through light/dark box passive  
175 avoidance apparatus (Ugo Basile Comerio, VA, Italy) (13). On the first day, the latency to enter the  
176 dark compartment from the light compartment was measured, with the door to the dark compartment  
177 opening 30 seconds after the start of the trial. Twenty four hours later, the latency to enter the dark  
178 compartment was repeated, which was followed by a mild 0.3mA, 2 second foot-shock in the dark  
179 compartment. The mice were then retested 6 hours later for the latency to enter the dark compartment,  
180 this time without a foot-shock. The latencies were measured automatically by the device following the  
181 opening of the door separating the light and dark compartments with a maximal time allowed of 300  
182 seconds. Mice that did not enter the dark compartment were eliminated from analysis.

183 **Y maze**

184 Y maze testing of spatial hippocampal memory was performed as previously described with a 2 hour  
185 inter-trial interval (ITI) on day 10 following the start of treatment in the aged UE2316 study (15). For  
186 the UE2316 icv administration study, Y maze testing was performed on day 8 of treatment. The  
187 amount of time spent in each arm was measured and analyzed using AnyMaze software (Stoelting,  
188 Dublin, Ireland).

189 **Open field**

190 The open field test was performed on day 23 of the short term Tg2576 study and week 38 of the long  
191 term Tg2576 study. Mice were placed in an open field (OF) box (60 x60 cm) marked off into 16 equal  
192 squares. The outer row of squares adjacent to the walls of the box are considered less anxiogenic than

193 the inner squares. For a 5 min period, the number of crossings, time, and distance (movement of all  
194 four legs into a new square) into each square was noted. Total movement in the maze reflects general  
195 activity and the relative movement in the inner zone is correlated to the anxiety state of the mouse.

### 196 **Spontaneous alternation**

197 Spontaneous alternation, a test of working hippocampal memory, was tested after 26 days of  
198 treatment in the short term treated Tg2576 mice and after 39 weeks of treatment in the long term diet  
199 study. Mice were placed in a Y maze apparatus consisting of three enclosed black Plexiglas arms (50  
200 cm long, 11 cm wide and 10 cm high), with prominent extramaze visual cues. Mice were allowed to  
201 explore the maze for five minutes after starting in a randomly chosen 'start' arm. The order and  
202 number of arm entries by each mouse in the 5 minute test period was recorded. Percentage  
203 spontaneous alternation was calculated using the following formula: % spontaneous alternation=  
204 [number of alternations (which is entries into 3 different arms consecutively)/(number of arms entered  
205 minus 2)] x 100.

### 206 **Morris water maze**

207 Morris water maze testing was performed as previously described on day 14 of the short term study  
208 and week 52 of the long term study (24). For the short term treatment study in Tg2576 mice, mice  
209 who did not swim (i.e. did not engage with the task) during the visible platform test, in which a visual  
210 cue (i.e. stacked Lego blocks) was placed on the submerged platform in the tank and no visuospatial  
211 clues were present (curtains were closed) were eliminated from analysis. The mice undertook 4 trials  
212 per day with a 20 minute inter trial interval and a maximum swim time of 90 seconds per trial for 4  
213 days. Latency, swim speed and the percentage of time spent in each quadrant of the pool were  
214 measured by Watermaze software (Actimetrics, Evanston, IL, USA). In the long term treatment study,  
215 mice were initially assessed after 12 months of treatment for their ability to engage in the visible  
216 platform test. In this instance, mice that were able to find the platform and improve their latencies  
217 after 2 days of 4 x 90 second trials were then tested in the spatial water maze, in which the platform  
218 remained submerged without a visual cue on top and the mice utilised spatial clues located around the

219 maze (curtains open). Mice were tested in 4 x 90 second trials per day over 6 days. Twenty four  
220 hours after the final spatial water maze trial, the mice were then tested in the 90 second probe test, in  
221 which the hidden platform was removed from the tank and the percentage of time spent swimming in  
222 the target quadrant was measured.

223 After behavioural testing, mice were sacrificed by cervical dislocation on day 29 of vehicle or  
224 UE2316 treatment in the short term study or after 44 and 57 weeks in the long term study. The brains  
225 were removed and hemisected coronally. One half of the brain was dissected and cortex, hippocampus  
226 and cerebellum were immediately frozen on dry ice and stored at -80°C for further analysis. The other  
227 half was fixed in 4% paraformaldehyde in PBS (4% PFA) (VWR, Lutterworth, UK) and  
228 cryoprotected in 30% sucrose (Sigma) overnight at 4°C before storage at -80°C for  
229 immunohistochemistry.

### 230 **Immunohistochemistry**

231 All immunohistochemistry was performed on free floating 25 µm sections stored at -20°C in  
232 cryoprotectant (50 mM phosphate buffer, 25% glycerol, 25% ethylene glycol, Sigma). Sections were  
233 transferred to a 12 well tissue culture plate with Netwell inserts (VWR) and washed in PBS. Antigen  
234 retrieval was performed by heating the sections in sodium citrate buffer, pH 6.0 (Sigma) at 95°C for  
235 15 minutes, followed by peroxidase treatment (1% H<sub>2</sub>O<sub>2</sub> in PBS; Sigma) for 30 minutes to remove  
236 endogenous peroxidase activity, washed and then blocked with the appropriate serum for 1 hour  
237 followed by overnight incubation at 4°C with the antibody of choice. For staining using the 6E10  
238 antibody for visualization of amyloid beta plaques, the sections were blocked using the mouse on  
239 mouse (MOM) Ig blocking reagent (Vector Laboratories, Peterborough, UK) followed by overnight  
240 incubation with a 1:1000 dilution of beta amyloid 1-16 mouse monoclonal antibody (6E10) (Covance,  
241 Cambridge Bioscience, Cambridge, UK). Following washing, sections were incubated with secondary  
242 antibody for 1 hour at room temperature. Staining was visualised using the Vectastain ABC kit and  
243 DAB peroxidase substrate kit (Vector Laboratories). The sections were then mounted on Superfrost  
244 Plus slides (VWR), dehydrated and coverslipped. The number of 6E10 positive plaques per brain area

245 was counted by an experimenter blinded to the treatment group using a Zeiss Axioskop and the  
246 KS300 imaging program (Zeiss, Eching, Germany). Plaque area, measured using the same program,  
247 was expressed as plaque area divided by the total area of the brain region. Iba-1 antibody was  
248 purchased from Abcam (Cambridge, UK). Goat and rabbit serum were purchased from Sigma.  
249 Biotinylated rabbit anti-goat IgG antibody and biotinylated rabbit anti-sheep IgG antibody were  
250 purchased from Vector Labs.

## 251 **Western blotting**

252 Protein extracts were prepared from brain areas by homogenization in Krebs buffer containing  
253 protease inhibitor (Roche, Burgess Hill, UK) followed by centrifugation at 3000 RPM for 5 minutes.  
254 Protein concentration of the supernatant was measured using the Bradford Assay (BioRad, Hemel  
255 Hempstead, UK). Proteins were separated by SDS-PAGE using NuPAGE Novex 4-12% bis-tris gels  
256 (Invitrogen, Paisley, UK) and transferred to nitrocellulose membranes (0.2 $\mu$ m pore size; Invitrogen).  
257 Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk blotting grade  
258 blocker (BioRad) in PBS, pH 7.4, containing 0.1% Tween 20 (PBS-T) and then incubated overnight  
259 with shaking at 4°C with the primary antibody diluted in blocking reagent. This was followed by  
260 incubation at room temperature in the appropriate secondary antibody. IDE, PSD95, ADAM10,  
261 synaptophysin and CD31 antibodies were purchased from Abcam. The anti-BACE 1 N terminus (46-  
262 62) antibody was sourced from Sigma. Mouse beta-tubulin antibody was purchased from Merck-  
263 Millipore (Watford UK). Goat-anti rabbit IgG antibody was obtained from Licor Biosciences UK  
264 (Cambridge UK). Alexa Fluor 680 donkey anti-sheep IgG (H+L) and Alexa Fluor 680 rabbit anti-goat  
265 IgG antibodies were purchased from Invitrogen (Paisley, UK). Proteins were visualized and band  
266 intensities were quantified using the Odyssey Infrared Imaging System (LiCor Biosciences UK,  
267 Cambridge, UK).

## 268 **Statistical analysis**

269 Data are expressed as mean  $\pm$  SEM. Groups were compared by ANOVA. When ANOVA was  
270 significant post hoc tests were performed as indicated in the Figure legends. Differences were  
271 considered significant when  $p < 0.05$ .

## 272 **Results**

### 273 **UE2316 inhibits 11 $\beta$ -HSD1 in the brain**

274 We previously reported the discovery and pharmacological effects of the selective 11 $\beta$ -HSD1  
275 inhibitor UE1961, which was based on a thiophene amide scaffold (15). However, this molecule has  
276 sub-optimal potency and pharmacokinetic properties for progression to late-stage preclinical  
277 development. Further medicinal chemistry optimisation of this compound, replacing the  
278 decahydroquinoline and substituted piperidine groups flanking the thiophene core, led to the  
279 identification of UE2316 (Figure 1A). UE2316 displays greater potency than UE1961, excellent  
280 selectivity and an improved drug metabolism and pharmacokinetic profile for use in in vivo studies  
281 (Figure 1B). Pharmacodynamic inhibition of 11 $\beta$ -HSD1 in tissues was confirmed following  
282 administration by oral and subcutaneous (SC) routes. Single dose oral administration of UE2316 to  
283 C57BL/6 mice induced significant ex vivo inhibition of 11 $\beta$ -HSD1 in the brain for at least 4 hours  
284 (Figure 1C), while constant infusion of 10mg/kg/day of UE2316 over 14 days by SC Alzet osmotic  
285 minipumps also produced  $34.2 \pm 8.3$  % inhibition of 11 $\beta$ -HSD1 in the brain (data not shown). The  
286 results from these studies were in agreement with those from previous studies (19,20). UE2316 was  
287 thus chosen to investigate the effects of chronic 11 $\beta$ -HSD1 inhibition on cognitive impairment and  
288 AD pathology in mouse models using either SC or oral administration.

### 289 **UE2316 acts in the brain to improve cognition in aged wild type mice**

290 To assess the effects of UE2316 on memory in cognitively impaired mice, C57BL/6 male mice aged  
291 22 months were randomly assigned to treatment with 0, 5 or 15 mg/kg/day of UE2316 via SC  
292 implanted Alzet minipumps for 14 days. In the Y-maze, a non-stressful test of hippocampal-  
293 associated spatial memory, there was a significant increase in time spent exploring the novel arm after  
294 a 120 min inter-trial interval in mice receiving 15 mg/kg/day UE2316 compared to vehicle-treated

295 controls (Figure 2A). Cognition was also assessed in the passive avoidance task, which tests  
296 emotional and fear associated memories (13). During the retention phase of the passive avoidance  
297 test, UE2316 increased latency to enter the dark compartment at both 5 and 15 mg/kg/day compared  
298 with vehicle-treated mice, indicating improved memory (of the footshock) (Figure 2B).

299 To investigate whether brain-specific inhibition of 11 $\beta$ -HSD1 was responsible for these improvements  
300 in cognition, we delivered UE2316 directly to the brain of aged (24 month old) C57BL/6 mice at an  
301 appropriate concentration via icv administration for 9 days. Post-mortem analysis of whole brain  
302 samples revealed  $39.9 \pm 5.5$  % inhibition of 11 $\beta$ -HSD1 was achieved with a 100 ng/h infusion.  
303 Vehicle-treated aged controls showed impaired spatial memory in the Y maze (similar times spent in  
304 all 3 arms) as previously reported (15). Mice treated with icv UE2316 spent more time exploring the  
305 novel arm of the Y-maze than vehicle-treated mice, indicating an improvement in spatial memory  
306 after 8 days of treatment (Figure 2C).

### 307 **UE2316 improves cognition in a murine model of Alzheimer's disease**

308 Following confirmation of the effects of 11 $\beta$ -HSD1 inhibition using UE2316 in age-related cognitive  
309 impairment, we examined effects of short-term UE2316 administration in the Tg2576 mouse model of  
310 AD. Tg2576 mice carry a transgene with mutations at amino acids 670 and 671 in the human APP  
311 gene under the control of the hamster prion promoter, which leads to accumulation of A $\beta$  plaques in  
312 the brain from 9-12 months of age with consequent cognitive impairment (25). Singly housed age-  
313 matched male mice were separated into 4 groups of 10 mice per group: wild type or Tg2576 mice  
314 administered vehicle or UE2316. Mice were treated by 2 SC implanted Alzet minipumps from 14  
315 months of age for 29 days. No treatment-related adverse effects on final body weight, daily food  
316 intake or adrenal size were observed. However, as previously reported, Tg2576 mice weighed less  
317 than their wild type littermates throughout the study despite consuming more food (Supplementary  
318 Figure 1A-B) , which may reflect their increase in locomotor activity and hypothalamic dysfunction  
319 (26,27).

320 Tg2576 mice perform poorly in the Y-maze spatial memory test due to retinal degeneration, therefore,  
321 fear-associated memory was assessed in the passive avoidance task, which is not dependent on visual  
322 acuity. In this test, performed on days 27 and 28 of drug or vehicle administration, UE2316 treatment  
323 increased latencies in re-entry to the dark compartment at 6 hours post footshock in both control and  
324 Tg2576 mice (Figure 3A), suggesting an improvement in fear associated memory with drug treatment.  
325 The effect of UE2316 treatment was particularly pronounced in Tg2576 mice, which may reflect a  
326 difference in sensitivity to the electrical shock in this mouse strain. In a separate open field test, no  
327 difference was observed in the time spent in the inner zone in either wild type or Tg2576 mice with or  
328 without drug (two way ANOVA, treatment effect:  $p=0.98$ ), suggesting that UE2316 does not affect  
329 anxiety (Figure 3B). No difference in speed was observed between either strain, in the presence or  
330 absence of drug (two way ANOVA, treatment effect:  $p=0.94$ , data not shown).

331 In spontaneous alternation, a test of working hippocampal memory, Tg2576 mice tended to enter  
332 more arms than the control mice ( $p=0.06$ ), which again may be due to their increased locomotion  
333 (data not shown) (26). Treatment with UE2316 led to a trend in increased percentage alternation in  
334 both wild type and Tg2576 mice compared to vehicle (two way ANOVA, treatment effect:  $p=0.06$ )  
335 (Figure 3C).

### 336 **UE2316 prevents cognitive decline in a murine model of Alzheimer's disease**

337 Effects of long-term UE2316 treatment were examined by administering UE2316 in the diet to 6-7  
338 month old Tg2576 mice over a period of 57 weeks. The mice on the UE2316-supplemented diet  
339 maintained an average daily dose of approximately 30 mg/kg/day throughout the experiment and did  
340 not exhibit any adverse effects (Supplementary Figure 2A). UE2316-treated mice tended to weigh less  
341 than vehicle-treated mice up to 44 weeks (ANOVA,  $p<0.01$ ) despite eating more food (ANOVA,  
342  $p<0.001$ ) (Supplementary Figure 2B-C). In contrast to the short-term study, mice were pre-screened  
343 for retinal degeneration (RD) and only those which were RD negative were included. Memory was  
344 assessed at intervals using passive avoidance, spontaneous alternation and Morris Water Maze tests.

345 Fear associated memory was assessed using the passive avoidance test after 15 and 41 weeks of  
346 treatment. As expected, at 15 weeks, when mice were aged 10-11 months, similarly increased  
347 latencies were observed following training in both the vehicle and UE2316-treated groups, consistent  
348 with preserved cognitive function at this age (Figure 4A). In contrast, after 41 weeks of treatment,  
349 when the mice were aged 16-17 months, vehicle-treated mice exhibited cognitive impairment as  
350 demonstrated by lack of prolongation of latency after training, but Tg2576 mice treated with UE2316  
351 maintained an increase in latency to enter the dark compartment 6 hours post shock, indicating that  
352 UE2316 prevents an age-associated decline in fear associated memory (Figure 4B). There was an  
353 increase in latency for training in vehicle-treated mice, suggesting an impairment in their ability to  
354 effectively to engage with the task. No difference in anxiety was observed with UE2316 treatment in a  
355 separate open field test (two way ANOVA, treatment effect:  $p=0.25$ ), but Tg2576 mice were less  
356 anxious compared to wild type mice (two way ANOVA, genotype effect:  $p<0.01$ ) (Figure 5A). There  
357 was also an increase in locomotion in Tg2576 mice when compared to wild type mice (two way  
358 ANOVA, genotype effect:  $p<0.01$ ), but no effect of treatment (two way ANOVA, treatment effect:  
359  $p=0.69$ ).

360 Working memory was assessed at 39 weeks of treatment using the spontaneous alternation test.  
361 UE2316 increased spontaneous alternation (Figure 5B), although there was no difference in the total  
362 number of arm entries (data not shown).

363 Spatial memory was assessed in the Morris Water Maze after 52 weeks of treatment (mice aged 18-19  
364 months). Swim speeds were not affected by UE2316 treatment and there was no difference between  
365 strains (data not shown). UE2316 reduced the time taken to find the hidden platform across the testing  
366 period (Figure 5C), and increased time spent in the target quadrant during the probe test performed 24  
367 hours after the final trial (vehicle:  $34.2\pm 3.2\%$ , UE2316:  $52.1\pm 5.1\%$ ,  $p=0.02$ ), consistent with  
368 improved spatial learning and retention (Supplementary Figure 3A).

369 **Effect of UE2316 on A $\beta$  plaques in the brain**



370 Immunohistochemistry with 6E10 antibody (28), which detects A $\beta$ 1-16, was performed on Tg2576  
371 brains to determine whether UE2316 affected the number and volume of A $\beta$  plaques (wild type  
372 control brains were not analysed as these mice do not develop amyloid plaques) (Figure 7A).

373 Short-term 4-week UE2316 treatment decreased A $\beta$  plaque number in the cortex and amygdala but  
374 not the hippocampus of 15m old Tg2576 mice (Figure 6A). The total plaque number in the brains of  
375 Tg2576 mice was 54% lower in UE2316-treated than vehicle-treated mice. Plaque areas were  
376 correspondingly reduced (Figure 6B).

377 After chronic 44-week treatment with UE2316 (mice aged 17-18 months) there were a similar number  
378 of A $\beta$  plaques in the cortex and hippocampus as in mice treated with UE2316 for only 4 weeks  
379 (Figure 6C). However, there were fewer A $\beta$  plaques in the brains of the vehicle-treated group from  
380 the 44-week study than in the vehicle-treated animals from the 4-week study. In the 44-week study  
381 there was no significant effect of UE2316 on number of A $\beta$  plaques.

382 To explore possible mechanisms mediating the effects of short-term UE2316 treatment on A $\beta$  plaque  
383 burden in the cortex we conducted western blot analyses of selected proteins involved in A $\beta$   
384 generation and metabolism, the expression of which are known to be regulated by glucocorticoids  
385 (Figure 7B). UE2316 treatment did not modulate BACE protein expression in either WT or Tg2576  
386 mice (Table 2). Nor did UE2316 alter ADAM10, a metalloproteinase possessing  $\alpha$ -secretase activity  
387 involved in the non-amyloidogenic pathway of APP processing (29) (Table 2). However, UE2316  
388 significantly increased, by 31% and 34% respectively, insulin degrading enzyme (IDE) protein  
389 expression in the cortex of both control and Tg2576 mice (Table 2). No difference in IDE expression  
390 was found in the brains from mice treated with UE2316 for 44 weeks (IDE to beta-tubulin ratio:  
391 vehicle: 0.016 + 0.004 vs UE2316: 0.011 + 0.002; Student's t-test, p=0.22).

392 PSD95 and synaptophysin, markers of synaptic density (30,31), were unaffected by UE2316 (Table  
393 2). In addition, microglial density was not increased as evidenced by no change in Iba1 levels (32)  
394 (Table 2). CD31 staining was also conducted to probe for potential changes in cerebral vascular

395 density, as 11 $\beta$ -HSD1 null mice exhibit enhanced angiogenesis (33), but no effect of UE2316 was  
396 observed (Table 2).

397

## 398 **Discussion**

399 We have generated UE2316, which is a potent and selective inhibitor of 11 $\beta$ -HSD1 in mouse brain.  
400 UE2316 administered either systemically or directly to the brain induces improvements in memory in  
401 cognitively impaired rodents. In aging mice the effects of UE2316 recapitulate those of other selective  
402 11 $\beta$ -HSD1 inhibitors (15, 16) and provide further evidence that short term inhibition of 11 $\beta$ -HSD1 in  
403 the brain improves memory impairments associated with aging. Moreover, our data demonstrate that  
404 these improvements are associated with inhibition of 11 $\beta$ -HSD1 specifically in the brain since mice  
405 treated with icv administration with sub-systemic doses display memory improvements comparable to  
406 those observed in mice treated systemically. The effects are evident across a range of behavioral tasks  
407 that involve the hippocampus, in contrast with the attenuation of contextual fear-associated memory  
408 that we previously reported with UE2316 which is likely mediated in different brain regions (19). It is  
409 likely that these short term improvements in memory are due to the effects of reduced intracellular  
410 corticosterone in regions of the brain such as the hippocampus, which are sufficient to reverse the  
411 memory-impairing effects of glucocorticoids in aged mice (17). Structural changes to the  
412 hippocampus, such as synaptic and dendritic atrophy may be reversed by reduced intracellular  
413 glucocorticoid levels over a period of hours and weeks respectively, and could be responsible for the  
414 short term memory improvements observed in these studies (34,35). However, we also observed  
415 significant improvements in memory with long term 11 $\beta$ -HSD1 inhibition which may be mediated by  
416 structural hippocampal changes. It should be noted that behavioral testing was only carried out in  
417 male mice and the exploration of any potentially sexually dimorphic effects of 11 $\beta$ -HSD1 inhibition  
418 will require separate, comparative studies in male and female mice.

419 There is now substantial evidence from studies in rodents and in humans that reductions in 11 $\beta$ -HSD1  
420 activity in the brain provides beneficial effects on the cognitive decline associated with aging (15, 16,

421 18, 23). However, to date no studies have been published that assess the effects on 11 $\beta$ -HSD1  
422 inhibition in rodent models of AD. We found that short term (29 days) treatment of already  
423 cognitively-impaired 14-month old Tg2576 mice with UE2316 led to improvements in memory  
424 during the passive avoidance task. UE2316 also improved latency in wild type mice but to a lesser  
425 extent than in Tg2576 mice. Our data also demonstrate that long term inhibition of 11 $\beta$ -HSD1 in  
426 Tg2576 mice maintains cognitive performance with aging since age-matched mice without UE2316  
427 treatment are cognitively impaired in tests performed from 16 months of age onwards. Moreover,  
428 cognitive improvement with 11 $\beta$ -HSD1 inhibition is maintained in the presence of significant  
429 Alzheimer's pathology.

430 In the Tg2576 mouse strain, A $\beta$  plaques have been shown to develop from 9 months of age onwards,  
431 associated with impairments in cognitive ability in memory tests from 10-12 months (25). As  
432 expected, when we examined the brains of the Tg2576 mice A $\beta$  plaques were observed in the cortex,  
433 amygdala and hippocampus. Short term UE2316 treatment substantially decreased A $\beta$  plaque number  
434 and area in the cortex and amygdala of Tg2576 mice. In contrast, in a separate cohort of mice from  
435 which those with retinal impairment were excluded, we observed less A $\beta$  plaque burden and no  
436 statistically significant effects of chronic UE2316 administration. This likely reflects the differences  
437 in animals with and without visual impairment. Overall, the results suggest that treatment with  
438 UE2316 has a disease-modifying effect on amyloid plaque deposition in Tg2576 mouse brains by  
439 impairing plaque accumulation. However, our findings dissociate cognitive improvement from A $\beta$   
440 plaque pathology after chronic treatment, suggesting the mechanism of improved cognition with 11 $\beta$ -  
441 HSD1 inhibition is not mediated solely through reduced plaque burden. Whatever the mechanism, the  
442 effect on cognition is likely associated with lowered intracellular glucocorticoid levels in the brain,  
443 and the consequent altered balance of glucocorticoid and mineralocorticoid receptor action (23).

444 Additionally, we observed an increase in cortical IDE protein levels with short term UE2316  
445 treatment. This increase may explain, in some part, the reduction in plaque numbers and plaque area  
446 in the drug-treated mice, as previous studies have demonstrated that overexpression of IDE or  
447 neprilysin in the neurons of transgenic mice significantly reduced brain amyloid beta levels and

448 slowed or completely prevented amyloid plaque formation in APP TG mice (36), while IDE null mice  
449 have excess cerebral accumulation of A $\beta$  (37). In humans, genome-wide association studies report a  
450 higher susceptibility to AD in Finnish patients with polymorphisms of IDE, suggesting that the rate of  
451 A $\beta$  degradation may be an important factor in the development of human AD (38). However, the lack  
452 of IDE induction with chronic UE2316 administration, in the face of persisting benefits for cognitive  
453 function, suggests that other pathways are involved in cognitive protection with 11 $\beta$ -HSD1 inhibition.  
454 Alternatively, Tg2576 mice selected for intact vision may be relatively resistant to glucocorticoid  
455 effects on IDE and A $\beta$  turnover.

456 These pre-clinical results support the concept that 11 $\beta$ -HSD1 inhibition may be efficacious for  
457 memory impairments not only with aging, but also in AD. A recent phase 2 clinical trial in patients  
458 with mild-moderate AD was halted, however, when the selective 11 $\beta$ -HSD1 inhibitor ABT-384 failed  
459 to show non-inferiority against donepezil for the primary endpoint of ADAS-Cog score (39).

460 Although a pharmacodynamic study of ABT-384 using stable isotope d4-cortisol tracer has been  
461 reported (40), it remains uncertain whether ABT-384 inhibited 11 $\beta$ -HSD1 adequately in brain since:  
462 data were presented for only two control subjects without administration of ABT-384; after ABT-384  
463 administration, d3-cortisol levels (generated by 11 $\beta$ -HSD1) (41) were very low in plasma, consistent  
464 with systemic enzyme inhibition, and this may account for the undetectable levels of d3-cortisol in  
465 CSF; and the maximum CSF concentrations of ABT-384 achieved, which were present for only a  
466 short time after dosing, were not high enough to inhibit 11 $\beta$ -HSD1 by more than 10%, according to  
467 published potency of the compound. The benefits of 11 $\beta$ -HSD1 inhibition may only be apparent in the  
468 treatment of early disease, when the combination of symptomatic cognitive improvement and  
469 potential for disease modification we have observed in the mouse model of AD may be most useful.

470

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475

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593

## 594 **Tables.**

### 595 **Table 1: Treatment table.**

596 **Table 2: UE2316 increased IDE protein levels in the cortex of control and Tg2576 mice.** Data are  
597 mean  $\pm$  SEM from Western blot densitometry, normalized to beta-tubulin. 2-way ANOVA analysis  
598 was performed and the treatment effect of UE2316 is shown. For IDE \* $p$ <0.04 for effect of UE2316  
599 within each genotype by post hoc Fisher's LSD tests.

600

## 601 **Figures and Legends.**

602 **Figure 1: UE2316 Characteristics.** A: Structural comparison of UE1961 and UE2316 B: Potency  
603 and selectivity of UE2316. C: Male C57BL6 mice were treated with a single 10 mg/kg oral dose of  
604 UE2316 (n=3 animals per time point) and inhibition by scintillation proximity assay was assessed 1, 4  
605 and 6 hours post dosing and expressed as % inhibition compared with values obtained in vehicle-  
606 treated mice. (One-way ANOVA,  $p$ =0.009; Bonferroni's post-hoc comparisons, \* $p$ <0.05 vs vehicle).

607 **Figure 2: UE2316 improved spatial and fear-associated memory in aged C57Bl6 mice.** Aged 22  
608 month-old C57Bl6 mice were treated with 0 (n=6), 5 (n=8) or 15 (n=8) mg/kg/day UE2316 for 23  
609 days via SC implanted osmotic minipumps. A: Spatial memory was assessed by Y maze on day 10 of  
610 treatment. The initial 1 minute ITI was performed prior to surgery. Treatment with 15 mg/kg/day  
611 UE2316 increased the time spent in the novel arm in the 2 hour ITI compared to vehicle treated  
612 animals (\* $p$ <0.05 by Student's t-test with Bonferroni correction), and a trend for improvement was

613 seen with the 5 mg/kg/day dose. B: Passive avoidance was analyzed on days 13 and 14 of treatment.  
614 Both 5 mg/kg/day (\*p=0.02 vs vehicle by Student's t-test) and 15 mg/kg/day (\*p=0.03 by Student's t-  
615 test) UE2316 improved latency in the retention trial compared to the vehicle treated group (by two-  
616 way repeated measures ANOVA drug interaction with training vs retention p<0.05). C: Similarly aged  
617 C57Bl/6 mice were treated with an infusion of either vehicle (artificial CSF) (n=9) or 100ng/h  
618 UE2316 (n=8) via ICV cannulas. Spatial memory was assessed by Y maze on day 9 of treatment. The  
619 initial 1 minute ITI was performed prior to surgery. Treatment with UE2316 increased the time spent  
620 exploring the novel arm during the 2 hour ITI compared with vehicle-treated controls (\*\*p=0.005 by  
621 Student's t-test).

622 **Figure 3: UE2316 improved fear-associated behaviour in the passive avoidance test in Tg2576**  
623 **mice.** Tg2576 and wild type mice (n=10 per group) were treated with vehicle or UE2316 at 10  
624 mg/kg/day by SC Alzet minipump infusion from the age of 14 months for 29 days. A: Tg2576 mice  
625 were assessed in the passive avoidance task on days 27 and 28 of drug treatment. The latency to enter  
626 the dark compartment was assessed in the training trial (pre) and the retention trial (post). UE2316  
627 increased latency to enter the dark compartment 6 hours post shock (2-way repeated measures  
628 ANOVA drug effect p<0.04; \*\*p<0.01 by Student's t test) to a greater extent in Tg2576 mice (by an  
629 increment of  $171.6 \pm 28.0$  seconds compared to  $42.6 \pm 21.5$  seconds in vehicle-treated mice,  
630 \*\*p=0.004 by Student's t-test). B: Open-field was performed after 23 days of treatment in vehicle and  
631 UE2316 treated wild type and Tg2576 mice. The percentage of time during the 5 minute trial that  
632 was spent in the inner zone of the open field apparatus was measured. There was no significant effect  
633 of treatment or genotype. C: Spontaneous alternation was assessed at day 26 of treatment. There was a  
634 trend for increased alternation with UE2316 treatment (2-way ANOVA; treatment: p=0.06).

635 **Figure 4: Long term administration of UE2316 improves cognition in Tg2576 mice.** Tg2576 mice  
636 were treated with either control diet (RM1, n=16) or RM1 supplemented with 175 ppm UE2316 for  
637 an estimated dose of 30 mg/kg/day (n=32) from the age of 6- 7 months for 57 weeks. A: Passive  
638 avoidance was analysed after 15 weeks of treatment with vehicle (n=13) or UE2316 (n=23). Both  
639 groups exhibited significantly increased latency to enter the dark compartment in the retention test,

640 indicating preserved cognitive function but there was no effect of UE2316 (by two-way ANOVA  
641 training vs retention effect  $p < 0.001$ , drug effect  $p = 0.26$ ; by Student's t-test  $*p = 0.03$ ,  $***p = 0.0007$ ). B:  
642 Passive avoidance was retested after 41 weeks of treatment with vehicle ( $n = 13$ ) or UE2316 ( $n = 23$ ) in  
643 chow. UE2316 but not vehicle treated mice demonstrated a significant increase in latency to enter the  
644 dark compartment in the retention test (by Student's t-test  $**p = 0.004$ ).

645 **Figure 5. Behavior of Tg2576 mice following long term administration of UE2316.** A: Open field  
646 was performed in control (vehicle:  $n = 5$ , UE2316:  $n = 5$ ) and Tg2576 mice (vehicle:  $n = 13$ , UE2316:  
647  $n = 23$ ) after 38 weeks of treatment with either normal chow or diet containing UE2316. The Tg2576  
648 mice spent more time in the inner zone than their wild type counterparts; however, there was no effect  
649 of drug administration (by two-way ANOVA,  $*p < 0.05$ ). B: Spontaneous alternation was assessed in  
650 Tg2576 mice after 39 weeks of vehicle ( $n = 13$ ) or UE2316 treatment ( $n = 23$ ). UE2316-treated mice  
651 exhibited a significant increase in percent alternation compared to control mice (by Student's t-test  
652  $*p = 0.04$ ). C: The spatial Morris Water Maze test was performed after 52 weeks of treatment. Mice  
653 that were able to find the platform in a visible platform test were tested in their ability to find the  
654 submerged platform using spatial cues located around the testing room. UE2316 treated mice ( $n = 9$ )  
655 exhibited significant decreases in latency to find the hidden platform across the testing period  
656 compared to vehicle treated animals ( $n = 6$ ) (2-way repeated measures ANOVA, drug effect  $p = 0.02$   
657 and interaction of drug with time  $p < 0.01$ ; by Student's t-tests  $*p < 0.05$  at days 3, 5 and 6).

658 **Figure 6: Effect of short- and long-term UE2316 administration on amyloid plaque burden in**  
659 **Tg2576 mice.** A: Short term plaque number. 6E10 positive amyloid plaques were counted in at least 5  
660 non-sequential sections per mouse treated for 29 days with vehicle or UE2316 ( $n = 10$  per treatment)  
661 via SC minipumps using the KS300 imaging program and the total number of positive plaques was  
662 expressed per area of the brain. UE2316 had no effect on plaque number in the hippocampus but  
663 decreased 6E10 staining in the cortices (Student's t-test,  $**p = 0.002$ ), amygdala (Student's t-test,  
664  $*p = 0.05$ ) and whole brain (Student's t-test,  $*p = 0.01$ ) in comparison to vehicle. B: Short term plaque  
665 area. Total plaque area of short term (29 day) treated mice was measured using the KS300 imaging  
666 program and was expressed as plaque area divided by the total area of the brain region in question.

667 The total plaque area in the cortex was decreased by UE2316 in comparison to vehicle (Student's t-  
668 test, \*\*\*p=0.0001). C: Long term plaque number. 6E10 positive amyloid plaques were counted in at  
669 least 5 non-sequential sections per mouse treated for 44 weeks with vehicle or UE2316 (n=5 per  
670 treatment) in the diet using the KS300 imaging program and the total number of positive plaques was  
671 expressed per area of the brain. UE2316 had no statistically significant effect on plaque number in the  
672 hippocampus or cortex in comparison to vehicle.

673 **Figure 7: Effects on brain pathology in Tg2576 mice treated with UE2316.** A: Representative  
674 brain sections from Tg2576 mouse showing amyloid plaques in brain regions stained with 6E10  
675 antibody. (a) cortex, vehicle treated (b) cortex, UE2316-treated (c) amygdala, vehicle-treated (d)  
676 amygdala, UE2316-treated (e) hippocampus, vehicle-treated (f) hippocampus, UE2316-treated. B:  
677 Representative western blot of cortex protein (30µg/sample) from 29 day treated mice. Quantitation  
678 was performed using the Odyssey Infrared imaging system and adjusted for beta-tubulin. IDE levels  
679 were increased in UE2316 treated cortices compared to vehicle treated tissues in both wild type and  
680 Tg2576 animals (see Table 1).

681

## 682 **Supplementary Data.**

683 **Supplementary Figure 1: Effects of short-term (29 day) treatment with UE2316 in wild type and**  
684 **Tg2576 mice on food intake and body weight.** Wild-type and Tg2576 mice were treated via 2  
685 subcutaneously implanted Alzet minipumps with either vehicle or 10 mg/kg/day UE2316 for 29 days  
686 (n=10 per treatment). A: Body weights were measured weekly in all mice. There was no effect of  
687 UE2316 on body weight although the Tg2576 mice weighed less than the controls (two-way repeated  
688 measures ANOVA, genotype effect p<0.001 and interaction with time p=0.001). B: Food was  
689 weighed weekly in all mice. Food intake was averaged as grams of food consumed/day. Tg2576 mice  
690 consumed more food but there was no effect of UE2316 (two-way repeated measures ANOVA  
691 genotype effect p=0.004).

692

693 **Supplementary Figure 2: Effects on food intake and body weight with long-term inhibition of**  
694 **11 $\beta$ -HSD1 in Tg2576 mice by UE2316 supplemented diet.** A: Estimated daily drug dosage was  
695 calculated weekly by measuring the food intake and comparing with body weight. The dose of  
696 UE2316 remained constant throughout the experiment. B: Individually housed mice treated with  
697 either control diet (RM1; n=16) or RM1 diet supplemented with 175 ppm UE2316 (RM1 + UE2316;  
698 n=32) were weighed weekly. 5 animals per group were culled after 45 weeks and the remainder  
699 continued until 57 weeks. By repeated measures ANOVA, there was an interaction of drug treatment  
700 with time only before 45 weeks ( $p < 0.01$ ) with UE2316 treated animals tending to be lighter in the  
701 early weeks of the study. C: Food intake was measured weekly in both groups and was averaged as  
702 grams of food/day. Food intake was higher in mice fed diet supplemented with UE2316 in the first  
703 few weeks of the study (by repeated measures ANOVA, drug interaction with time  $p < 0.001$ ; by  
704 Student's t-test  $*p < 0.05$  at week 10).

705 **Supplementary Figure 3: Effects on behavior and brain pathology in Tg2576 mice treated with**  
706 **UE2316.** The water maze probe test was performed 24 hours after the final spatial water maze trial.  
707 UE2316-treated Tg2576 mice (n=9) spent significantly more time exploring the target quadrant of the  
708 water maze than the vehicle treated mice (n=6) ( $*p = 0.02$ ).

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