

# 1 The therapeutic and diagnostic potential of amyloid $\beta$ oligomers (A $\beta$ Os) 2 selective antibodies to treat Alzheimer's disease

3 Running title: A $\beta$ O-antibodies for therapeutics and diagnostics

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20 Abbreviations: AD-Alzheimer's disease; A $\beta$ O-Amyloid  $\beta$  oligomer; CSF-cerebrospinal fluid; GFAP-glial  
21 fibrillar acidic protein; ICV-intracerebroventricular; MNS-magnetic nanostructures; MRI-magnetic  
22 resonance imaging; PET-positron emission tomography; NOR-novel object recognition; NLR-novel  
23 location recognition; PiB-Pittsburgh Compound B; pTau-phosphorylated tau; ThioS-thioflavin S; Tg-  
24 transgenic; WT-wild-type

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## Abstract

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Improvements have been made in the diagnosis of Alzheimer's disease (AD), manifesting mostly in the development of *in vivo* imaging methods that allow for the detection of pathological changes in AD by MRI and PET scans. Many of these imaging methods, however, use agents that probe amyloid fibrils and plaques - species that do not correlate well with disease progression and are not present at the earliest stages of the disease. Amyloid  $\beta$  oligomers (A $\beta$ Os), rather, are now widely accepted as the A $\beta$  species most germane to AD onset and progression. Here we report evidence further supporting the role of A $\beta$ Os as pathological instigators of AD and introduce promising anti-A $\beta$ O diagnostic probes capable of distinguishing the 5xFAD mouse model from wild type mice by PET and MRI. In a developmental study, A $\beta$  oligomers in 5xFAD mice were found to appear at 3 months of age, just prior to the onset of memory dysfunction, and spread as memory worsened. The increase of A $\beta$ Os is prominent in the subiculum and correlates with concomitant development of reactive astrocytosis. The impact of these A $\beta$ Os on memory is in harmony with findings that intraventricular injection of synthetic A $\beta$ Os into wild type mice induced hippocampal dependent memory dysfunction within 24 hours. Compelling support for the conclusion that endogenous A $\beta$ Os cause memory loss was found in experiments showing that intranasal inoculation of A $\beta$ O-selective antibodies into 5xFAD mice completely restored memory function, measured 30-40 days post-inoculation. These antibodies, which were modified to give MRI and PET imaging probes, were able to distinguish 5xFAD mice from wild type littermates. These results provide strong support for the role of A $\beta$ Os in instigating memory loss and salient AD neuropathology, and they demonstrate that A $\beta$ O selective antibodies have potential both for therapeutics and for diagnostics.

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KEYWORDS: A $\beta$  oligomers; Alzheimer's disease; 5xFAD; MRI; PET; diagnostics; therapeutics

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## Introduction

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### General Alzheimer's disease

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More than 6 million Americans are currently living with Alzheimer's disease (AD), and Alzheimer's-related deaths have increased 145% from 2000 to 2019 (2021). The financial burden is even more staggering - Alzheimer's and other dementias have cost the US more than \$600 billion in medical expenses and unpaid care in 2021 (2021). Despite the great personal and economic burden, progress toward developing effective diagnostics and therapeutics remains slow. Aduhelm® (also known as Aducanumab) was recently approved as a treatment for AD (Investor Relations, 2021), the first in more than a decade, but it still focuses on A $\beta$  elimination rather than specific A $\beta$ O targets. As AD burden is expected to increase drastically with the aging population, improved diagnostics and therapeutics are more urgent now than ever.

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### A $\beta$ Os as a biomarker for early Alzheimer's disease

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The primary pathological hallmarks of Alzheimer's disease are extracellular amyloid plaques and intraneuronal tangles of hyperphosphorylated tau (Masters et al., 1985). It is well known, however, that amyloid plaques do not correlate well with cognitive decline in AD (Terry et al., 1991; Hsia et al., 1999; Lee et al., 2004) and are not present in the earliest stages of the disease (Nyborg et al., 2013). Research from the previous two decades strongly indicates that soluble amyloid beta oligomers (A $\beta$ Os), not plaques, are the more appropriate amyloid beta species to target in AD (Ashe, 2020; Hampel et al., 2021).

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A $\beta$ Os are potent neurotoxins that show AD-dependent accumulation in the brain of AD patients (Gong et al., 2003; Kayed et al., 2003; Lacor et al., 2004) and transgenic (Tg) rodent AD models (Chang et al., 2003; Lesne et al., 2006; Ohno et al., 2006). For reviews of other perspectives regarding AD molecular etiology, see (Braak and Del Tredici, 2011; Robakis, 2011; Lasagna-Reeves et al., 2012). A $\beta$ Os begin to accumulate early in AD, decades prior to symptoms, and are widely held to be the neurotoxic instigators of AD (Rodgers, 2005; Gandy et al., 2010; Schnabel, 2011; Mucke and Selkoe, 2012). A $\beta$ Os have been shown to exert their toxic effects by instigating failure of synaptic plasticity and memory (Lambert et al., 1998; Wang et al., 2002; Lesne et al., 2006; Townsend et al., 2006). Recently, soluble cortical extracts were examined by ELISA and showed that the ratio of A $\beta$ O levels to plaque density fully distinguished demented from non-demented patients (Esparza et al., 2013); simply put, those with high A $\beta$ O to plaque ratios were demented and low A $\beta$ O to plaque ratios were not.

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### The 5xFAD mouse model

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The 5xFAD transgenic mouse is an increasingly used AD model that harbors gene mutations in amyloid  $\beta$  protein precursor (A $\beta$ PP) (K670N/M671L + I716V + V717I) and presenilins (PS1/2) (M146L + L286V) (Oakley et al., 2006). These mutations are known to increase production of A $\beta$ 42, characteristic of familial AD, and exhibit expedited plaque development compared to other transgenic mice (Oakley et al., 2006). The Mutant Mouse Resource Research Center (MMRRC) found that A $\beta$  accumulation occurred at different rates, depending on the breeding background, with mice bred on a B6SJL background developing pathology at a significantly more rapid rate (unpublished, available at [MMRRC 5xFAD strain data](#)) than those bred on a C57 background. The 5xFAD mouse model is well characterized for memory impairments (Oakley et al., 2006; Kimura and Ohno, 2009; Ohno, 2009; Girard et al., 2013; Girard et al., 2014; Zhang et al., 2021a), neuron loss (Jawhar et al., 2012; Oblak et al., 2021), and A $\beta$  plaque accumulation (Devi et al., 2010; Jawhar et al., 2012; Ashe, 2020; Zhang et al., 2021a). Comprehensive studies on the 5xFAD model have also looked at cholesterol and glucose levels (Oblak et al., 2021), activity levels (Oblak et al., 2021), neuroinflammation-related protein levels (Ou-Yang and Van Nostrand, 2013; Oblak et al., 2021), tau phosphorylation (Shao et al., 2011; Kanno et al., 2014), and visual acuity (Zhang et al., 2021a).

## 96 Alzheimer's disease diagnostics

97 Recommended tests ([Alzheimer's Disease Diagnostic Guidelines | National Institute on Aging \(nih.gov\)](#))  
98 for diagnosing Alzheimer's disease include a standard health evaluation and MMSE evaluations. If  
99 indicated, these tests are typically followed with cerebrospinal fluid (CSF) assays for tau and A $\beta$  levels,  
100 MRI for brain volume and functionality, and positron emission tomography (PET) scans for A $\beta$  plaques,  
101 glucose metabolism, and/or tau fibrils in the brain (Albert et al., 2011; Jack et al., 2011; McKhann et al.,  
102 2011; Sperling et al., 2011). These analyses may rule out other dementia etiologies and help to  
103 determine disease severity, but they cannot detect AD at its earliest stages or closely predict disease  
104 progression, as they do not probe for AD's earliest biomarkers.

## 105 Current diagnostic methods in development

106 Spinal taps are invasive, but cerebrospinal fluid assays show promise (Georganopoulou et al., 2005;  
107 Toledo et al., 2013b). Nonetheless, assays using CSF analytes have presented challenges with respect  
108 to accuracy and reliable disease-state discrimination (Slemmon et al., 2012). More recently, assays for  
109 A $\beta$ O levels in the blood plasma have been developed with promising results (Meng et al., 2019). These  
110 assays show a correlation between A $\beta$ O levels and declining memory scores that appear not to be  
111 influenced by age, gender, or ApoE4 status. A promising addition to diagnostic methodology is the  
112 detection of AD pathology using targeted *in vivo* brain imaging. The introduction of PET probes for  
113 amyloid plaques has been a great technical advance (Klunk et al., 2004) and has established  
114 precedent for the usefulness of brain molecular imaging as a diagnostic tool and for proof of efficacy  
115 studies in drug development (Johnson et al., 2013). Still, these new imaging tools focus on late-stage  
116 by-products of AD such as plaques, rather than early stage instigators such as A $\beta$ Os.

117 Prior studies using 5xFAD mice have examined early- and late-stage disease development, but none  
118 have looked at the progressive development of A $\beta$ Os in this model. Here, we present an analysis of  
119 memory impairment from 2-9 months of age and the progressive accumulation of A $\beta$ Os across the  
120 same age-span. Our studies presented here use an A $\beta$ O-selective antibody to characterize the  
121 spatiotemporal development of A $\beta$ Os in the 5xFAD mouse model and demonstrate a correlation with  
122 memory impairment. Strikingly, intranasal inoculation of the A $\beta$ O-selective antibody rescued memory  
123 performance in 6-month-old 5xFAD mice. We demonstrate the capability of detecting A $\beta$ O pathology *in*  
124 *vivo* in the 5xFAD mouse by introducing molecular imaging modalities (MRI and PET) with probes for  
125 A $\beta$ Os. We additionally present immunofluorescent evidence of a remarkable association between  
126 A $\beta$ Os and GFAP-positive reactive astrocytes in the 5xFAD mice. Taken together, we provide further  
127 data implicating A $\beta$ Os as essential diagnostic indicators and therapeutic targets, and show evidence  
128 suggesting a mechanism through which A $\beta$ Os instigate pathological abnormalities: by induction of  
129 reactive astrogliosis.

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131 **Materials and Methods**

132 **Materials**

133 ACU193 humanized anti-A $\beta$ O antibody was a generous gift from Acumen Pharmaceuticals, Inc. A $\beta$ <sub>1-42</sub>  
134 (TFA preparation) was sourced from multiple suppliers (California Peptide, Peptides International,  
135 American Peptide). Primary hippocampal cultures were prepared from tissue obtained from BrainBits,  
136 LLC, using media and reagents also obtained from BrainBits. All chemicals were purchased from  
137 Sigma unless otherwise specified.

138 **Animals**

139 The 5xFAD Tg mouse model (B6SJL-Tg(APPSwF1Lon,PSEN1\*M146L\*L286V)6799Vas)(Oakley et al.,  
140 2006) (Jackson Laboratories) was bred on a non-transgenic background (B6SJLF1/J mice (Jackson  
141 Laboratories, RRID: IMSR\_JAX:100012)). Aged transgenic and wild-type littermates, 2-20 months old,  
142 were used. All mice were kept under a 12/12 h light/dark cycle (7 AM/7 PM) at 22 ± 2 °C. Mice had free  
143 access to food and water, including during behavioral experiments, were housed at ≤5/cage (NexGen  
144 IVC, Allentown) with enriched environment and daily veterinarian assessment, according to NU's  
145 standard procedures. Procedures complied with NIH's Guide for the Care and Use of Laboratory  
146 Animals (NIH publication No. 80-23, 1996) and were approved by IACUC (protocol #IS00004010).  
147 Behavioral experiments were conducted between 12-6 PM.

148 For intracerebroventricular (icv) experiments, B6SJLF1/J mice (Jackson Laboratories, RRID:  
149 IMSR\_JAX:100012) were utilized at ages ranging from 6 months of age (30-50 g).

150 **A $\beta$  Oligomer Preparation**

151 Unlabeled (A $\beta$ Os) and fluorescently-labeled A $\beta$  oligomers (FAM-A $\beta$ Os) were prepared essentially  
152 according to the protocol published by Klein and colleagues (Lambert et al., 2007; Velasco et al., 2012).  
153 Briefly, A $\beta$ <sub>1-42</sub> (American Peptide or Peptides International) or FAM-A $\beta$ <sub>1-42</sub> (Anaspec) was dissolved in  
154 hexafluoro-2-propanol (HFIP) and distributed into microcentrifuge tubes. Hexafluoro-2-propanol was  
155 removed by evaporation and traces removed under vacuum; the tubes were stored at -80°C. For  
156 unlabeled A $\beta$ Os, an aliquot of A $\beta$ <sub>1-42</sub> was dissolved in anhydrous dimethyl sulfoxide (DMSO) to ~5 mM,  
157 and diluted in ice-cold Ham's F12 medium without phenol red (Caisson Laboratories) to 100  $\mu$ M. For  
158 FAM-A $\beta$ Os, an aliquot of each peptide was dissolved in anhydrous dimethyl sulfoxide (DMSO) to ~5  
159 mM, mixed 5:1 (mol: mol) A $\beta$ : FAM-A $\beta$ , and diluted in ice-cold Ham's F12 medium without phenol red  
160 (Caisson Laboratories) to 100  $\mu$ M. For both A $\beta$ O preparations, this solution was incubated at 4°C for 24  
161 hr. and centrifuged at 14 000 g for 10 min. The supernatant, defined as the A $\beta$ O or FAM-A $\beta$ O  
162 preparation, was transferred to a clean microfuge tube and stored at 4°C until use. Protein  
163 concentration was determined using Coomassie Plus protein assay kit (Pierce).  
164 A modification of this protocol was used to produce crosslinked A $\beta$ Os (Cline et al., 2019b).  
165 All preparations were tested for quality using SDS-PAGE on a 10-20% Tris-Tricine gel followed by both  
166 silver stain and Western blot with NU2 anti-A $\beta$ O antibody (Lambert et al., 2007; Velasco et al., 2012).

167 **Cell Culture**

168 Hippocampal cells were prepared and maintained for at least 18 days as previously described (Gong et  
169 al., 2003) by using (0.002%) poly-L-lysine coated coverslips plated at a density of 1.04 x 10<sup>4</sup> cells per  
170 cm<sup>2</sup> in Neurobasal media (Brainbits, LLC) with B27 supplements and L-glutamine (2.5  $\mu$ M).

171 **A $\beta$  Oligomer Incubation and Immunolabeling of Cells**

172 Cells were incubated at 37°C in conditioned media collected from the cell cultures containing  
173 crosslinked A $\beta$ Os or FAM-A $\beta$ Os or an equivalent dilution of vehicle. Following incubation with A $\beta$ Os or  
174 vehicle for 60 min, the cells were rinsed rapidly 3 times with warm media then fixed by adding an equal

175 volume of warm 3.7% formaldehyde (in PBS) to the third rinse in each well/dish and allowing it to sit at  
176 RT for 5 min. The media/formaldehyde was completely removed and replaced with a volume of 3.7%  
177 formaldehyde for 5 min at RT. Cells were blocked in 10% normal goat serum (NGS) in PBS or HBSS  
178 for 45 min at RT then incubated overnight at 4°C on an orbital shaker with fluorescent-tagged antibody  
179 or anti-A $\beta$ O probe diluted in blocking buffer. The cells were washed 3 times for 5 min each with PBS or  
180 HBSS. After secondary antibody incubation, coverslips were mounted onto glass slides using ProLong  
181 Gold Anti-fade reagent with DAPI (Invitrogen) and imaged using an epifluorescence (TE2000, Nikon), a  
182 widefield fluorescence microscope (Leica DM6B, Leica Corp.), or confocal microscope (Leica SP2,  
183 Leica Corp).

#### 184 A $\beta$ O intracerebroventricular (icv) administration in mice

185 Icv injections and behavior testing were performed in 4 independent experiments of 13-21 mice each.  
186 Littermates were arbitrarily assigned to different injection groups, targeting 5-10 mice/group for  
187 statistical power ( $n = ((Z_{\alpha/2} * \sigma)/E)^2$  at  $\alpha = 0.05$ ;  $\sigma = 10.55$  and  $E = 6.67$  derived from pilot studies).

188 Mice were lightly anesthetized (2% isoflurane) during injection (~1 min). A $\beta$ Os (1, 10 pmol in 3  $\mu$ l) or  
189 vehicles were administered icv free-handed (Bicca et al., 2015). Separate needles were used for each  
190 vehicle, progressing from low-high A $\beta$ O concentration to minimize carryover. No analgesics or anti-  
191 inflammatory agents were necessary. Mice were monitored constantly for recovery of consciousness  
192 and ambulation, then periodically for food-and-water intake until behavior analysis. Needle placement  
193 was confirmed by brain dissection after behavioral experiments (euthanization: CO<sub>2</sub> then decapitation).  
194 Mice showing needle misplacement (3 mice) or cerebral hemorrhage (2 mice) were excluded from  
195 analysis; final  $n = 5-7$  mice/group.

#### 196 Object Recognition/Location Recognition (NOR/NLR) Tasks

197 Tasks were performed essentially as described (Bicca et al., 2015), to evaluate mouse ability to  
198 discriminate between familiar and new, or displaced, objects within an area, measured by object  
199 exploration (sniffing, touching). The open-field testing arena was constructed of gray polyvinyl chloride  
200 at 21x21x12" (WxLxH), with a 5x5 square grid on floor and visual cue on wall. 24 h post-injection, mice  
201 underwent 6 min sessions of habituation and training, with 3 min between. All sessions were video  
202 recorded and analyzed by two researchers blind to experimental groups. During habituation and  
203 training, mice were screened for ability to move about the arena and explore the objects, two activities  
204 required for accurate memory assessment in subsequent testing sessions. Locomotive inclusion criteria  
205 (>100 grid crossings and >15 rearings; evaluated in habituation) were based on extensive previous  
206 experiments with the same mouse strain and arena; 3/65 mice did not meet this criterion. During  
207 training, mice were placed at the arena center with two objects, which were plastic and varied in shape,  
208 color, size and texture. Exploration inclusion criteria were low exploration (<3 sec total) or object  
209 preference (>50% of total time for either object); 7 of remaining 62 mice did not meet this criterion.

210 Hippocampal-related memory function was assessed 24 h post-training by displacing one of the two  
211 training objects. Cortical-related memory function was assessed 24 h later by replacing the displaced  
212 object with a novel object. Hippocampal-related memory function was re-tested 31-38 days post-  
213 injection by displacing the novel object. Memory dysfunction was defined as an exploration of the  
214 familiar object for >40% total time. Mice were arbitrarily assessed by cage. The arena and objects were  
215 cleaned thoroughly between sessions with 20% (v/v) alcohol to minimize olfactory cues.

#### 216 Immunolabeling of slices

217 Free floating 45  $\mu$ m thick sagittal sections were cut using a Leica SM2010 R sliding microtome and  
218 transferred to sterile TBS for storage. Sections were gathered and placed sequentially into wells (~4  
219 per well). Sections were then randomly selected from each well to perform antibody staining using the  
220 primary antibodies ACU193 (0.2  $\mu$ g/ml), Alexa Fluor® 555-conjugated NU4 (0.92  $\mu$ g/ml), Cy3-  
221 conjugated anti-GFAP (1:800, Sigma) and the secondary antibody Alexa Fluor® 633 goat anti-human

222 IgG (1:2000, Invitrogen). Floating slices were rinsed 3x10 min with TBS and blocked with blocking  
223 buffer (10% NGS with 0.3% Triton X-100 in TBS) for 60 min at room temperature. Slices were then  
224 incubated with the respective antibodies in blocking buffer overnight at 4°C with gentle rotation.  
225 Sections were washed 3 x 10 mins in TBS and incubated with secondary antibody for 3 hours at room  
226 temperature (RT) with orbital agitation in the dark. Secondary was prepared in blocking buffer diluted  
227 10-fold with TBS. Sections were then washed 3 x 10 mins in TBS, mounted using ProLong Diamond®  
228 antifade mounting media with DAPI (Invitrogen) and 24x60mm No.1.5 glass coverslips (Thermo  
229 Scientific). Z-stacks of the brain sections were collected at 10x or 100x on a Leica SP5 confocal  
230 microscope and analyzed with ImageJ.  
231

### 232 **Thioflavin S counterstain.**

233 Thioflavin-S counterstaining to NU4 immunofluorescence labeling was performed as previously  
234 described (Guntern et al., 1992) with a few modifications (Viola et al., 2015). 5xFAD and WT brains  
235 were sliced at a thickness of 50µm and immunolabeled following the same protocol described above  
236 (immunolabeling of slices). Slices were incubated with antibody as described above. The slices were  
237 then washed with PBS for 5 times 5 min each and incubated with 0.002% of Thioflavin-S solution in  
238 TBS-T (diluted from a stock solution 0.02% of Thioflavin-S in distilled water) for 10min. Slices were  
239 then washed 3 times for 1 min in 50% ethanol and 2 times in TBS-T for 5 min. The slices were mounted  
240 with ProLong Gold Antifade reagent for examination by fluorescence microscopy. Images were  
241 acquired at 40x magnification and analyzed by ImageJ software.

### 242 **Radiolabeling and Quality Control**

243 Antibodies, NU4 and non-specific mouse IgG or ACU193 and non-specific human IgG were  
244 radiolabeled with positron emitter  $^{64}\text{Cu}$  ( $^{64}\text{CuCl}_2$  in 0.1 M HCl; radionuclide purity >99%, Washington  
245 University). For radiolabeling, Wipke and Wang's method was applied (Wipke et al., 2002). Basically,  
246 antibodies mentioned above were conjugated with DOTA-NHS-ester (Macrocyclics, Dallas, TX) and  
247 then radiolabeled with  $^{64}\text{Cu}$ .

#### 248 **Conjugation.**

249 Antibody solutions were buffer exchanged with PBS using YM-30 Centricon® centrifugal filters  
250 (Millipore, Billerica, MA). For conjugation, antibodies were reacted with DOTA-NHS-ester in 0.1 M  
251  $\text{Na}_2\text{HPO}_4$  buffer of pH 7.5 at 4°C for 12 - 16 h in a molar ratio of DOTA-NHS-ester:antibody = 100: 1.  
252 After conjugation, the reaction mixture was centrifuged repeatedly (5 times) through a YM-30  
253 Centricon® centrifugal filter with 0.1M pH 6.5 ammonium citrate buffer to remove unconjugated small  
254 molecules. The concentrations of purified antibody-conjugate was determined by measuring the  
255 absorbance at 280 nm in a UV spectrophotometer.

#### 256 **Labeling.**

257 When labeling with  $^{64}\text{Cu}$ , 1 mg DOTA-conjugated NU4 and 5 mCi (185 MBq) of  $^{64}\text{Cu}$  as incubated in  
258 0.1 M ammonium citrate buffer, pH 6.5, at 43°C for 1 hour. Labeled antibody was separated by a size-  
259 exclusion column (Bio-Spin6, BIO-RAD Laboratories).

#### 260 **Quality Control.**

261 Radiochemical purity of antibody was determined by integrating areas on the Fast Protein Liquid  
262 Chromatography (FPLC) equipped with a flow scintillation analyzer. This analysis was conducted on a  
263 Superose 12 10/300 GL (Cytiva) size-exclusion column and characterized by the percentage of  
264 radioactivity associated with the 150 kDa protein peak. The stability of the  $^{64}\text{Cu}$  radiolabeled mAbs was  
265 determined by bovine serum challenge at 44 hours.

#### 266 **Conjugation efficiency.**

267 Based on our preliminary data, > 90% of conjugation rate, >70% of labeling rate is achieved by  
268 following prescribed protocol.

269 **Overall details of micro PET and micro CT acquisition**

270 Mice were placed in a 37.5 °C heated cage 20-30 minutes prior to radiotracer injection and moved to a  
271 37.5 °C heated induction chamber 10 minutes prior to injection where they were anesthetized with 2-  
272 3% isoflurane in 1000 cc/min O<sub>2</sub>. A dose of 40 µg/200 µCi in 100 µL of proposed PET tracers was  
273 administered intravenously through the tail vein. Each animal was administered a dose ranging from  
274 30-40 µg NU4PET, ACU193PET, or non-immune IgGPET. Probes were administered in a single dose.  
275 PET/CT imaging was conducted at 0, 4, 24, and 48 h to measure for changes in distribution and time  
276 required for probe clearance or decay.

277 NU4PET scans were acquired using a Genisys<sup>4</sup> PET (Sofie Biosciences, Culver City, CA) system and  
278 CT scans were acquired using a Bioscan NanoSPECT/CT (Washington, D.C.). When scanning, all  
279 mice were placed prone on the bed of the scanner. A 10 minute static acquisition was used for PET  
280 imaging followed immediately by a 6.5 minute CT acquisition both utilizing the mouse imaging chamber  
281 from the Genisys<sup>4</sup>. PET reconstruction was performed without attenuation correction using 3D  
282 Maximum Likelihood Expectation Maximization (MLEM) with 60 iterations and CT reconstruction used  
283 Filtered Back Projection with a Shepp-Logan Filter. PET and CT reconstructions were exported in  
284 dicom image format and fused using custom software developed by the Small Animal Imaging Facility  
285 at Van Andel Institute. Fused PET/CT images were analyzed using VivoQuant Image Analysis Suite  
286 (inviCRO, LLC, Boston, MA). Standardized Uptake Values (SUV) were calculated using the mouse  
287 body weight and corrected for residual dose in the injection syringe and the injection site, as applicable.  
288 The formula used to calculate SUV was

289 
$$SUV = \frac{\text{Activity}_{\text{tissue}}/\text{Volume}_{\text{tissue}}}{\text{Injected Activity}/\text{BodyWeight}}.$$

290 **Evaluation NU4PET (<sup>64</sup>Cu-NU4) in AβOs detection**

291 Two groups (n = 3/ group) of 6 months old 5xFAD Tg AD mouse model and 2 groups (n = 3/ group) WT  
292 mouse model were used for evaluating the capability of AβOs detection. NU4PET (<sup>64</sup>Cu-NU4) or non-  
293 specific IgGPET (<sup>64</sup>Cu-IgG) was injected into each 5xFAD Tg AD mouse model and WT mouse model  
294 groups, respectively.

295 Target (AβOs)-Background (normal tissue) contrasts in PET images were used to distinguish the  
296 difference of the capability of AβOs detection between NU4PET and IgGPET in different mouse  
297 models. Tracer uptake of high intensity (hot) areas and background tissues in the brain were chosen by  
298 drawing regions-of-interest (ROI) along the edges of the areas from the PET images. Average pixel  
299 values of each ROIs were acquired and use in Target (AβOs)-Background (normal tissue) contrasts  
300 calculation. The formula used to calculate Target-Background contrast was

301 
$$T - B \text{ Contrast} = \frac{\text{Target Average Pixel Value}}{\text{Background Average Pixel Value}}.$$

302 **Tissue Biodistribution Assessment**

303 Animals were sacrificed immediately after the 44 hour post injection image was acquired. Blood was  
304 collected, while brains and 13 other organs and tissues were harvested and weighed. After the blood  
305 sample was taken from the heart (~500-1000µl), 10 ml of saline was injected into left ventricle while the  
306 heart was still beating to flush out the residual blood in the organs. Radioactivity in each tissue (cpm)  
307 wa measured using the γ-scintillation counter. Percentages of the injected dose/gram (%ID/g) were  
308 calculated for each tissue/ organ by the following formula.

$$\% ID/g = \frac{(\text{Sample Activity} - \text{Background})}{(\text{Injected Activity} - \text{Background})(\text{Sample weight}(g))} \times 100\%$$

309 Student's t-test was conducted to the results between different groups. P<0.05 is considered  
310 statistically significant.

311 **Synthesis of Magnetic Nanostructures (MNS)**

312 16 nm magnetite nanoparticles were synthesized by decomposition of iron-oleate at 320°C as  
313 described in an earlier report.(Park et al., 2004)

314 *Synthesis of Iron-oleate complexes:* 10.8 g of iron (III) chloride hexahydrate and 36.5 g sodium oleate  
315 were dissolved in a mixture of 60 ml distilled water, 80 ml ethanol and 140 ml hexane and heated at  
316 60°C for 4 hr. The organic layer of the biphasic mixture becomes dark, indicating phase transfer of iron  
317 (III) ions and formation of iron oleate complex. The resulting dark solution is separated and washed with  
318 water three times.

319 *Synthesis of 16 nm magnetite nanoparticles:* 18 g of iron oleate complex and 2.58 g of oleic acid were  
320 dissolved in 100 g of octadecene at room temperature and heated to 320°C at a rate of 3.3°C per  
321 minute. The reaction mixture is kept at 320°C for 40 min., then cooled down to room temperature.  
322 Resulting nanoparticles are separated from the solution by addition of ethanol and ethyl acetate  
323 followed by centrifugation.

324 **Preparation of Dopamine-TEG-COOH and Phase Transfer**

325 To make the organic phase synthesized MNS suitable for biological application, we functionalized the  
326 MNS using an in-house synthesized ligand with carboxylate as terminal group (for antibody  
327 conjugation), tetraethylene glycol(TEG) as a stabilizer, and nitrodopamine (nDOPA) as an anchor due to  
328 its high affinity for Fe (Nandwana et al., 2016).

329 Synthesis of carboxylate terminated nDOPA ligand and functionalization of the MNS was carried out  
330 according to the following protocol. Tetraethylene diacid, N-hydroxysuccinimide (NHS), N,N'-  
331 Dicyclohexylcarbodiimide (DCC), nDOPA hydrochloride and anhydrous sodium bicarbonate was  
332 dissolved in chloroform under argon atmosphere and stirred for 4 hr. Hexane stabilized MNS were  
333 added and stirred for another 24 hr. The precipitate formed was separated by magnet, dispersed in  
334 water and purified by dialysis.

335 **Conjugation of antibody to MNS**

336 The conjugation of buffer stabilized MNS with antibody was done using a conventional carboxyl-amine  
337 crosslinking method. We first activated the carboxyl terminated MNS by sulfo-N-hydroxy succinimide  
338 (SNHS) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) followed by incubation with  
339 corresponding antibody (NU4 or IgG<sub>1</sub>, with or without fluorescent label) overnight. Conjugated MNS  
340 were separated by magnet to remove excess reagent and antibody then re-dispersed in working media.  
341 Conjugation efficiency was estimated using UV spectroscopy (absorbance at 280nm) of the  
342 magnetically separated supernatant.

343 Ab conc. = (total mg added Ab) - (mg Ab in supernatant)

344 **Intranasal immunization.**

345 Mice were anesthetized with isoflurane and then placed on their backs with their heads positioned to  
346 maximize the residency time for the delivered material to remain on the olfactory surface. Each naris  
347 was administered with ACUMNS or non-immune IgGMNS (10  $\mu$ l/naris), using a sterile micropipette,  
348 slowly over a period of 1 min, keeping the opposite naris and mouth closed to allow complete aspiration  
349 of delivered material. Steps were repeated up to 5 times, maintaining anesthetization in between  
350 inoculations, for maximum doses of up to 50 $\mu$ l/naris

351 **Magnetic Resonance Imaging of Tg and WT mice in vivo**

352 Following intranasal inoculation, the probe was allowed to distribute for 4 hours before MR imaging was  
353 performed according to imaging methodology described in Mundt et al.(Mundt et al., 2009) T1, T2, and  
354 T2\* weighted MR images were acquired on a Bruker BioSpec 9.4T magnet, using a 25 mm RF  
355 quadrature coil. The in-plane resolution was 75  $\mu$ m with slice thickness 0.4 mm. T1- and T2-weighted  
356 images provide anatomical guidance as well as some localization of the ACUMNS and were acquired

357 with a fat suppressed spin echo sequence (Rapid Acquisition with Relaxation Enhancement, RARE)  
358 with the following parameters for T1-weighted (TR=1000 ms, TEeff=13.2 ms, rare factor 2, number of  
359 excitations, NEX=4) and for T2-weighted (TR=3500 ms, TEeff=58.5 ms, rare factor 4, NEX=4). T2\*-  
360 weighted imaging provides more of the localization of the NU4MNS as the iron causes local changes in  
361 magnetic susceptibility which T2\* weighted images can be sensitive to. A gradient echo sequence was  
362 used with the following parameters (gradient echo fast imaging, GEFI; TR=1200 ms, TE=5.6 ms, flip  
363 angle 35° and NEX=4).

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## Results

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### Memory dysfunction in 5xFAD mice begins shortly after A $\beta$ O emergence and progressively worsens with concomitant A $\beta$ O accumulation in the hippocampus

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#### Tg 5xFAD NOR/NLR

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Amyloid plaque development and intraneuronal A $\beta$ 42 accumulation are well-established in the 5xFAD transgenic (Tg) mouse model of Alzheimer's disease. There is robust plaque buildup around 5-6 months of age (Ohno et al., 2006) and intraneuronal A $\beta$ 42 accumulation begins as early as 2 months (Oakley et al., 2006). The majority of neuropathological studies in 5xFAD mice have used probes that show amyloid plaque development; how 5xFAD memory impairment coincides with A $\beta$ Os pathology and development is much less well-characterized. In order to characterize how memory loss correlates with A $\beta$ Os in the 5xFAD mice, we used the well-established novel object recognition task (NOR) for non-spatial (cortical) memory (Cohen and Stackman, 2015; Denninger et al., 2018) and the novel location recognition task (NLR) for spatial (hippocampal) memory (Antunes and Biala, 2012; Bengoetxea et al., 2015; Grayson et al., 2015; Denninger et al., 2018). We assessed memory in mice aged 2-18 months. 5xFAD mice showed no evident memory impairment at 2 to 3 months old (Figure 1a). By 4 to 5 months old, most transgenic mice showed memory impairment, and by 6 to 7 months of age memory impairment was apparent in all 5xFAD mice. Importantly, at 4 months old, the majority of 5xFAD mice were impaired in both the hippocampal-dependent and cortical-dependent tasks; there were, however, some mice that showed only cortical-impairment. Though less obvious than their Tg littermates, memory loss was detected at 9 months of age in wild-type mice. In summary, we showed that 5xFAD mice first present memory impairment between 3 and 4 months of age. This memory dysfunction afflicts more mice as their age increases until, at 6 to 7 months, all of the Tg mice are impaired in both hippocampal-dependent and cortical-dependent tasks. These data indicate that memory impairment begins before observed amyloid plaque build-up in the 5xFAD mice.

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#### Immunohistofluorescence validation of A $\beta$ O development

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The development of amyloid plaque pathology is well-established in the 5xFAD mouse model (Oakley et al., 2006; Ohno et al., 2006). Amyloid plaques, however, are no longer considered the most germane A $\beta$  species to AD pathology (Overk and Masliah, 2014; Viola and Klein, 2015; Selkoe and Hardy, 2016; Cline et al., 2018; Li and Selkoe, 2020). Characterizing the development of the most relevant species, putatively A $\beta$ Os, and their association with other pathological changes in AD, such as glial activation or pTau accumulation, is necessary to better understand disease progression in this model. Sagittal sections of brain tissue, collected and fixed from WT and 5xFAD mice at ages 2, 3, 4, 6, and 8 months of age, were immunolabeled with ACU193 and imaged using confocal microscopy. ACU193, a humanized monoclonal antibody that targets A $\beta$ Os, has been shown to selectively bind oligomers *in vitro* (Krafft et al., 2013; Goure et al., 2014; Savage et al., 2014) and in the TG2576 mouse model. Here, using ACU193 to probe for A $\beta$ Os, we show the progressive, spatio-temporal accumulation of A $\beta$ Os in the hippocampus of 5xFAD mice (Figure 1b). A $\beta$ Os first appear in the subiculum as early as 2 months of age in some mice and are detectable by 3 months in all 5xFAD Tg mice examined. In the transgenic mice, A $\beta$ Os show a continued accumulation in the subiculum and a spreading of pathology to CA1, CA2 and the dentate gyrus. This timing suggests that A $\beta$ Os are associated with the observed memory loss.

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#### ACU193 detects A $\beta$ Os bound to primary neurons with high specificity

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To validate the specificity of ACU193 for A $\beta$ Os, the antibody was used *in vitro* to detect synthetic preparations of oligomers introduced into primary hippocampal neurons in culture (Supplemental Figure 1). Primary hippocampal neurons were treated with cross-linked A $\beta$ Os, which have been shown to preserve A $\beta$ O structure *in vitro* (Cline et al., 2019b), or vehicle control. The cells were subsequently fixed and labeled with ACU193 at increasing dosages. Confocal imaging of the cells showed somatic staining of A $\beta$ Os in addition to small, nanoscale puncta along dendritic processes (labeled with MAP2).

413 These ACU193-positive puncta are likely A $\beta$ Os binding to dendritic spines, as seen in previously  
414 published work (Lacor et al., 2007; De Felice et al., 2009; Pitt et al., 2017). Minimal ACU193 labeling  
415 was observed on vehicle-treated neurons, indicating its specificity for A $\beta$ Os.

#### 416 **ACU193 and NU4 detect A $\beta$ Os**

417 Additional support for the specificity of ACU193 can be seen in comparing the distribution of ACU193 in  
418 brain sections with the distribution of NU4, a well-established A $\beta$ O monoclonal antibody (Lambert et al.,  
419 2007; Xiao et al., 2013; Viola et al., 2015). Using ACU193 and NU4 conjugated to Alex Fluor 555 we  
420 found that both antibodies similarly detected A $\beta$ Os in the subiculum and other areas of the  
421 hippocampus (Figure 2) including CA1, CA2 and the dentate gyrus. ACU193- (cyan) and NU4-positive  
422 (magenta) cells were observed accumulating in a nearly identical pattern, from 3 months to nine  
423 months of age. ACU193 and NU4 selectively detect A $\beta$ Os in the 5xFAD mice with virtually no signal in  
424 WT mice.

#### 425 **Alzheimer's-associated astrocyte pathology develops concomitantly with A $\beta$ Os**

426 To determine whether other Alzheimer's related pathologies show developmental regulation or  
427 accumulation in the 5xFAD mouse model for AD in association with A $\beta$ Os, we examined  
428 immunohistochemical patterns of glial fibrillary acidic protein (GFAP), activated microglia (Iba1), and  
429 phosphorylated tau (pTau). Immunolabeling for pTau yielded difficult to interpret results which varied  
430 amongst the different antibodies for the same epitope and often did not match the literature. Instead,  
431 we focused on the inflammatory pathways, stimulated by the strong interest in the involvement of  
432 inflammatory responses in AD, in particular a new and growing interest in astrocytes (Wang et al.,  
433 2021). Immunolabeling for activated microglia (Iba1) (Supplemental Figure 2) indicated that the WT  
434 mice have more ramified microglial cells (resting) while 5xFAD littermates have more amoeboid and  
435 activated-shaped microglial cells. Notably, microglial activation was evident at 2 months, with no  
436 obvious increase in abundance seen in older animals. In contrast, sagittal sections from 5xFAD or wt  
437 mice, aged 3-9 months, were immunolabeled with antibodies against GFAP and co-labeled with  
438 ACU193, then imaged by confocal microscopy. We found a marked spatiotemporal association of  
439 GFAP pathology with ACU193-positive A $\beta$ Os in the 5xFAD mice. GFAP (Figure 3, magenta) pathology  
440 first appeared in the subiculum at 3 months of age concurrent with the first appearance of A $\beta$ Os (cyan)  
441 in the subiculum and in close proximity to one another. As the mice aged, GFAP and ACU193-positive  
442 pathology concomitantly spread throughout the subiculum and hippocampus (Figure 3, B & E). At 9  
443 months, WT mice have minimal GFAP expression (Figure 3C) and no A $\beta$ Os (Figure 3F). These  
444 patterns are consistent with possible induction of reactive astrogliosis by A $\beta$ Os. At higher magnification,  
445 we observed GFAP-positive reactive astrocytes surrounding an ACU193-positive neuron and projecting  
446 their processes onto the cell soma (Figure 3I). In addition, we observed micron-wide ACU193-positive  
447 puncta adjacent to astrocytic processes distant from the cell soma.

#### 448 **A $\beta$ Os given to WT littermates induces memory impairment within 24 hours**

##### 449 ICV A $\beta$ Os induce impairment in NLR/NOR

450 While the previous data indicate a relationship between A $\beta$ O accumulation and memory dysfunction in  
451 the 5xFAD mice, the question remained whether A $\beta$ Os cause the observed memory loss. We therefore  
452 asked whether injection of A $\beta$ Os into WT littermate mice would induce similar behavioral dysfunction.  
453 Wild-type littermates from the 5xFAD colony were injected with either 10 pmol synthetic A $\beta$ Os or  
454 volume equivalent of vehicle control into the right lateral ventricle, following our previously established  
455 protocol (Lambert et al., 2007; Velasco et al., 2012; Cline et al., 2019b). After 24 hours, the mice were  
456 assessed by the NLR task, and later, the NOR assay at 48 hours post-injection. We found that ICV  
457 injection of A $\beta$ Os induce memory dysfunction within 24 hours and impacts both cortical (NOR) and  
458 hippocampal (NLR) memory (Figure 4). As in the 5xFAD mice, A $\beta$ O injected mice showed no  
459 preference to either new or old objects and explored both equally. Vehicle-injected mice scored no

460 different from wild-type in these tasks. These data show that A $\beta$ Os are sufficient to induce memory  
461 impairment within 24 hours post-injection in wild-type mice. We next sought to establish the functional  
462 effect of neutralizing these A $\beta$ Os in the 5xFAD mice.

### 463 **Oligomer-selective antibodies engage and neutralize A $\beta$ Os responsible for memory 464 dysfunction in 5xFAD mice**

#### 465 ACU193-based probes ameliorate memory dysfunction

466 We have previously observed no short-term detrimental impact after inoculation of our A $\beta$ O antibodies  
467 into 5xFAD mice, but no studies have been done to determine the long-term positive or negative effects  
468 in these mice. To determine the impact of A $\beta$ O-neutralization in 5xFAD mice, 6- and 7-month-old mice  
469 were first assessed for memory impairment using the NLR/NOR assay. Mice were then inoculated with  
470 ACU193-based probes and imaged 24 hours later *in vivo* to ensure target engagement (see next  
471 section). The mice were then housed for 30-40 days to monitor any adverse effects or changes in  
472 behavior before being reassessed for memory impairment in the NLR/NOR tasks. Strikingly, we found  
473 that 6-month-old 5xFAD mice inoculated with the ACU193-based MRI probe had reversal of memory  
474 dysfunction, with performance the same as WT controls in the NOR task 30 days post-inoculation  
475 (Figure 5). The ACUPET probe similarly ameliorated memory impairment, measured 40 days post-  
476 injection. As controls, 5xFAD mice injected with human IgGMNS or IgGPET probe showed no memory  
477 improvement. Results from 4 trials of 10-12 animals each show that the ACU193 antibody engages  
478 A $\beta$ Os *in vivo*, completely reversing memory dysfunction in the 5xFAD mice with no evidence of health  
479 issues or side effects. The data establish A $\beta$ Os as the primary instigators of cognitive dysfunction in  
480 5xFAD mice and support the therapeutic relevance of A $\beta$ O-selective probes.

#### 481 **A $\beta$ Os imaged *in vivo* using ACU193-based probes distinguish 5xFAD from wild-type 482 mice**

#### 483 MRI signal from ACUMNS distinguishes 5xFAD from wild-type mice.

484 Our previous work showed that A $\beta$ Os can be detected *in vivo* in the 5xFAD mouse model using  
485 antibody-based MRI probes which were conjugated to magnetic nanostructures (MNS) (Viola et al.,  
486 2015). These prior studies used NU4 as the A $\beta$ O-targeting antibody, which as shown above, binds  
487 similarly to ACU193. Here we show that ACU193 can also be developed into a molecular probe for  
488 A $\beta$ O detection *in vivo*. After baseline imaging by MRI, 12-month-old mice were intranasally inoculated  
489 with MNS-conjugated ACU193 and allowed to recover overnight (about 16 hours) before imaging again  
490 (Figure 6). MRI data shows an accumulation of the ACUMNS probe in the hippocampus and cortex of  
491 the 5xFAD mice that is absent in WT controls. ImageJ quantification of signal intensity in the  
492 hippocampi of inoculated mice shows a ~ 30-fold increase in 5xFAD mice over their WT littermates.  
493 Using the ACUMNS probe in 18-month-old mice showed similarly robust AD-dependent MRI signal in  
494 the hippocampus of the 5xFAD animals, but signals obtained in younger animals (6-months old) were  
495 less consistent. These data add to previous studies with the NU4 probe and show that non-invasive *in*  
496 *vivo* imaging of A $\beta$ Os is possible using the ACUMNS probe, suggesting its potential diagnostic value  
497 and ability to confirm target engagement.

#### 498 Development of an ACU193-based PET imaging probe for early A $\beta$ O detection.

499 While the spatial resolution of MRI is excellent, its sensitivity is lower than other imaging modalities  
500 such as positron emission tomography (PET). Given PET sensitivity is at least 100 times greater than  
501 MRI, we thought it might detect very low levels of A $\beta$ Os during early stages of AD development.  
502 ACU193 was conjugated to DOTA, a chelator, as the initial step in the PET probe development. To  
503 ensure that this conjugation did not interfere with the antibody's ability to target A $\beta$ Os, sagittal brain  
504 slices from 5xFAD mice were probed with the ACU193-DOTA probe and counterstained with Thioflavin  
505 S (ThioS) for amyloid plaques (Supplemental Figure 3). Results show that ACU193-DOTA detected  
506 A $\beta$ Os in the 5xFAD brain and did not co-localize with ThioS, consistent with previously obtained results  
507 showing that ACU193 does not bind amyloid plaques cores (Cline et al., 2019a).

508 **ACUPET detects pathology in the brains of 4-month and older 5xFAD mice.**

509 The next step was to determine if radiolabeled ACU193-DOTA (ACUPET) detects AD-related A $\beta$ Os in  
510 the 5xFAD mouse brain at an early age. ACU193-DOTA was incubated with  $^{64}\text{Cu}$  and free isotopes  
511 were removed prior to tail vein injection into mice of either 4 or 18 months old. Mice were then imaged  
512 at 1, 4, and 24 hours post-injection for ACUPET distribution. At 4 hours post-injection, ACUPET  
513 accumulation in the brain was detectable, but not robust. By 24 hours, accumulation of the ACUPET  
514 probe in the brains of the 5xFAD animals was evident in both the 4-month-old animals (Supplemental  
515 Figure 4A) and the 18 month old animals (Supplemental Figure 4B-D). Animals at 6, 7, 8 and 12  
516 months were also examined and similarly were able to distinguish 5xFAD from WT mice (data not  
517 shown).

518 **A $\beta$ Os are specifically detected in vivo by NU4PET**

519 **NU4-based PET probe development**

520 Given the success of the NU4-based MRI probe (Viola et al., 2015), an NU4-based probe was  
521 synthesized for PET imaging. NU4 was conjugated to DOTA and tested to ensure that this conjugation  
522 did not interfere with the antibody's ability to target A $\beta$ Os. Primary hippocampal neurons, pre-treated  
523 with fluorescently conjugated A $\beta$ Os (FAM-A $\beta$ Os) and were probed with NU4-DOTA (Supplemental  
524 Figure 5). Data show that nearly all FAM-A $\beta$ Os (magenta) were also labeled with the NU4-DOTA probe  
525 (colocalization seen as dark blue) and no free NU4-DOTA (cyan) was detected. Vehicle treated cells  
526 showed no NU4-DOTA binding. Data confirm the specificity of the NU4-DOTA probe for A $\beta$ Os,  
527 necessary for its use for *in vivo* imaging.

528 **NU4PET detects AD-related pathology *in vivo* in 5xFAD mice, distinguishing them from WT**

529 Validation of the A $\beta$ O-PET probes as effective for early AD diagnostics requires verification that they  
530 produce an *in vivo* signal that depends on the presence of A $\beta$ Os. To validate our new probe, NU4  
531 (Lambert et al., 2007; Acton et al., 2010) and non-specific IgG antibodies were conjugated to DOTA  
532 and then radiolabeled with positron emitter  $^{64}\text{Cu}$  using Wipke and Wang's method (Wipke et al., 2002).  
533 Our next step was to image for A $\beta$ Os by PET following probe delivery. Animals (12 total), 7 months of  
534 age, were injected via tail vein with either NU4PET or IgGPET and then imaged at T=1, 2, 4, 8, 20, 30,  
535 40, and 44 hours after injection. After 44 hours, the animals were euthanized and their brains removed  
536 for a final *ex vivo* image of all 12 brains simultaneously (3 animals per group). Results showed the  
537 NU4PET specifically identified 5xFAD animals (Figure 7). No signal was detected in all three control  
538 groups (5xFAD with IgGPET; WT with NU4PET; WT with IgGPET).

539 The fraction of NU4PET probe retained (Supplemental Figure 6) showed good uptake into the brains of  
540 the 5xFAD mice but not the WT littermates (quantification of uptake; see Methods). For all mice, the  
541 IgGPET probe showed negligible signal. Quantification showed uptake into the brain was comparable  
542 to levels of uptake seen with the commercially available Pittsburgh Compound B (PiB) tracer (Mathis et  
543 al., 2003; Klunk et al., 2004). To corroborate the presence of A $\beta$ Os in the animals used for these  
544 studies, we analyzed the brain tissue with immunofluorescence. After final PET imaging, the brains  
545 were fixed and stored in 10% sucrose until no longer radioactive. Brains were then sliced sagittally at  
546 50  $\mu\text{m}$  and probed with ACU193. Images were collected and analyzed for ACU193 signal intensity  
547 (Supplemental Figure 7). Data showed that only 5xFAD mice, and not WT littermates, had A $\beta$ O  
548 pathology. Results confirm the NU4 PET probe gives a signal selective for A $\beta$ O-positive mice.

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## DISCUSSION

551 Alzheimer's disease is costly and marked by accumulation of pathological hallmarks such as amyloid  
552 plaques and neuronal tangles of hyperphosphorylated tau. Because A $\beta$  plaques have shown poor  
553 correlation with AD progression, there has been a rise in the exploration and development of  
554 therapeutics that are not based on amyloid (Cummings et al., 2021). This shift in focus has resulted in  
555 numerous potential therapies that have made it into clinical trials, but so far there have been limitations  
556 on their impact. As an alternative, focusing on A $\beta$ Os as the target for diagnostics and therapeutics  
557 appears to be a promising strategy for developing disease modifying treatments and early diagnosis.  
558 Here, we confirm that A $\beta$ Os can induce memory dysfunction in wild type mice and that A $\beta$ Os build up in  
559 5xFAD mice in a manner concomitant with astrocyte pathology and with memory dysfunction.  
560 Importantly, targeting this buildup with A $\beta$ O-selective antibodies rescues memory performance.  
561 Furthermore, we demonstrate that antibody-based brain imaging probes that target A $\beta$ Os can be used  
562 to identify animals that present with AD pathology, indicating the value of A $\beta$ O-selective antibodies both  
563 for diagnostics and therapeutics.

564 Recent interest in inflammatory processes and their involvement in AD has grown. Our data showed a  
565 striking association between GFAP-positive astrocytes and ACU193-positive A $\beta$ Os. This association  
566 and concomitant increase indicates a potential mechanism for A $\beta$ O-induced behavioral abnormalities.  
567 These findings are particularly intriguing given recent studies indicating AD's dependence on astrocytes  
568 (Huang et al., 2017; Monterey et al., 2021; Nisa et al., 2021; Preeti et al., 2021; Zhou et al., 2021). One  
569 especially interesting study showed that when apolipoprotein E (ApoE), a protein expressed in  
570 astrocytes which A $\beta$ Os associate with at synapses, was knocked out in astrocyte-only populations of  
571 P301S mice, AD pathology markedly improved (Wang et al., 2021). As ApoE4 is the greatest genetic  
572 risk factor of late onset AD, we propose that it may mediate A $\beta$ O-induced reactive astrogliosis and the  
573 subsequent neuropathology instigated by reactive astrocytes. Another study showed that astrocytes  
574 were activated into their reactive state via the JAK/STAT3 pathway in 6 month-old 5xFAD mice (Choi et  
575 al., 2020). Consistent with the idea that reactive astrogliosis is necessary for behavioral dysfunction in  
576 5xFAD mice, STAT3 phosphorylation inhibition restored cognitive function in the 5xFAD mice. Taken  
577 together with our data, we propose that A $\beta$ Os may induce JAK/STAT3 pathway-dependent reactive  
578 astrogliosis in astrocytes which is necessary for observed cognitive dysfunction in 5xFAD mice. In  
579 addition to astrocytes, microglia play a major role in AD pathology. The Triggering Receptor Expressed  
580 on Myeloid cells 2(TREM2)- expressed in microglia- has already been shown to be involved in AD, with  
581 mutations being neuroprotective and TREM2 accumulation being detected in AD patients (Jiang et al.,  
582 2013; Benitez et al., 2014; Guven et al., 2020). Previous studies have shown that A $\beta$ Os associate with  
583 TREM2 (Zhao et al., 2018; Zhong et al., 2019; Price et al., 2020), but TREM2 has no impact on  
584 established pathology (Yuan et al., 2021).

585 While interest increases in alternatives to the Amyloid Hypothesis, we are still left with no effective  
586 diagnostic tools for identifying AD at its earliest stages when therapeutics have the greatest impact.  
587 Currently recommended tests may rule out other dementia etiologies and help to determine disease  
588 severity, but they cannot detect AD at its earliest stages or closely predict disease progression. While  
589 AD diagnosis has significantly improved with the incorporation of a multiple assay evaluation currently  
590 being recommended, the tests still cannot predict disease progression or diagnose AD at its earliest  
591 stages because they are not quantifying the earliest biomarkers of the disease. However, alternative  
592 detection assays are being developed. Pre-tangle Tau, thought to be the toxic form of tau, has now  
593 been detected in MCI and AD and has been found to be one of the earliest tau lesions that correlates  
594 with cognitive status (Mufson et al., 2014). Synapse loss (Bastin et al., 2020; Buchanan et al., 2020;  
595 Camporesi et al., 2020; Mecca et al., 2020; Pereira et al., 2021), changes in hormone levels (Cheng et  
596 al., 2021), changes in blood biomarker levels (Guzman-Martinez et al., 2019; Montoliu-Gaya et al.,  
597 2021), electroencephalogram (EEG) readings (Hulbert and Adeli, 2013; Siwek et al., 2015; Lin et al.,  
598 2021), retinal assays (Ashok et al., 2020; Mirzaei et al., 2020), and changes in specific protein levels

599 (Buchanan et al., 2020; Colom-Cadena et al., 2020) are some of the myriad assays being developed to  
600 try to detect AD earlier and predict when and if the change from mild cognitive impairment (MCI) to AD  
601 will occur (Zhang et al., 2021b). All of these new developments are focused towards enabling earlier  
602 therapeutic intervention when chances for success would be greatest.

603 A $\beta$ Os as a diagnostic resource are currently unavailable. Cerebrospinal fluid assays show promise  
604 (Georganopoulou et al., 2005; Toledo et al., 2013a; Savage et al., 2014; Yang et al., 2015; Yang et al.,  
605 2019), but spinal taps are invasive and assays using CSF analytes have presented challenges with  
606 respect to accuracy and reliable disease-state discrimination (Slemmon et al., 2012). Other assays for  
607 A $\beta$ O levels are under development and show promise as well (Meng et al., 2019). For example, A $\beta$ O  
608 quantification in blood plasma shows a correlation between A $\beta$ O levels and declining memory scores  
609 that appear to not be influenced by age, gender, or ApoE4 status. Recently, the examination of soluble  
610 cortical extracts by ELISA found a link between the ratio of A $\beta$ Os and fibrils with disease. “The ratio of  
611 A $\beta$ O levels to plaque density fully distinguished demented from non-demented patients, with no overlap  
612 between groups in this derived variable.” (Esparza et al., 2013)

613 Because A $\beta$ Os are regarded as the first toxin to appear in disease progression, they should provide an  
614 excellent target for diagnostic imaging (Hefti et al., 2013; Goure et al., 2014). The usefulness of  
615 targeting A $\beta$ Os is indicated by human neuropathology studies in which A $\beta$ Os initially appear bound to  
616 discrete neurons, localizing to synapses in dendritic arbors (Lacor et al., 2004) through putative  
617 association with clustered cell surface receptors (Ferreira and Klein, 2011). FAM-A $\beta$ Os bind at discrete  
618 sites on dendrites, showing saturable, concentration-dependent synaptic binding (Viola et al., 2015),  
619 further suggesting their potential as a suitable target for an antibody-based diagnostic probe.  
620 Pronucleon<sup>TM</sup> imaging used engineered peptides that deliver a readout when associated with beta-rich  
621 A $\beta$  fibers and oligomeric A $\beta$  (Nyborg et al., 2013). Several PET probes have also been developed  
622 including a probe from curcumin<sup>18</sup>F (Rokka et al., 2014), a probe created by modifying 6E10 antibody  
623 with PEG and <sup>64</sup>Cu that distinguished Tg from control mice (McLean et al., 2012), and a probe  
624 developed from an <sup>124</sup>I-labeled mAb158 against A $\beta$  protofibrils (Magnusson et al., 2013). Still, none of  
625 these probes specifically target A $\beta$ Os.

626 Previously, we described a molecular MRI probe that is targeted against A $\beta$ Os (Viola et al., 2015).  
627 Based on the success of our initial MRI probe and the antibody-based probes being explored by others,  
628 it follows that A $\beta$ O-specific antibodies can be used to target probes and provide better signal-to-noise  
629 ratios. Here we showed that anti-A $\beta$ O antibodies can be used to develop molecular MRI and PET probes  
630 that distinguish WT mice from their 5xFAD littermates at ages as early as 4 months old. These probes  
631 have proven to be non-toxic over the periods examined and, in fact, showed *in vivo* efficacy. These  
632 studies, however, are limited to the 5xFAD mouse model for AD and have not yet been tested in other  
633 animal models or in human subjects. Our paper in essence establishes proof of concept that oligomers  
634 can be detected by antibody-based probes for PET and MRI. This is a first step, and a great deal of  
635 work remains. A case in point, while *ex vivo* PET imaging is robust in its ability to distinguish AD from  
636 control brains, the conditions for *in vivo* imaging require significant optimization.

637 Early diagnostics are critical to combating this devastating disease, but without effective therapeutics,  
638 they have limited value. The first FDA-approved drug to treat Alzheimer’s disease (AD) in nearly two  
639 decades, Aduhelm®, shows a preferential affinity for all aggregated forms of amyloid beta (A $\beta$ ), rather  
640 than targeting only the toxic A $\beta$ Os. Currently, there are more than 126 agents in clinical trials, with most  
641 aimed at disease modification (Cummings, 2021; Cummings et al., 2021). While less than 10% of these  
642 target A $\beta$ , there remains evidence that A $\beta$  is a significant target for therapeutic development. Lowering  
643 A $\beta$ O levels by enhancing fibril formation has been shown to be protective (Mucke et al., 2000). This is  
644 supported by previous antibody-based studies (Lambert et al., 2007; Xiao et al., 2013). The data  
645 presented here importantly show that A $\beta$ O-selective antibodies rescue memory performance in a  
646 widely used AD model. These antibodies, which have been modified for use in brain imaging of A $\beta$ O,  
647 show great promise as potential agents for AD therapeutics and diagnostics; the potential of one A $\beta$ O-  
648 selective antibody is now being assessed in a recently begun clinical trial.

649

650

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## Contribution to the field statement

671 Alzheimer's disease is costly and marked by pathological damage and progressive memory loss. While  
672 there has been progress made towards developing better therapeutics and diagnostics, it has been  
673 limited. Diagnostic improvements have primarily been in the development of better imaging methods,  
674 mostly using agents that probe amyloid fibrils and plaques- species that do not correlate well with  
675 disease progression and are not present at the earliest stages of the disease. Amyloid  $\beta$  oligomers  
676 (A $\beta$ Os) are now widely accepted as the A $\beta$  species most germane to AD onset and progression. Here  
677 we report evidence further supporting the role of A $\beta$ Os in Alzheimer's disease and introduce a  
678 promising anti-A $\beta$ O diagnostic probe capable of distinguishing the 5xFAD mouse model from wild type  
679 mice by PET and MRI. Our studies also showed a concomitant development of memory impairment  
680 with the accumulation of A $\beta$ Os and reactive astrocytes. Compelling support for the conclusion that  
681 A $\beta$ Os cause memory loss was found in experiments showing that A $\beta$ O-selective antibodies into 5xFAD  
682 mice completely restored memory function. These antibodies, modified to give imaging probes, were  
683 able to distinguish 5xFAD mice from wild type littermates. These results demonstrate that A $\beta$ O  
684 selective antibodies have potential both for therapeutics and for diagnostics.

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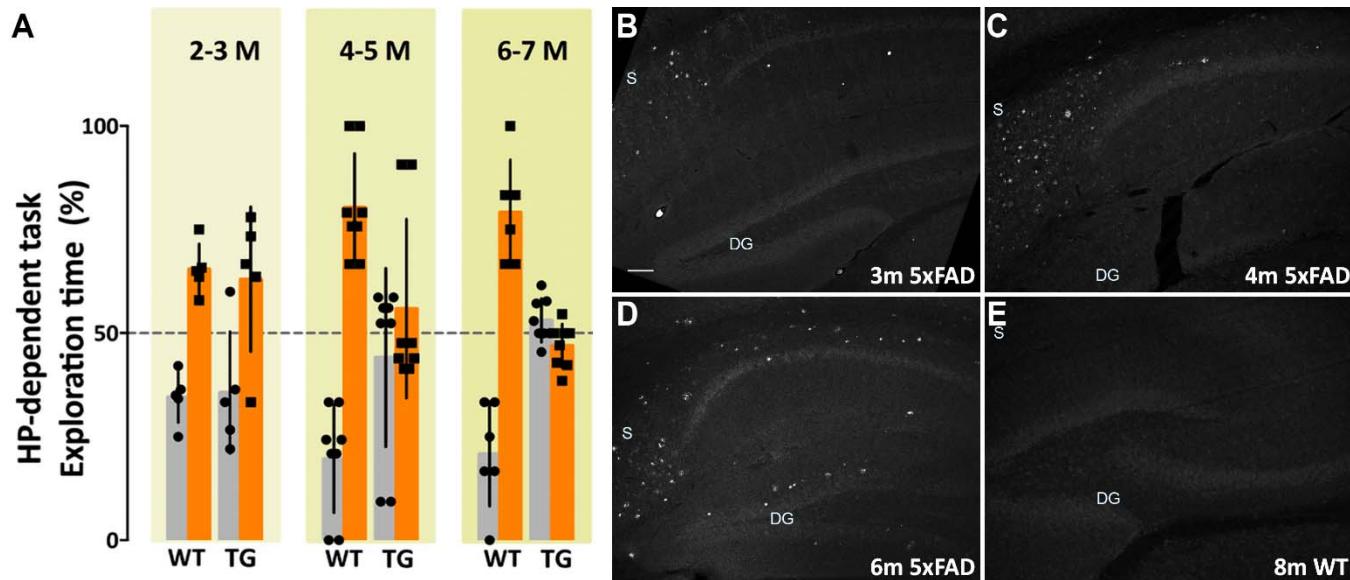
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## Figures, Titles and Legends

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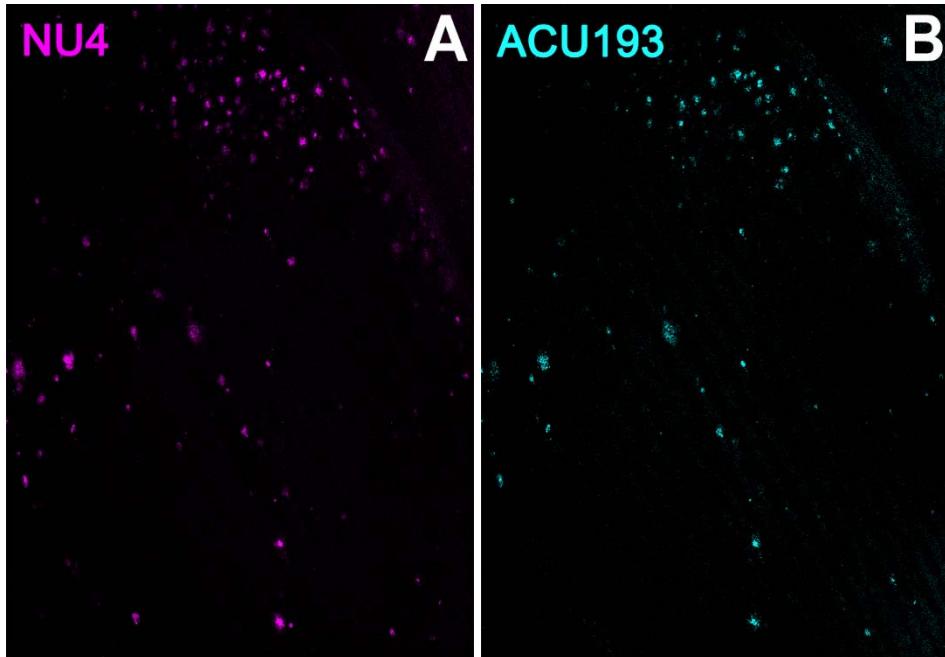
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1077 **Figure 1**

1078 **Memory dysfunction in 5xFAD mice is substantial by 4 months and is preceded by A<sub>β</sub>O**  
1079 **pathology, detectable by 3 months of age.** (Left) 5xFAD mice and wild-type littermates were  
1080 assessed for memory dysfunction using novel location recognition (NLR; hippocampal-dependent task)  
1081 and novel object recognition tasks (NOR; cortical-dependent task). Ages ranged 2-12 months. Data  
1082 shown here are for the hippocampal-dependent NLR assay. In 5xFAD mice, memory impairment was  
1083 negligible at 2-3 months, substantial by 4-5 months, and fully penetrant by 6 months of age. Statistical  
1084 analysis shows that there was no significant difference between the behaviors of the WT mice and the  
1085 5xFAD mice at ages 2-3 months, but a statistically significant difference was evident between the  
1086 recognition task behaviors of the WT mice and 5xFAD mice for ages 4-5 months ( $p<0.001$ ) and 6-7  
1087 months ( $p<0.0001$ ). (Right) Sagittal brain sections were obtained from 5xFAD and WT mice at ages 2,  
1088 3, 4, 6, and 8 months and probed for A<sub>β</sub>O pathology using a humanized A<sub>β</sub>O monoclonal antibody.  
1089 Fluorescent signal was barely detectable at 2 months of age in some mice, more readily detectable by  
1090 3 months in all Tg mice, and robust by 6 months. Wild-type littermates presented no signal. Scale bar  
1091 = 100  $\mu$ m.

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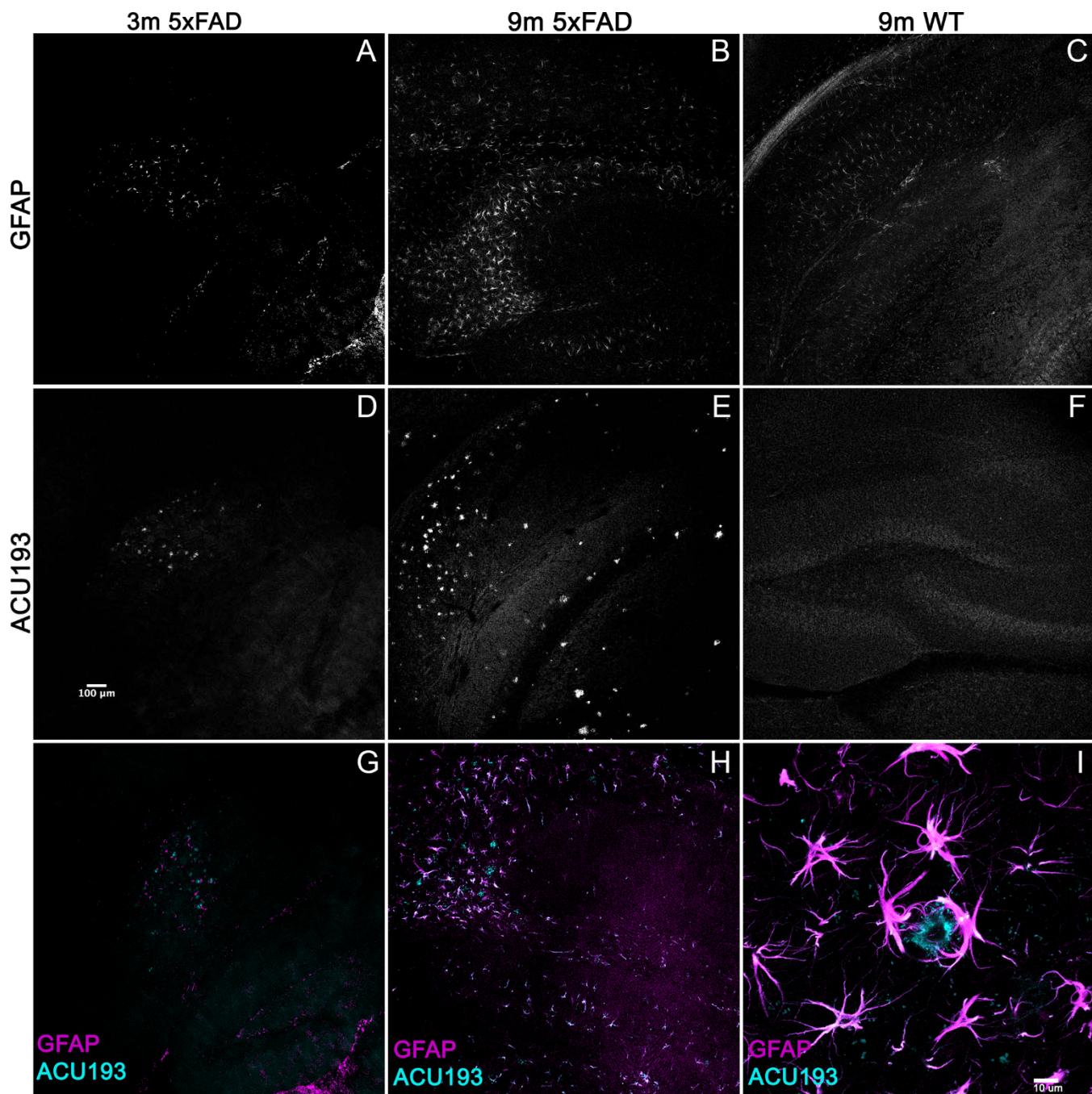
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1095 **Figure 2**

1096 **ACU193 and NU4 detect A $\beta$ Os *ex vivo*.** Sagittal sections from 9-month-old 5xFAD mice were  
1097 immunolabeled with 2 different anti-A $\beta$ O antibodies, NU4 and ACU193, to determine the extent to  
1098 which A $\beta$ O pathology is detected by both antibodies. Data show that A $\beta$ Os accumulate and that  
1099 ACU193 and NU4 show very similar detection of A $\beta$ Os.

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1101



1103 **Figure 3**

1104 **Alzheimer's-associated astrocyte pathology develops concomitantly with A $\beta$ Os.** Sagittal sections  
1105 from 5xFAD mice, aged 3-9 months, and their wild-type littermates were immunolabeled with antibodies  
1106 against GFAP and ACU193, then imaged on the Leica SP5 confocal microscope at 10x and 100x. Data  
1107 show that, like the ACU193, GFAP positive glial cells accumulate in an age dependent manner. Sale  
1108 bar = 100  $\mu$ m for panels A-H ad 10  $\mu$ m for panel I.

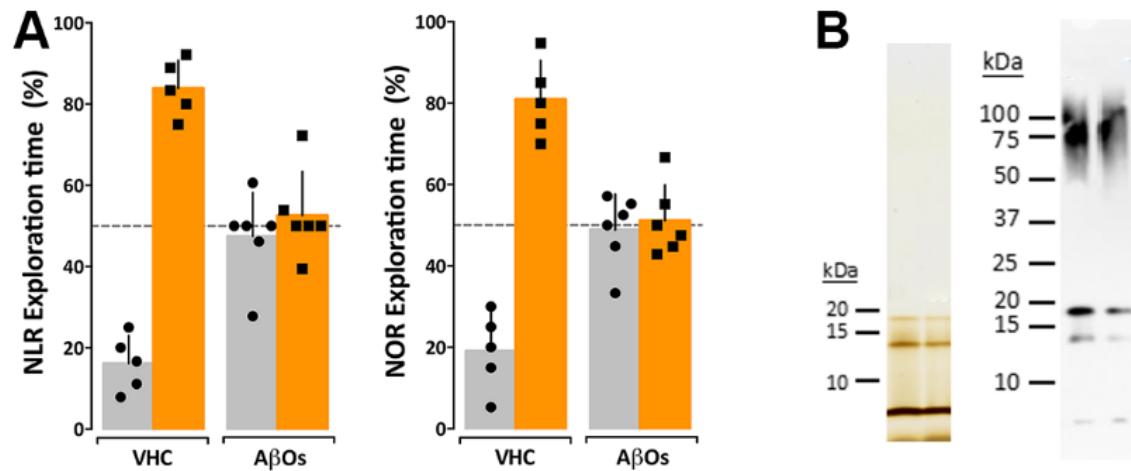


Figure 4

**Intraventricular A $\beta$ O injection causes memory impairment in wild type mice within 24 hours.** (A) Wild type mice were tested for performance in recognition tasks beginning 24 hours after receiving vehicle (VHC) or A $\beta$ O injections (A $\beta$ Os) (10 pmols in 3  $\mu$ l) into the right lateral ventricle. Mice first were assessed for novel location recognition (NLR; 24 hr post-injection) and subsequently for novel object recognition (NOR; 48 hr post-injection). A $\beta$ O-injected mice were unable to perform either recognition task. Statistical analysis shows that there is a statistically significant difference between the recognition task behaviors of the WT mice and the A $\beta$ O injected mice ( $p<0.0001$ ). (B) Silver stain (left) and Western blot (right) analysis of the A $\beta$ Os used for injections and other assays in this study shows preparations contain trimer, tetramer, and higher molecular weight species as has been shown before (Lacor et al., 2007; Lambert et al., 2007; Velasco et al., 2012).

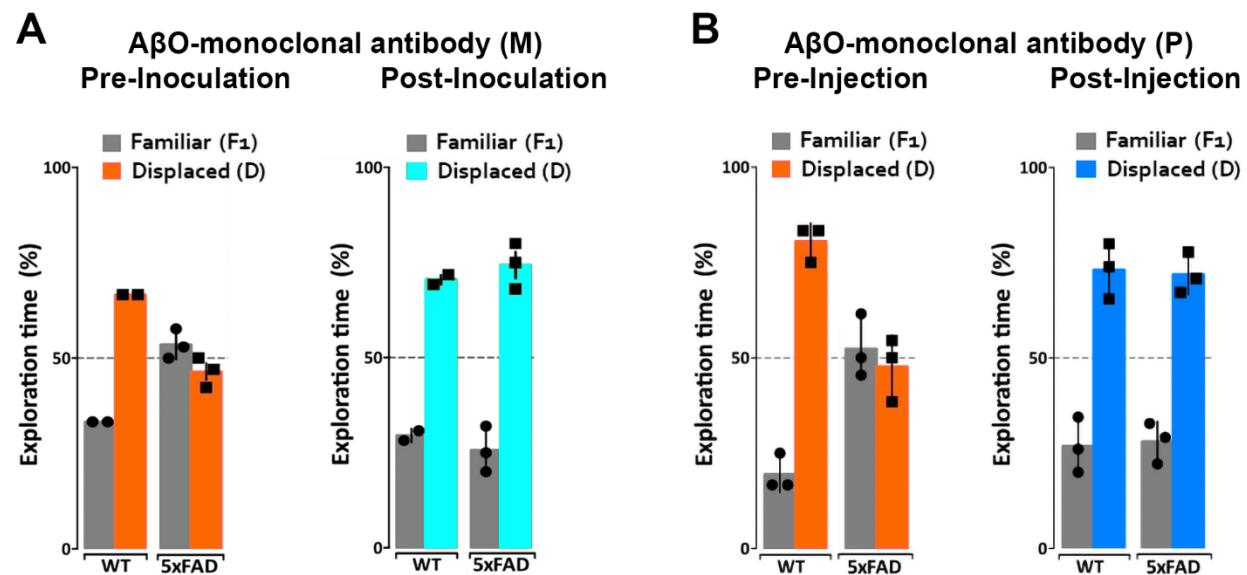
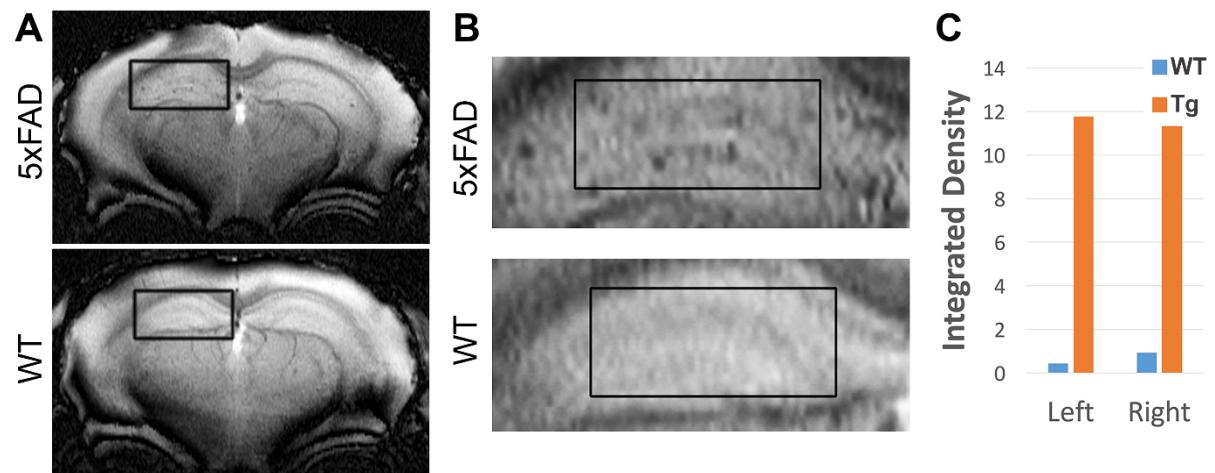


Figure 5

ACUMNS delivered intranasally or ACUPET given iv each rescue memory function in 6- to 7-month-old mice.

Tg and WT mice, aged 6 months (A), were tested by NLR and NOR assays to ensure predicted behavioral deficits. Mice were then intranasally inoculated with ACUMNS and imaged for probe distribution and detection of A<sub>β</sub>O pathology *in vivo*. After imaging, animals were monitored for 30 days for signs of adverse reactions to the probe (none detected), then re-tested by NOR. The 6-month-old animals showed a significant recovery of memory impairment 30 days after inoculation. Human IgGMNS showed no impact on memory recovery. (B) To test the impact of the ACUPET probe on memory function, Tg and WT mice, aged 7 months, were tested by NLR and NOR assays prior to imaging as before. Mice were then injected, via tail vein, with ACUPET or non-specific IgGPET and imaged for up to 24 hours to monitor probe distribution. After imaging, animals were monitored for 40 days for signs of adverse reactions to the probe. Animals were re-tested by NOR at 40 days recovery. 5xFAD animals injected with ACUPET showed a persistent recovery of memory impairment that was not seen in the 5xFAD animals injected with IgGPET. ACU-based probes have no impact on wt behavior. Results are representative of 4 separate trials that showed beneficial impact of these antibody-based probes on memory.



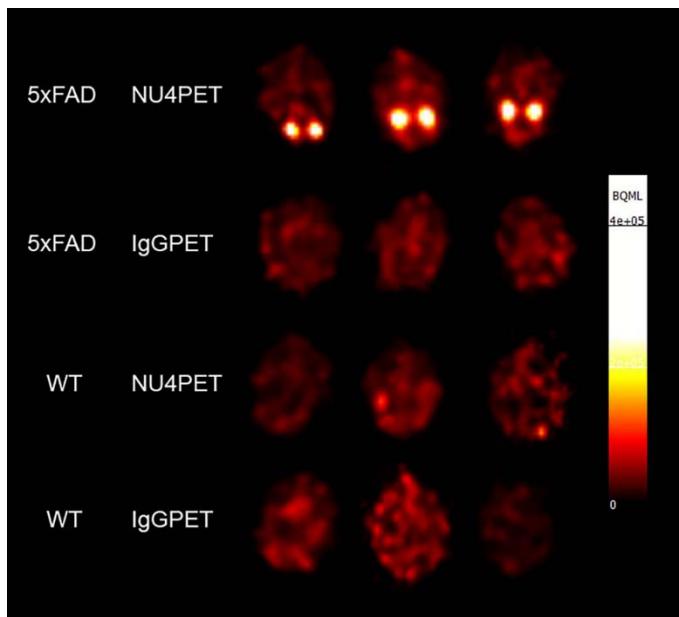
1145  
1146 **Figure 6**

1147 **ACUMNS gives AD-dependent MRI signal in hippocampus of 12-month-old 5xFAD mice.**

1148 *In vivo* studies with ACUMNS probe show robust AD-dependent MRI signal in the hippocampus of 12  
1149 month-old mice.

1150

1151



1152  
1153 **Figure 7**

1154 **NU4PET probe gives 5xFAD- specific CNS signal.**

1155 Signal obtained after IV injection of NU4PET showed probe accumulation in the hippocampus of 5xFAD  
1156 mice (aged 5-7 months). Controls (IgGPET in AD mice; NU4PET in wild type littermates; IgGPET in  
1157 wild type littermates) showed no signal (3 animals per group).

1158

1159