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## Maintenance of membrane organization in the aging mouse brain as the determining factor for preventing receptor dysfunction and for improving response to anti-Alzheimer treatments.

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1           **Maintenance of membrane organization in the aging mouse brain as**  
2           **determining factor for preventing receptor dysfunction and for improving**  
3           **response to anti-Alzheimer treatments**

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18  
19          **Abbreviations:** A $\beta$ , amyloid- $\beta$  peptide; AD, Alzheimer’s disease; ARA, arachidonic acid; CNTF,  
20          ciliary neurotrophic factor; CNTF-R, CNTF receptor; DHA, docosahexaenoic acid; icv,  
21          intracerebroventricular; ip, intraperitoneal; PBS, phosphate-buffered saline; PUFA,  
22          polyunsaturated fatty acid; StD, standard diet

25 **Abstract**

26 Although aging is a major risk factor for Alzheimer's disease (AD), it is not considered in  
27 preclinical validation of anti-AD drugs. Here, the ciliary neurotrophic factor (CNTF)-associated  
28 pathway was chosen as a model to explore how aging affects neuronal reactivity to anti-AD  
29 agents. Comparison of the neuroprotective properties of CNTF in 6- and 18-month-old mice  
30 revealed an age-dependent CNTF resistance in the older animals associated with the exclusion  
31 of the CNTF-receptor subunits from raft to non-raft cortical membrane domains and their  
32 subsequent dispersion. This membrane remodelling prevented the formation of active CNTF-  
33 receptor complex and the activation of the pro-survival STAT-3 and ERK1/2 pathways in the  
34 aging brain. Moreover, CNTF-receptor distribution associated with CNTF signalling responses  
35 were improved in older mice receiving dietary docosahexaenoic acid, with CNTF-receptor  
36 functionality being similar to those of younger mice. In conclusion, age-altered membranes  
37 impair the reactivity of potential therapeutic targets. Dietary intervention could represent a  
38 promising adjuvant strategy to maintain functional neuronal membranes, thus allowing  
39 associated receptors to respond appropriately to anti-AD agents.

40

41 **Keywords:** brain aging, ciliary neurotrophic factor, dietary lipids, docosahexaenoic  
42 acid, lipid rafts, neuronal membranes

43

44 **Highlights:**

- 45 • Cortical membranes exhibit structural and functional changes in aging brain
- 46 • Membrane lipid composition and organization are key determinants for brain function
- 47 • Aging is associated with membrane protein relocation and loss of function
- 48 • Dietary DHA favors proper location and activity of membrane proteins in aging brain
- 49 • Preclinical Alzheimer's models should consider aging for improved relevance

50 **1. Introduction**

51 Aging of the brain is a physiological process that reflects the long-term exposure of the  
52 individual to a number of environmental factors, including diet and lifestyle (smoking,  
53 alcohol consumption, etc.). Although age is an established risk factor for Alzheimer's disease  
54 (AD), preclinical testing for drug development is often based on pathological AD animal  
55 models without consideration of the aging process, which needs to be clearly understood in  
56 order to develop effective therapeutics for the elderly AD patient.

57 Numerous studies have provided strong evidence for associations between chronic  
58 conditions such as metabolic disorders and dyslipidaemia with AD-related cognitive decline  
59 (Frisardi et al., 2010), suggesting that disruption of lipid homeostasis is linked to risk of AD.  
60 Physiological brain aging has indeed been associated with alterations of lipid metabolism  
61 and bioavailability, including that of polyunsaturated fatty acids (PUFAs) such as the omega-  
62 6 arachidonic (ARA) and omega-3 docosahexaenoic (DHA) acids (Gao et al., 2013; Taha et al.,  
63 2012), both of which influence membrane fluidity with consequences on brain functions  
64 including neurotransmission and synaptic plasticity. Furthermore, both qualitative and  
65 quantitative age-associated alterations in neuronal membrane lipid composition (Martín et  
66 al., 2010; Ohno-Iwashita et al., 2010) may affect a variety of cellular functions such as  
67 membrane fusion, receptor function, and activation of signalling pathways (Ledesma et al.,  
68 2012), all of which have been reported to be altered in AD. Lipid rafts serve as signalling  
69 platforms for these membrane-dependent cell activities, providing appropriate  
70 microenvironments for specific associations of receptors and signalling proteins, and the  
71 means to regulate cell functions including survival/death balance and neuroprotection (Davis  
72 et al., 2007; Sui et al., 2006). These highly-ordered cholesterol- and sphingolipid-rich  
73 membrane microdomains are distinct from non-raft regions, and their specific architecture can

74 significantly influence receptor activation, signal transduction and related pathways, as well as  
75 A $\beta$  peptide release and cerebral vulnerability to neuronal stress conditions (Colin et al., 2016).  
76 We therefore initiated this study to evaluate age-dependent changes in these signalling  
77 platforms, as well as the functional consequences with regards to AD susceptibility.

78 To do this, we selected as a model the ciliary neurotrophic factor (CNTF), whose  
79 neuroprotective properties against A $\beta$ -induced neuronal damage and behavioural impairment  
80 have previously been demonstrated in our *in vivo* AD model (Garcia et al., 2010). CNTF is of  
81 particular interest for this study since its effects result from its interaction with a tripartite  
82 CNTF-R receptor (CNTFR $\alpha$ /LIFR $\beta$ /gp130) and the associated JAK-STAT transduction pathway,  
83 which depends on lipid raft architecture and composition (Sehgal et al., 2002). Furthermore,  
84 the CNTF-R activation depends on its two subunits gp130 and LIF-R that are located in  
85 possibly distinct microdomains (Buk et al., 2004), but that must cluster together with  
86 CNTFR $\alpha$  in lipid rafts in order for transduction of CNTF signalling to occur (Port et al., 2007).

87 We show here that aging modulated the structural organization of neuronal membranes,  
88 resulting in altered functional properties of CNTF-R and significantly attenuated  
89 neuroprotective effect of CNTF. In contrast, the CNTF neuroprotective activity was maintained  
90 in older mice upon dietary DHA supplementation, demonstrating that dietary lipid-based  
91 preventive management could improve the efficiency of therapeutic agents by regulating the  
92 direct microenvironment of their targets in neuronal membranes.

93

## 94 **2. Materials and Methods**

### 95 **2.1. Chemicals and reagents**

96 All chemicals and reagents were obtained from Sigma, unless otherwise specified. A $\beta$  was  
97 obtained from Bachem, and soluble oligomers of A $\beta$  were prepared at the concentration of

98 0.5 mM in sterile 0.1 M PBS, pH 7.4 as previously described (Kriem et al., 2005), and aliquots  
99 were stored at -20°C until use. Analysis of oligomeric A $\beta$  preparations by electrophoresis in  
100 non-denaturing conditions indicated the presence of monomers, trimers and tetramers  
101 (Garcia et al., 2010). Soluble human CNTF was prepared at 1  $\mu$ g/ $\mu$ L in sterile 0.1 M phosphate  
102 buffered saline (PBS), pH 7.4, and aliquots were stored at -20°C until use.

## 103 **2.2. Animals and diets**

104 Adult male C57BL/6J mice aged 3 or 15 months were purchased from Janvier Breeding (Le  
105 Genest-St Isle, France). Animals were housed in pathogen-free, certified animal facility with  
106 access to food and water *ad libitum*. They were kept in a controlled environment ( $22 \pm 1^\circ\text{C}$ ,  
107  $50 \pm 20\%$  relative humidity) with a 12-h light/dark cycle. At the beginning of the experiments,  
108 body masses of animals were (mean  $\pm$  SD)  $24.1 \pm 0.2$  and  $32.3 \pm 0.1$  grams, respectively. The  
109 group of young mice received a standard diet (StD, 3.08 kcal/g), containing 18% lipids (Harlan  
110 Laboratories, Gannat, France), while the older mice were randomly divided into 2 groups. One  
111 group received the StD diet, and the other group was placed on a DHA+ diet (3.11 kcal/g),  
112 corresponding to the StD diet supplemented with 0.3% (w/w) DHA-ethyl ester (KD Pharma,  
113 Bexbach, Germany). Animals received StD or DHA+ diet for 10 weeks. Animal handling and  
114 experimental protocols were authorized in accordance with the European Communities  
115 Council Directive (86/609/EEC) and the French Directive for animal experimentation  
116 (2013/118) for the use and care of laboratory animals and in conformity with PHS policy on  
117 Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory  
118 Animal Research Guide for Care and Use of Laboratory Animals. The experimental protocol  
119 was approved by the institutional ethic committee (CELMEA-2012-0026).

## 120 **2.3. Surgical procedures**

121 All surgical procedures were performed on mice anesthetized by intraperitoneal injection of  
122 0.8% (w/v) ketamine (Virbac, Carros, France) in physiological serum containing 0.1% (w/v)  
123 xylazine (Bayer HealthCare), at the dose of 1 mg ketamine/10 g body weight. One  $\mu$ L of soluble  
124 A $\beta$  oligomers (0.5 nmol), CNTF (1  $\mu$ g), or saline were injected into the right ventricle  
125 (stereotaxic coordinates from the bregma in mm: AP-0.22, L-1.0 and D 2.5) using a 10- $\mu$ L  
126 Hamilton microsyringe fitted with a 26-gauge needle as previously described (Garcia et al.,  
127 2010). After surgery, the mice were returned to their cages until the day of Y-maze testing,  
128 which was 4 days after ICV injections. At the end of the experimental period, all animals were  
129 anesthetized with isoflurane, followed by decapitation to remove the brain. Cortical tissues  
130 were removed and immediately placed on ice and stored at  $-80^{\circ}\text{C}$ .

#### 131 **2.4. Evaluation of short-term memory**

132 Immediate spatial working memory performance was evaluated by recording spontaneous  
133 alternation behaviour in a Y maze 4 days after icv injection, as described by Garcia et al. (2010).

#### 134 **2.5. Duolink<sup>®</sup> labelling**

135 Mouse brains freshly dissected were immediately placed in pre-cooled ( $-20^{\circ}\text{C}$ ) methylbutane  
136 for 30 min at  $-20^{\circ}\text{C}$ , removed and then stored at  $80^{\circ}\text{C}$ . Brain sections (14- $\mu$ m thickness) were  
137 prepared using a cryostat HM550 (Microm-Microtech, Francheville, France) and then mounted  
138 on gelatin-coated slides. Brain sections were fixed by treating with 4% (w/v) paraformaldehyde  
139 in PBS for 15 min at room temperature, after which all steps were performed at room  
140 temperature. After washing in PBS, the sections were stained using the Duolink<sup>®</sup> *in situ* kit  
141 (Olink Bioscience, Uppsala, Sweden) according to manufacturer's instructions. This method is a  
142 proximity ligation assay which allows the detection of two closely located proteins. To  
143 evaluate the number of fluorescence spots corresponding to protein interaction,

144 5 independent fields were counted using a confocal microscope (Fluoview Fv10i, Olympus), for  
145 brain sections from 3 mice, and quantification was performed using Image J image-processing  
146 software (<http://imagej.nih.gov>).

#### 147 **2.6. Preparation of protein homogenates**

148 Brain cortex tissues were dissected and immediately placed in RIPA lysis buffer (Millipore)  
149 containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and  
150 cOmplete™ protease inhibitor cocktail (Roche). After homogenisation with a syringe through  
151 a 23G x 1¼" needle, the samples were lysed using 2 cycles of freezing and thawing, and  
152 centrifuged at 4°C for 30 min at 10,000 × g to collect supernatants. Protein concentrations in  
153 the supernatants were determined by BCA (Thermo Fisher Scientific).

#### 154 **2.7. Isolation of lipid rafts**

155 Preparation of membrane extracts and raft isolation were optimised based on the method  
156 by Ledesma et al. (2003) and all steps were carried out at 4°C. Brain cortex tissues were  
157 homogenised in 10 mM Tris buffer, pH 7.4, containing 2 mM EDTA, 0.32 M sucrose, 1 mM  
158 PMSF and cOmplete™ protease inhibitors, using a Potter homogeniser followed by  
159 10 passages through a 23G needle. After centrifugation at 750 × g for 10 min, the resulting  
160 postnuclear supernatants were transferred to new tubes and total membrane fractions were  
161 pelleted by ultracentrifugation at 100,000 × g for 1 h, prior resuspension in ice-cold MEST  
162 buffer, pH 7.4, containing 25 mM MES, 5 mM DTT, 2 mM EDTA, 1 mM PMSF, cOmplete™  
163 protease inhibitors and 1% Triton X-100. Samples were incubated 1 h on ice, homogenised  
164 again by 20 passages through a 23G needle and transferred into a discontinuous 5-45%  
165 sucrose gradient in 5-mL Ultra-Clear™ (Beckman Coulter) centrifugation tubes. Gradients  
166 were then subjected to buoyant density ultracentrifugation at 150,000 × g for 18 h, and



167 9 fractions of 500  $\mu$ L were collected consecutively from the top of each tube, with the order  
168 being from 1 to 9 as the lowest to highest density. An aliquot from each fraction was  
169 immediately mixed with 2 $\times$  Laemmli buffer and denatured by boiling for immunoblot  
170 analyses to assess the distribution of various proteins, while the remainder was stored at  
171  $-20$   $^{\circ}$ C for measurement of cholesterol levels using the Amplex<sup>®</sup> Red Cholesterol assay  
172 (Invitrogen). Lipid rafts were considered as those fractions containing both flotillin-1 and  
173 cholesterol peak levels.

#### 174 **2.8. Immunoblot analyses**

175 Samples were mixed with 2 $\times$  Laemmli buffer prior denaturation at  $95^{\circ}$ C for 5 min. Samples  
176 were then subjected to SDS-PAGE and immunoblotting. Reagents for electrophoresis were  
177 purchased from BioRad and polyvinylidene fluoride membranes were from Millipore. The  
178 following antibodies (1:1000 dilution) were used: rabbit monoclonal anti-CNTFR $\alpha$ , anti-LIFR $\beta$   
179 and anti-gp130 antibodies (Santa Cruz Biotechnology), anti-P-STAT3 (STAT3 phosphorylated  
180 on Tyr705) and anti-P-ERK1/2 (ERK1/2 phosphorylated on Tyr202/Thy204) antibodies (Cell  
181 Signaling Technology), mouse monoclonal anti- $\beta$ -tubulin (Sigma), anti-flotillin (BD  
182 Biosciences), anti-STAT3 (Santa Cruz Biotechnology) and anti-ERK1/2 antibodies (Cell  
183 Signaling Technology). Goat anti-rabbit IgG and horse anti-mouse IgG peroxidase-conjugated  
184 secondary antibodies (Cell Signaling Technology) were detected using Supersignal  
185 chemiluminescence (ECL kit; Millipore). Approximate molecular weights were estimated  
186 using PageRuler prestained Protein Ladder Plus (Euromedex). Densitometric analysis of  
187 immunoblot images was performed using the Fusion Fx5 Imaging System (Vilber Lourmat)  
188 and ImageJ software.

#### 189 **2.9. Statistical analysis**

190 StatView software (SAS Institute) was used for the statistical analysis. Multiple pairwise  
191 comparisons among the groups of data were performed using ANOVA, followed by a  
192 Scheffe's post-hoc test. For data concerning the memory tests, statistical comparisons  
193 among the datasets were made by one-way ANOVA, followed by Scheffe's post-hoc test.  
194 Values of  $p < 0.05$  indicate significant differences between experimental groups. All reported  
195 values represent the mean  $\pm$  SEM.

196

### 197 **3. Results**

#### 198 **3.1. Impaired CNTF neuroprotective effect and CNTF-induced signalling pathways in aged mice**

199 CNTF has previously been reported to protect mice from neuronal damage and  
200 behavioural impairments that occur following a single *icv* administration of soluble A $\beta$   
201 oligomers (Garcia et al., 2010). To study the effect of age on the neuroprotective effect of  
202 CNTF against AD-like symptoms, male C57Bl/6J mice aged 6 (6-mo) or 18 (18-mo) months  
203 (n=6 per 6-mo group and n=8 per 18-mo group) were injected *icv* with soluble A $\beta$  oligomers  
204 in the absence or presence of CNTF. Short-term spatial memory was then evaluated as %  
205 alternation in the Y-maze test 4 days post-injection. We compared the results between  
206 young and old mice, with or without CNTF co-administration, using vehicle-injected mice as  
207 controls. No difference was observed in short-term memory performance in 6-mo and 18-  
208 mo vehicle-injected mice (Fig1A). However, alternation was significantly lower in mice  
209 exposed to A $\beta$  oligomers than to vehicle, in mice of both age groups. This decrease was  
210 greater ( $p < 0.05$ ) in the older mice ( $69 \pm 1\%$  vs.  $50 \pm 2\%$ ), as compared to the younger  
211 animals ( $73 \pm 4\%$  vs.  $57 \pm 2\%$ ), suggesting that A $\beta$ -induced stress and subsequent short-term  
212 spatial memory impairment were more pronounced in the older animals (Fig1A). In 6-mo  
213 mice receiving co-administration of CNTF with A $\beta$ , the % alternation was not different than

214 that measured in the control mice of the same age, demonstrating the neuroprotective  
215 effect of CNTF against A $\beta$ -induced spatial memory impairment. In contrast, CNTF provided  
216 only partial protection against amyloid stress in 18-mo mice, with the % alternation of A $\beta$ -  
217 exposed aged animals being significantly lower ( $p < 0.01$ ) than in the corresponding aged  
218 controls (Fig1A). Treatment of CNTF alone did not induce any visible differences in %  
219 alternation when compared to that of saline-injected mice of the corresponding age group.  
220 Also, the number of arm entries did not differ significantly among all experimental groups  
221 (data not shown), indicating that the differences observed between the 2 groups were not  
222 due to changes in exploratory, locomotor or motivational activities, nor from visual,  
223 olfactory or tactile sensorial acuity.

224 A subgroup of the animals were euthanized 3 h following CNTF injection to determine the  
225 effect of age on CNTF-associated signal transduction. Despite identical amounts of CNTF  
226 injected into both young and old mice, the relative levels of phosphorylated forms of both  
227 STAT3 and ERK1/2 in cortical homogenates were significantly lower by  $37 \pm 11\%$  ( $p < 0.05$ )  
228 and  $75 \pm 5\%$  ( $p < 0.01$ ), respectively, in 18-mo mice as compared to 6-mo animals (Fig1B and  
229 fig1C), suggesting impaired CNTF signalling and response in the older animals.

### 230 **3.2. Loss of collocation of tripartite CNTF-R complex associated with exclusion from lipid rafts in** 231 **brain cortex of 18-mo mice**

232 Green fluorescent Duolink<sup>R</sup> signals reflecting collocation and direct interactions between  
233 CNTF-R subunits were quantified and compared in brain cortex slices from 6-mo and 18-mo  
234 mice. Duolink<sup>R</sup> spots indicating CNTFR $\alpha$ -LIFR $\beta$ , CNTFR $\alpha$ -gp130 or LIFR $\beta$ -gp130 collocation  
235 were significantly lower by  $57 \pm 7\%$  ( $p < 0.01$ ),  $80 \pm 2\%$  ( $p < 0.001$ ) and  $61 \pm 4\%$  ( $p < 0.001$ ),  
236 respectively, in the older mice as compared to younger animals (Fig2A). These subunits no  
237 longer located in close vicinity to each other, suggesting dramatically reduced interactions of

238 the three CNTF-R subunits in brain cortex of older mice as compared to those of younger  
239 animals. The Duolink<sup>R</sup> assay was next used on cortical brain sections to determine the  
240 location of the CNTF-R subunits relative to the raft-specific protein, flotillin-1 (Flot). Analysis  
241 revealed numerous CNTFR $\alpha$ -Flot, LIFR $\beta$ -Flot and gp130-Flot interactions in 6-mo mice,  
242 suggesting that CNTF-R subunits were primarily colocalized in raft domains (Fig2B). In  
243 contrast, Duolink<sup>R</sup> signals in 18-mo mice for CNTFR $\alpha$ -Flot, LIFR $\beta$ -Flot and gp130-Flot were  
244 strongly reduced by  $88 \pm 4\%$  ( $p < 0.001$ ),  $83 \pm 2\%$  ( $p < 0.001$ ) and  $47 \pm 8\%$  ( $p < 0.01$ ),  
245 respectively, which demonstrated that in the cortex of older mice, the CNTF-R subunits were  
246 excluded from lipid rafts and dispersed in other membrane domains.

### 247 **3.3. Exclusion of CNTF-R subunits from lipid rafts associated with membrane remodelling in aged** 248 **brain cortex**

249 We next sought to determine potential differences in membrane domain composition  
250 and organisation using brain cortical samples obtained from 6-mo and 18-mo mice. Lipid raft  
251 fractions separated on sucrose gradients were considered as those containing the maximal  
252 levels of flotillin-1 and cholesterol detected by immunoblot and fluorimetry, respectively. In  
253 the cortical membranes from 6-mo mice, the three fractions (# 6, 7 and 8) identified as  
254 having the highest cholesterol and flotillin-1 levels were pooled to constitute the raft  
255 subpopulation used in immunoblots. In contrast, raft domains in cortical membranes from  
256 18-mo mice were identified in only one cholesterol- and flotilline-1-rich fraction (#4), at a  
257 lower density than those found in 6-mo mice (Fig3A and fig3B).

258 Cholesterol levels measured in the membrane fractions also appeared to be increased in  
259 18-mo mice as compared to 6-mo mice, both fed the same StD diet (Fig3A). This was  
260 confirmed by the detection of 2.2-fold higher ( $p < 0.001$ ) cholesterol levels in the total

261 membrane extracts from brain cortical tissues of older animals as compared to young  
262 animals (Fig3C).

263 We used the cholesterol- and flotillin-1-enriched isolated raft fractions to measure the  
264 levels of the CNTF-R subunits by immunoblot. No difference was observed in the levels of  
265 these proteins in the total membrane extracts between both age groups (data not shown),  
266 confirming that the decreased levels in the rafts (Fig2) was due to their exclusion from raft  
267 domains. Indeed, a significant reduction of CNTFR $\alpha$  and LIFR $\beta$  levels by  $58 \pm 4\%$  ( $p < 0.001$ )  
268 and  $41 \pm 3\%$  ( $p < 0.01$ ), respectively, was revealed in the raft fraction of 18-mo mice as  
269 compared with 6-mo mice (Fig4). The third subunit of CNTF-R, gp130, could not be detected  
270 in raft fractions due to limited sensitivity of immunoblot analysis, although its presence was  
271 clearly demonstrated by immunohistochemical analyses (Fig2). These observations clearly  
272 demonstrated that brain cortical membranes underwent significant age-related remodelling  
273 that altered raft domain properties and lateral distribution of associated proteins such as the  
274 CNTF-R subunits that no longer cluster together in lipid rafts.

#### 275 **3.4. Limitation of age-related remodelling and persistence of CNTF-R location in brain membranes** 276 **upon dietary DHA supplementation**

277 Preliminary studies led us to measure a 15.3% enrichment of DHA ( $p < 0.001$ ) in cortical  
278 membranes of mice fed for 10 weeks with the DHA+ diet as compared to animals on StD diet.  
279 In the present experiment, lipid analyses of brain cortical membranes obtained from 6-mo and  
280 18-mo mice on StD diet showed no significant differences in either DHA levels relative to total  
281 fatty acid content, omega-6/omega-3 or ARA/DHA ratios (data not shown), which clearly  
282 indicates that the observed age-related changes were not due to discrepancies in DHA or  
283 PUFA levels between the young and older animals.

284 A group of 18-mo mice was placed on DHA+ diet for 10 weeks, and then euthanized.  
285 Cholesterol levels in total cortical membrane samples were found to be 37% lower ( $p < 0.01$ ) in  
286 these mice as compared to those of mice of the same age on StD diet, and not significantly  
287 different from those of 6-mo mice fed the StD diet (Fig3C). Analysis of the membrane fraction  
288 profile of 18-mo mice on the DHA+ diet (Fig3A and fig3B) revealed raft domains of higher  
289 density (fractions 5 to 8) and lower cholesterol levels as compared to those of 18-mo mice on  
290 StD diet, demonstrating the ability of this fatty acid to influence membrane organisation and  
291 raft profile. Although cholesterol levels and flotillin-1 distribution in 18-mo mice on DHA+ diet  
292 were not identical to those of younger mice, the DHA-induced remodelling was visible enough  
293 to suggest an anti-aging effect by DHA on brain membranes. Indeed, an increase of CNTFR $\alpha$   
294 and LIFR $\beta$  protein levels by  $80 \pm 17\%$  ( $p < 0.01$ ) and  $44 \pm 5\%$  ( $p < 0.05$ ), respectively, was  
295 observed in the raft fractions of 18-mo mice on the DHA+ diet, as compared to 18-mo mice on  
296 the StD diet (Fig4). Taken together, these data suggest that dietary DHA could not only prevent  
297 the increase of brain cholesterol in 18-mo mice, but also contributed towards limiting cortical  
298 membrane remodelling and preserving a CNTF-R subunit distribution profile in raft domains  
299 similar to that found in younger mice.

### 300 **3.5. Sustained CNTF signalling and improved CNTF protective effects on learning and memory** 301 **abilities in A $\beta$ -exposed 18-mo mice upon dietary DHA supplementation**

302 The responsiveness of CNTF-activated pathways was evaluated in 18-mo mice that received  
303 either StD or DHA+ diets. Three hours after icv administration of CNTF, CNTF-induced P-STAT3  
304 and P-ERK1/2 levels were found to be significantly higher in 18-mo mice that received the  
305 DHA+ diet, by 2.7 and 3.5 fold ( $p < 0.01$ ), respectively (Fig5A), as compared to those on StD  
306 diet. To determine whether CNTF neuroprotective effects could be improved in 18-mo mice  
307 that received the DHA+ diet, we also measured cognitive performance in the Y-maze following

308 icv injection of soluble A $\beta$  oligomers. While there was no difference in the short-term memory  
309 capacity of vehicle-injected mice on StD or DHA+ diet (Fig5B), A $\beta$ -induced decrease in  
310 alternation behaviour was significantly lower ( $p < 0.05$ ) in the DHA+ group. Co-icv  
311 administration of A $\beta$  oligomers and CNTF significantly reduced A $\beta$ -induced memory  
312 impairment in both diet groups. However, CNTF neuroprotection from the A $\beta$  toxic effect was  
313 significantly higher ( $p < 0.01$ ) in DHA+ mice than in StD mice. More importantly, in 18-mo mice  
314 exposed to A $\beta$ , the combined administration of DHA and CNTF maintained short-term memory  
315 capacities at the same levels as those found either in control 18-mo mice or in 6-mo A $\beta$ -  
316 injected mice (Fig1A), suggesting that dietary DHA provided the means to maintain efficient  
317 CNTF signalling, as well as complete neuroprotection from amyloid stress in the older mice.  
318 Again, no alteration in exploratory, locomotor, visual or motivational capacities was  
319 observed during behavioural evaluation of any group of mice (data not shown).

320

### 321 ***Discussion***

322 The present study demonstrates for the first time that neuronal membrane remodelling  
323 in the brains of 18-mo mice was associated with changes in CNTF receptor distribution and  
324 function, resulting in significant impairment in response to CNTF stimulus and in lower  
325 efficacy of this cytokine as neuroprotective molecule.

326 Cell signalling by neurotrophic factors, including neurotrophin-3, nerve growth factor and  
327 brain-derived neurotrophic factor as well as CNTF, is essential in the development of cognitive  
328 functions such as learning and memory (Croll et al., 1998). Impaired signalling in aged  
329 individuals and in pathological situations has been reported to be involved in memory deficits,  
330 and to result from altered expression of these neurotrophic factors rather than from changes  
331 in expression of their specific receptors (Kononen et al., 1995; Shi et al., 2010). Different

332 studies have shown age-related CNTF signalling impairment in the brain of Alzheimer's  
333 patients or animal models, due primarily to reduced CNTF expression accompanied by either  
334 unchanged or upregulated CNTF-R subunit levels (Nobbio et al., 2009; Soilu-Hänninen et al.,  
335 2010). Different studies based on treatment with neurotrophic factors also demonstrated that  
336 they improve deficit in neurogenesis, synaptic plasticity and cognition in various AD mouse  
337 models (Blanchard et al., 2010; Bolognin et al., 2014; Garcia et al., 2010; Kazim et al., 2014).  
338 In this paper, we report that although icv injection of exogenous CNTF could provide  
339 complete protection from A $\beta$ -induced neurotoxicity and memory impairment in young (6-  
340 mo) mice, this beneficial effect was comparatively less pronounced in older (18-mo) mice.  
341 This clearly indicates that therapeutic strategies focusing on age-related diseases including AD  
342 should be evaluated in appropriate preclinical models that take into account the complex  
343 aging parameter. Despite the considerable time and effort invested in the search for AD  
344 therapeutics, little progress has been made, and there is still no efficient AD treatment  
345 available. The results obtained here clearly demonstrate the need for further research to  
346 elucidate the largely unknown mechanisms involved in the aging process, and most  
347 particularly in the aging brain.

348 With this perspective, we sought to understand the underlying mechanisms focusing on  
349 CNTF-R. No difference was observed between protein levels of CNTF-R subunits of 6-mo and  
350 those of 18-mo mouse brains, thus ruling out the possibility that the altered response to CNTF  
351 could be due to lower CNTF-R levels. Since CNTF-R activation by CNTF was assessed by icv  
352 injection of identical amounts of exogenous CNTF, the impaired response to CNTF was most  
353 likely due to the modified distribution of CNTF-R in the raft and non-raft cortical membrane  
354 fractions. Indeed, as a result of age-related neuronal membrane remodelling suggested by the  
355 distinct membrane fraction profiles and composition, our data revealed that in the cortex of



356 18-mo mice, the three CNTF-R subunits were excluded from lipid rafts and dispersed in non-  
357 raft cortical membrane domains, thereby altering their ability to form an active complex,  
358 which may explain the impaired signalling in response to CNTF that was observed in these  
359 animals. Cellular response to CNTF is triggered in lipid rafts upon specific binding to the GPI-  
360 anchored CNTFR $\alpha$ , which induces the heterodimerisation of gp130 and LIFR $\beta$ , the two signal-  
361 transducing subunits in a receptor complex (Davis et al., 1993; Port et al., 2007) that  
362 transduces intracellular signalling cascades mainly via the activation of Janus kinases and  
363 transcription factors of the STAT family (Heinrich et al., 1998). Thus, resistance to CNTF in the  
364 older mice could result from inability of the subunits to build the active receptor, thereby  
365 impairing CNTF neuroprotective signalling necessary to protect against A $\beta$  oligomer-induced  
366 cytotoxicity in aged brain cortex. In view of these results, as well as the potential  
367 microarchitectural and functional alterations of membrane domains in the older mice  
368 compared to young animals, we would conclude that aging is a fundamental parameter to be  
369 considered when using preclinical animal models to evaluate the responsiveness of  
370 therapeutic targets located in neuronal membranes.

371 Brain cortical membranes of aged mice contained higher cholesterol levels as compared to  
372 those of young individuals, which may have caused deleterious changes. A recent study  
373 reported the redistribution of cholesterol in the membranes of hippocampal neurons from old  
374 mice and rats, which was correlated with biophysical and biochemical changes occurring  
375 within the lipid rafts favouring amyloidogenic APP processing (Marquet-de Rougé et al.,  
376 2013). We also recently reported an increased neurotoxicity of soluble A $\beta$  oligomers in a  
377 dyslipidemic mouse model that is heterozygote for the lipolysis stimulated lipoprotein  
378 receptor, LSR. Old LSR+/- mice exhibited morphological changes in brain membranes (Stenger  
379 et al., 2012), as well as increased cholesterol levels which were correlated with a higher

380 susceptibility to A $\beta$ -induced memory impairment (Pinçon et al., 2015). Taken together, these  
381 data indicate that the observed changes in membrane cholesterol content in the older mice  
382 are consistent with neurodegeneration and increased susceptibility to AD.

383 Our experiments revealed that dietary DHA provided a means to counter both the  
384 membrane cholesterol increase and the non-raft location of CNTF-R subunits in 18-mo mice.  
385 Indeed, 18-mo mice receiving DHA in the diet displayed both CNTF-R subunits clustering within  
386 the lipid raft domains or cortical membranes, as well as lower cortical cholesterol levels similar  
387 to those of the younger mice on StD diet. Lipid rafts are considered an important target for  
388 DHA whose effects are opposite to those of cholesterol with regards to raft physicochemical  
389 properties and organisation as well as to raft-associated protein activities in various cell types  
390 including neuronal cells (Grimm et al., 2011; Langelier et al., 2010; Schley et al., 2007; Shaikh  
391 et al., 2009). DHA treatment has been shown to affect the distribution of presenilin-1 in raft  
392 and non-raft fractions in neuroblastoma SH-SY5Y cells, leading to reduced A $\beta$  production  
393 (Grimm et al., 2011). On the contrary, A $\beta$  production was increased in neuronal cells in the  
394 presence of oxidized DHA, even at concentrations as low as 1% (Grimm et al., 2016).  
395 Therefore, DHA content in neuronal membranes appears to be an essential parameter for  
396 neuronal function and survival.

397 The beneficial effects of DHA supplementation in the diet of the older mice were  
398 demonstrated by the restoration of CNTF-mediated signalling and neuroprotection from A $\beta$   
399 oligomer-induced amyloid stress and cognitive impairment. This was consistent with previous  
400 reports showing that lipid status and metabolism are factors that influence both brain  
401 plasticity as well as susceptibility to A $\beta$  neurotoxicity (Florent-Bécharde et al., 2009, 2007;  
402 Naudí et al., 2015). Also, high dietary intake of fish is associated with a lower risk of AD (Wu et  
403 al., 2015). Fish oil omega-3 PUFAs, and in particular DHA, have been widely reported to play a

404 major neuroprotective role, due to their anti-inflammatory, pro-resolvin and anti-  
405 amyloidogenic properties and *via* multiple pleiotropic mechanisms (Grimm et al., 2011;  
406 Lorente-Cebrián et al., 2015; Oster and Pillot, 2010). Although the age-related alterations in  
407 neuronal membranes reported in this study were not due to changes in DHA concentrations, it  
408 is important to note the structural and functional impact of this PUFA as dietary  
409 supplementation. DHA is known to be incorporated into brain membrane phospholipids,  
410 mainly in phosphatidylethanolamine, and subsequently change the lateral distribution of  
411 cholesterol and proteins (Shaikh et al., 2015), although its preferential incorporation in raft  
412 domains is still under debate. Investigation is underway to determine the specific mechanisms  
413 underlying the DHA protective effect.

414 In conclusion, our results suggest that dietary DHA could provide the means not only to  
415 prevent neuronal membrane changes and associated dysfunction of the aging brain, but also  
416 to maintain reactivity to molecular therapeutic targets that would otherwise be absent or  
417 impaired in the elderly AD patient. DHA-based adjuvant strategies to sustain the appropriate  
418 membrane environment necessary for optimal function of membrane-associated proteins and  
419 receptor therapeutic targets could be used in a poly-therapeutic approach towards the  
420 treatment of AD.

421

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431

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547  
548



549 **Figure legends**

550 **Fig.1. Comparison of CNTF-mediated neuroprotection and related signalling pathways in**  
551 **6-mo and 18-mo mice**

552 **(A)** Mice received icv injections of A $\beta$  oligomer  $\pm$  CNTF or vehicle, followed by a Y-maze test  
553 4 days post-injection. Alternation data are presented as means  $\pm$  SEM (n=6 per 6-mo group  
554 and n=8 per 18-mo group). **(B, C)** Three hours post-injection, CNTF-treated mice were  
555 euthanized and cortical protein homogenates were prepared. STAT3, P-STAT3 **(B)**, ERK1/2 and  
556 P-ERK1/2 **(C)** proteins were analysed by immunoblot. Representative blots are shown and  
557 densitometry was performed. P-STAT3/STAT3 and P-ERK1/2/ERK1/2 relative ratios were  
558 calculated and results are shown as means  $\pm$  SEM (N=3 separate blots, n=3 mice chosen  
559 randomly per group). Significant differences between groups are indicated as \* ( $p < 0.05$ ), \*\*  
560 ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ).

561

562 **Fig.2. Comparison of CNTF-R subunit clustering and location in 6-mo and 18-mo mouse brain**  
563 **cortical membranes**

564 Interactions of CNTFR $\alpha$ , LIFR $\beta$  and gp130 **(A)** with each other or **(B)** with flotillin-1 (Flot) were  
565 studied in the cortex of 6-mo and 18-mo mice by Duolink<sup>R</sup> analysis, and spots indicating  
566 colocalization were detected using fluorescence microscopy and quantified with Image J. A  
567 representative image is presented for each interaction. Results are presented as means  $\pm$  SEM  
568 (N=5 separate fields, n=2 mice chosen randomly per group). Significant differences between 6-  
569 mo and 18-mo animals are indicated as \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ).

570

571 **Fig.3. Effects of age and dietary DHA on flotillin-1 and cholesterol distribution in brain**  
572 **cortical membranes**

573 Brain cortical membranes isolated from 6-mo and 18-mo mice fed StD diet or 18-mo mice fed  
574 DHA+ diet for 10 weeks were treated with Triton X-100 and separated on sucrose gradient as  
575 described in the Materials and Methods section. Distribution of flotillin-1 (**A, B**) and  
576 cholesterol (**A**) was assessed in the various samples and representative profiles are shown for  
577 each group (n=6 mice chosen randomly per group). (**C**) Membrane cholesterol was measured in  
578 total membrane extracts. Results are presented as means  $\pm$  SEM (n=6 mice chosen randomly per  
579 group). Significant differences between groups are indicated as \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ).

580

581 **Fig.4. Effects of age and dietary DHA supplementation on CNTF-R subunit location in raft**  
582 **domains of brain cortical membranes**

583 Raft subpopulations isolated on 5-45% sucrose gradient were identified in fractions exhibiting  
584 the highest flotillin-1 (Flot) and cholesterol levels in membranes isolated from brain cortex of  
585 6- and 18-mo mice fed StD diet or 18-mo mice fed DHA+ diet for 10 weeks. CNTFR $\alpha$  and LIFR $\beta$   
586 protein levels in the raft fractions were quantified by densitometry analysis of immunoblots  
587 relative to Flot. Results are presented as means  $\pm$  SEM (n=6 mice chosen randomly per group).  
588 Significant differences between 6-mo and 18-mo animals are indicated as \* ( $p < 0.05$ ),  
589 \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ).

590

591 **Fig.5. Effects of dietary DHA supplementation on CNTF-activated signalling pathways and on**  
592 **learning and memory abilities of 18-mo mice treated with A $\beta$  oligomers**

593 Mice aged 18-mo were fed StD or DHA+ diet for 10 weeks, followed by icv injection of A $\beta$   
594 oligomers  $\pm$  CNTF. (**A**) Three hours post-injection, CNTF-treated animals were euthanized, and  
595 brain cortical protein homogenates were prepared. Total and phosphorylated STAT3 and  
596 ERK1/2 protein levels were quantified by densitometry of immunoblots. Representative blots

597 are shown and P-STAT3/STAT3 as well as P-ERK1/2/ERK1/2 ratios are shown as means  $\pm$  SEM  
598 (N=3 separate immunoblots, n=3 mice chosen randomly per group). Significant differences  
599 between the diet groups are indicated as \*\* ( $p < 0.01$ ). **(B)** Four days post-injection, spontaneous  
600 alternation in the Y maze was measured as described for Fig1A. Results are shown as means  $\pm$   
601 SEM (n=8 mice per group). Significant differences between groups are indicated as \* ( $p < 0.05$ ),  
602 \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ).