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

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Abbreviations: A β , amyloid- β peptide; AD, Alzheimer's disease; ARA, arachidonic acid; CNTF,
ciliary neurotrophic factor; CNTF-R, CNTF receptor; DHA, docosahexaenoic acid; icv,
intracerebroventricular; ip, intraperitoneal; PBS, phosphate-buffered saline; PUFA,
polyunsaturated fatty acid; StD, standard diet

Abstract

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

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

Highlights:

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Chemicals and reagents

All chemicals and reagents were obtained from Sigma, unless otherwise specified. A β was obtained from Bachem, and soluble oligomers of A β were prepared at the concentration of

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

2.2. Animals and diets

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

2.3. Surgical procedures

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

2.4. Evaluation of short-term memory

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

2.5. Duolink[®] labelling

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

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

2.6. Preparation of protein homogenates

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

2.7. Isolation of lipid rafts

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

9 fractions of 500 μ L were collected consecutively from the top of each tube, with the order being from 1 to 9 as the lowest to highest density. An aliquot from each fraction was immediately mixed with 2 $\times$ Laemmli buffer and denatured by boiling for immunoblot analyses to assess the distribution of various proteins, while the remainder was stored at -20°C for measurement of cholesterol levels using the Amplex[®] Red Cholesterol assay (Invitrogen). Lipid rafts were considered as those fractions containing both flotillin-1 and cholesterol peak levels.

2.8. Immunoblot analyses

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

2.9. Statistical analysis

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

3. Results

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

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

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

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

3.2. Loss of colocation of tripartite CNTF-R complex associated with exclusion from lipid rafts in brain cortex of 18-mo mice

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

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

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

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

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

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

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

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

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

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

3.5. Sustained CNTF signalling and improved CNTF protective effects on learning and memory abilities in A β -exposed 18-mo mice upon dietary DHA supplementation

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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Figure legends

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

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

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

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

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

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

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

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

Fig.5. Effects of dietary DHA supplementation on CNTF-activated signalling pathways and on learning and memory abilities of 18-mo mice treated with A β oligomers

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

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