DHA Improves Cognition and Prevents Dysfunction of Entorhinal Cortex Neurons in 3xTg-AD Mice

Dany Arsenault¹,², Carl Julien¹,², Cyntia Tremblay², Frédéric Calon¹,²*

¹ Faculté de pharmacie, Université Laval, Québec, Québec, Canada, ²Centre de Recherche du CHUL (CHUQ) Québec, Québec, Québec, Canada

Abstract

Defects in neuronal activity of the entorhinal cortex (EC) are suspected to underlie the symptoms of Alzheimer’s disease (AD). Whereas neuroprotective effects of docosahexaenoic acid (DHA) have been described, the effects of DHA on the physiology of EC neurons remain unexplored in animal models of AD. Here, we show that DHA consumption improved object recognition (↑ 12%), preventing deficits observed in old 3xTg-AD mice (↓ 12%). Moreover, 3xTg-AD mice displayed seizure-like a kinetic episodes, not detected in NonTg littermates and partly prevented by DHA (↓ 50%). Patch-clamp recording revealed that 3xTg-AD EC neurons displayed (i) loss of cell capacitance (CC), suggesting reduced membrane surface area; (ii) increase of firing rate versus injected current (F-I) curve associated with modified action potentials, and (iii) overactivation of glutamatergic synapses, without changes in synaptophysin levels. DHA consumption increased CC (↑ 12%) and decreased F-I slopes (↓ 21%), thereby preventing the opposite alterations observed in 3xTg-AD mice. Our results indicate that cognitive performance and basic physiology of EC neurons depend on DHA intake in a mouse model of AD.

Introduction

The loss of cognitive function is the most devastating feature of Alzheimer’s disease (AD) and is likely to involve a dysfunction of entorhinal-hippocampal circuitry [1,2,3,4,5]. The entorhinal cortex (EC) and hippocampus are among the brain regions where neurofibrillary tangles and amyloid-beta (Aβ) plaques first develop in AD patients [6,7,8,9]. Functional and anatomic magnetic resonance imaging reveal a higher activation of hippocampal and EC circuits in patients with mild cognitive impairment compared to Controls, which is followed by a lower activity in AD [10]. The loss of synapses in EC-hippocampus network is also an important structural correlate of cognitive decline in AD patients at an early stage [11,12]. In accordance with human post mortem data, synapse abnormalities and Aβ deposition in these two brain regions have been reported in animal models of Aβ overexpression [13,14], whereas spontaneous nonconvulsive seizure activities in cortical and hippocampal networks of young APP transgenic mice have been documented [5,15]. The more recent triple transgenic model of AD (3xTg-AD) also develops both neurofibrillary tangles and Aβ plaques in the EC and the hippocampus, without significant neuron loss [16,17]. There are thus compelling arguments to hypothesize that the activity of entorhinal and hippocampal neurons is altered early in AD and is partly responsible for the first impairments in cognitive function.

Beneficial effects of docosahexaenoic acid (DHA) have been described in several transgenic animal models of AD [18,19], including improved performance in the Morris water maze paradigm [20,21], prevention of the hyperphosphorylation of tau [22], decreased Aβ levels [21,22,23,24,25] and protection from the loss of synaptic proteins [20,26]. Most epidemiological prospective studies also support an association between higher DHA consumption and lower risk of developing age-related dementia (see discussion). Recent data from clinical assays reveal a potential nutraceutical role for DHA in preventing or ameliorating cognitive decline [27]. However, the effects of DHA on the physiology of cortical neurons within the EC-hippocampus loop, which could underlie these cognitive benefits, remain unexplored in animal models of AD.

The aim of this study was thus to investigate the beneficial effects of DHA in 3xTg-AD mice, an animal model of AD displaying both neurofibrillary tangles and Aβ plaques [17]. To establish functional correlates, we also studied the intrinsic and synaptic properties of EC deep layer neurons from NonTg and 3xTg-AD mice. We selected these neurons because they are key components of the entorhinal-hippocampal network [28,29] while displaying a higher susceptibility to network excitation [28,30]. Our results demonstrate that DHA intake alters intrinsic and synaptic properties of EC deep layer neurons, maintains cell membrane surface area, ameliorates object recognition and reduces the number of seizure-like akinetic episodes observed in 3xTg-AD mice.
Results

High DHA intake increased DHA and decreased arachidonic acid (AA) concentrations in the cortex of NonTg and 3xTg-AD mice

This study included 4 groups of 19 mice, of which 8 were used for electrophysiological and behavioral experiments. As reported previously [31], 3xTg-AD mice were heavier than NonTg mice (31% for mice fed with control diet and 16% for mice fed with high-DHA diet, P<0.001), whereas DHA intake had no effect on animal weight (P = 0.31, Table S1). Consistent with previous reports [20,22,32], high DHA consumption induced an increase in frontal cortex DHA of 15% in NonTg mice and of 27% in 3xTg-AD mice, compared to mice fed control diet (P<0.001, Figure 1A and Table S1). In parallel, mice fed a high-DHA diet had lower brain levels of AA (~22% for NonTg mice and ~25% for 3xTg-AD; P<0.001, Figure 1B) and, therefore, a lower DHA/AA ratio (44% for NonTg and 68% for 3xTg-AD, P<0.001, Table S1). The DHA/AA ratio is important for essential neurobiological functions like neurotransmission and the equilibrium between PUFA metabolites involved in oxidative stress and inflammatory response [33,34,35]. Finally, we found

Figure 1. High DHA intake modulates the fatty acid profile of the frontal cortex of NonTg and 3xTg-AD mice and decreases soluble ptau. High DHA intake from 4 to 14 months of age increased DHA content (A) and decreased AA levels (B). (C) DHA consumption had no effect on the level of Aβ in soluble or insoluble fraction. (D) ptau was decreased in following DHA treatment, without any effect on total tau in soluble fraction and on tau (or ptau) in insoluble fraction. Illustrations of tau/ptau and actin bands in TBS-soluble fraction (E) and tau in FAE fraction (F). Values are expressed as mean ± SEM. Statistical comparisons were performed using two-way ANOVA and correlations were analyzed using a linear regression. Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; FA, fatty acid; FAE, formic-acid extract (insoluble fraction); iAβ, insoluble Aβ; itau, insoluble tau; iptau, soluble phospho-tau; ROD, relative optical density; sAβ, soluble Aβ; N, number of mice; stau, soluble tau; sptau, soluble phospho-tau; TBS, tris-buffered saline (soluble fraction). *P<0.05, ***P<0.001.

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an inverse relationship between AA and DHA concentrations ($r^2 = 0.61, P<0.001$, data not shown).

DHA treatment reduced the phosphorylation of TBS-soluble tau with no significant effects on Aβ

To determine whether DHA consumption altered the development of tau and Aβ pathologies, we quantified the concentration of tau (Western immunoblots) and Aβ (ELISA) in TBS-soluble and detergent-insoluble fractions from brain cortex homogenates (Table S2). We found no significant effects of DHA on Aβ pathologies (Figure 1C, Table S2), whereas DHA intake selectively decreased phosphorylated tau ($\tau_{\text{tau}}$) in TBS fractions ($P<0.05$, Figure 1D-F, Table S2). The $\tau_{\text{tau}}/\tau_{\text{tau}}$ ratio was inversely correlated with the content of DHA in the frontal cortex ($r^2 = 0.26, P<0.05$, data not shown). No significant effect on insoluble tau was observed.

DHA improved mnesic performance deficit caused by Aβ/tau pathologies

The loss of cognitive/memory functions is the earliest devastating feature of AD. To evaluate this in 3xTg-AD mice, we used the novel-object recognition task. These experiments demonstrated that (1) 3xTg-AD performed significantly worse than NonTg mice ($P<0.05$) and (2) the consumption of DHA improved the recognition performance of both NonTg and 3xTg-AD mice ($P<0.05$) (Figure 2A, Table S4). These observations indicate that consumption of DHA improved the object recognition of 3xTg-AD mice, reaching performances comparable to NonTg mice fed with control diet.

The time spent in seconds to explore the object during the first exposition was $88 \pm 3$ ($n = 8$) and $114 \pm 3$ ($n = 8$) for NonTg mice fed with control or high-DHA diet, whereas it was $72 \pm 6$ ($n = 7$) and $72 \pm 5$ ($n = 8$) for 3xTg-AD mice fed with control or high-DHA diet. The adaptativity ratio (time spent exploring at 1 h divided by the time spent exploring during the conditioning phase) was $0.33 \pm 0.05$ ($n = 8$) for NonTg mice fed with control diet, $0.35 \pm 0.03$ ($n = 7$) for NonTg mice fed with high-DHA diet, $0.27 \pm 0.05$ ($n = 7$) for 3xTg-AD mice fed with control diet and $0.22 \pm 0.05$ ($n = 8$) for 3xTg-AD mice fed with high-DHA diet.

DHA partly prevented the occurrence of akinetic episodes in 3xTg-AD mice

We next characterized the locomotor activity of animals in an open field during 10 minutes. We observed diminished horizontal ($P<0.001$) and vertical activities ($P<0.001$) in 3xTg-AD compared to NonTg mice (Figure 2C-D and Table S4), without a significant effect of DHA. The clinging test demonstrated an equal physical performance between each group, ruling out a defect in muscular ability as the main cause of decreased locomotor activity or exploratory behavior (Figure 2B). However, besides these gross alterations in locomotor activity, we observed peculiar discontuities in the behavior of 3xTg-AD mice not present in NonTg animals. Indeed, 3xTg-AD mice exhibited frequent short episodes of low activity. These akinetic moments occurred spontaneously and they lasted several seconds (5–30 s). To quantify these akinetic episodes, we fragmented our recordings of locomotor activity into smaller, 15-sec periods and fixed two thresholds of low activity (Figure 2E-J). Periods of less than 15 or 10 beam interruptions were frequent in 3xTg-AD mice, strongly suggesting the occurrence of seizure-like freezing behavior. Interestingly, DHA intake reduced the number of akinetic episodes seen in 3xTg-AD mice ($P<0.05$, low and very low activity episodes, Figure 2I-J). In summary, our results indicate that the lower locomotor activity of 3xTg-AD mice may be explained by frequent akinetic episodes, a defect partly prevented by chronic DHA treatment.

DHA alters passive properties of EC deep layer neurons and prevents the decrease of CC in 3xTg-AD mice

To identify underlying functional correlates of the behavioral effect of DHA, we next sought to determine whether DHA treatment altered the functional properties of EC neurons within the entorhinal-hippocampal circuitry (Figure S1). Indeed, EC neurons transmit information to the hippocampus [29], thereby playing an important role in memory [36,37,38]. We first studied intrinsic properties of EC neuron using patch-clamp recording in current clamp, focusing on passive electrophysiological parameters. We observed that CC, an indicator of total membrane area [39,40,41], was decreased in neurons from 3xTg-AD mice compared to NonTg ($P<0.05$; Figure 3A, 3B). DHA treatment prevented this decrease and had an overall increasing effect on CC in all animals ($P<0.05$). In contrast, IR was lower in 3xTg-AD neurons ($P<0.05$; Figure 3C and 3D) and higher in DHA-treated animals ($P<0.05$). The flux of ions crossing the membrane via ion channels contributes to total IR; increased movement of ions can lead to a lower resistance. Thus, the variation of IR observed here was likely a consequence of changes in CC since we found no significant difference in membrane conductance (Figure 3E). The strong correlation between IR and CC supports a tight link between the two passive properties of EC neurons ($r^2 = 0.42, P<0.05$ and $r^2 = 0.36$ for NonTg mice, $P<0.001$ and $r^2 = 0.28$ for 3xTg-AD mice). In addition, the relationship between CC and $\tau_{\text{tau}}$ content suggests an association between tau pathology and membrane surface atrophy (Figure 3F). Overall, these observations strongly suggest that DHA increased the surface area of EC neurons, preventing the decrease observed in 3xTg-AD mice.

In addition, we observed that DHA consumption led to the hyperpolarization of EC neurons of NonTg mice ($P<0.001$, Figure 3G), an effect not present in 3xTg-AD mice. Interestingly, the resting potential inversely correlated with DHA/AA concentration ratio ($P<0.05$; Figure 3H) in 3xTg-AD mice. Such positive relationship in 3xTg-AD mice between resting potential and Aβ concentrations (Figure 3H) suggests that accumulation of Aβ may have blocked the hyperpolarizing effect of DHA in 3xTg-AD mice. Consistent with a hyperpolarizing effect of DHA, we observed that the number of spontaneously active neurons in NonTg mice fed with DHA diet was approximately half of those from animal fed control diet (Table S6).

Expression of transgenes, but not DHA intake, alters action potential characteristics

To further investigate implications of DHA or APP/PS1/tau transgenes in the physiology of entorhinal cortex deep layer neurons, we quantified key characteristics associated with a single action potential (Figure 4A, 4B and 4C, Table S3). Firstly, we observed a decrease in the amplitude of action potentials ($P<0.01$, Figure 4D), an increase of undershoot voltage variation ($P<0.001$, Figure 4E) and a reduction of post-spike hyperpolarization ($P<0.05$, Figure 4F) in 3xTg-AD neurons, compared to NonTg mice. DHA intake had no effect on these parameters. In summary, action potentials of 3xTg-AD EC neurons displayed important alterations.

Increased firing activity of 3xTg-AD neurons: partial preventive effect of DHA

To characterize the effect of DHA on firing properties of EC neurons, we next studied in current clamp two fundamental
features of neuronal activity, (1) the F-I curves and (2) the intensity of depolarization required to deliver an action potential (rheobase). First, we found that the F-I curves were increased in 3xTg-AD mice [P<0.001], but decreased by DHA treatment (P<0.01, Figure 5A–C). This result indicates that firing activity of 3xTg-AD neurons was increased compared to NonTg neurons, whereas DHA had the opposite effects in all animals. We also found a negative relationship between CC and F-I curves (P<0.05 and r² = 0.01, data not shown). Second, an increase of rheobase was detected in DHA-enriched neurons from NonTg mice (P<0.01, Figure 5D), whereas DHA had no effect on rheobase in 3xTg-AD animals. This is consistent with the hyperpolarizing effect of DHA on resting potential restricted to NonTg mice presented above, and further supported by a significant negative correlation between both parameters (P<0.001, r² = 0.61). In summary, DHA consumption decreased the F-I curves of EC neurons through a CC-dependent mechanism, while Aβ/tau pathologies increased firing activity.

Functional and molecular dysfunctions of glutamatergic synapses in 3xTg-AD mice

Cerebral hyperactivity and defective network activity were reported in APP transgenic models of AD [3,15] and AD patients [42,43,44]. Moreover, molecular impairments within glutamatergic synapses were found in AD patients and mouse models of AD [2,20,26,45,46]. To further investigate synaptic dysfunctions in 3xTg-AD mouse, we measured the sEPSC of EC neurons. First, we found that 3xTg-AD neurons displayed more sEPSC than NonTg (Figure 6A–C, P<0.001), suggesting the presence of a persistent hyperactivity of glutamatergic synapses in EC from 3xTg-AD mice and consistent with a network dysfunction. Interestingly, DHA treatment did not prevent synaptic hyperactivity of EC neurons, but rather increased the frequency of sEPSC in all animals (Figure 6C, P<0.01). Amplitude of sEPSC was not modulated by dietary DHA and expression of transgenes, suggesting that the depolarizing strength of each synapse remained unchanged for all groups (Figure 6D). The higher frequency of sEPSC is likely related to the ability of DHA to increase neurite outgrowth [47] and dendritic spine density [48,49], which would promote synapse formation, thereby increasing detection of sEPSC [50]. The increase of DHA-induced CC and the significant correlation between CC and sEPSC in both genotypes support the latter idea (Figure 6E–F, P<0.05 and r² = 0.10 for NonTg P<0.01 and r² = 0.13 for 3xTg-AD). To assess possible molecular correlates, we quantified the level of synaptophysin and PSD95 proteins, respectively pre- and postsynaptic markers of glutamatergic synapses [51]. PSD-95 is involved in the regulation of the ratio of excitatory versus inhibitory presynaptic contacts through neurexin-dependent pathways, suggesting that PSD-95 is involved in network activity [52,53]. The apparent translocalisation of PSD-95 from membrane to cytosolic fractions (Figure 6G) observed here might thus be a compensatory mechanism resulting from the hyperactivity of glutamatergic synapses. Such loss of PSD-95 is consistent with previous analyses in the membrane fraction from the cortex of AD patients or Tg2576 mice [20]. On the other hand, absence of synaptophysin alteration in 3xTg-AD mice (Figure 6H) is consistent with report on other animal model of AD [20,54] and suggest that the rise of sEPSC was not related to a massive change in synapses number. DHA intake had no effect on both pre- and postsynaptic markers.

Acute application does not mimic the effect of chronic DHA treatment

We then verified whether an acute exposure to DHA could reproduce the effects of the chronic DHA treatment. In vitro or in vivo data show that DHA incorporates into cell membrane phospholipids within seconds [55,56,57]. We thus incubated brain slices with DHA during 10 minutes followed by the same patch-clamp experiments described above (Figure 7A–B and Table S5). Previous studies have shown that DHA incorporates within seconds into membrane phospholipids, where its distribution reaches equilibrium within 10 minutes [53]. We found that acute application of DHA did not alter the resting potential (Figure 7C), CC (Figure 7D), input resistance (Figure 7E) or F-I curves (Figure 7F). These experiments strongly suggest that the effects of DHA treatment described above were not caused by binding to an ionic receptor or by modifying membrane properties. Rather, long-term mechanisms such as morphology alteration, gene transcription or long-term consequence of lipid profile modification (such as a decrease in AA and its derivatives) are likely to be necessary to explain the chronic effect of DHA on passive and active properties of EC deep layer neurons.

Discussion

Neuronal network dysfunction within the EC - hippocampus loop has been hypothesized to underlie cognitive dysfunction in AD. The results presented here indicate that EC neurons from mice genetically programmed to develop extensive Aβ and tau pathologies display alterations of intrinsic properties consistent with reduced membrane surface (atrophy) and show increased neuronal activity. The effects of DHA intake on classical markers of AD neuropathology in 3xTg-AD mice were limited to a decrease of phosphorylated tau, in accordance with a previous report using the same transgenic animals fed during a similar amount of time, but with almost twice the daily dose of DHA [22]. On the other hand, DHA exerted powerful protective effects against alterations of intrinsic properties and deterioration of cognitive performance in 3xTg-AD mice.

Defects in intrinsic properties of EC neurons in 3xTg-AD mice: protective effect of DHA against membrane surface atrophy

Neuronal atrophy and reduction in synapses and/or dendrites are well-recognized features of AD, which have been often found in animal models as well [12,14,58,59]. Since cellular atrophy is associated with decreased cell surface, we quantified membrane surface area of EC neurons, using electrophysiological measure-
ment of CC [39,40,41]. Therefore, the loss of CC in 3xTg-AD mice observed here is likely attributed to a diminution of cellular surface. Conversely, chronic intake of DHA increased CC in all animals, thereby fully correcting the decrease detected in 3xTg-AD mice. Such a hypertrophic effect of DHA is in agreement with studies reporting that DHA increases the density of synaptic spines in vivo [48,49] and stimulated the growth of neurites in vitro [60], an effect attributed to binding to syntaxin-3 [61]. Expected

Figure 3. Effects of DHA intake on passive properties of EC deep layer neurons from NonTg and 3xTg-AD mice. (A) CC of EC neurons increased with DHA intake and decreased with 3xTg-AD expression. The slope of the injected current x time constant versus voltage variation plot was used to estimate CC. Typical examples of slopes are illustrated in panel B. (C) DHA decreased, whereas 3xTg-AD transgenes increased the input resistance following the injection of a hyperpolarized current. Input resistance was determined from the slope of the voltage variation versus injected current plot and examples of current-voltage slope from one cell per group are illustrated in panel D. (E) Membrane conductance was not modulated by diet or transgene expression. (F) Inverse relationship between cell capacitance and ptau in 3xTg-AD mice. (G) DHA intake hyperpolarized EC neurons by altering their resting potential from ~60 mV to ~70 mV, an effect only present in NonTg mice. (H) Positive relationship between resting potential and TBS-soluble total Aβ (Aβ40 + Aβ42) in 3xTg-AD mice. Values are expressed as mean ± SEM. Statistical comparisons were performed using two-way ANOVA (CC and input resistance) and one-way ANOVA followed by Tukey-Kramer posthoc test (resting potential; variable interaction). Correlation was performed using a linear regression. Recorded neurons were obtained from 8 mice per group. Abbreviations: CC, cell capacitance, n, number of recorded neurons. *P<0.05; **P<0.01; ***P<0.001.

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consequence of such DHA-induced morphologic modulations would logically include enhanced glutamatergic synaptic activity and increase sEPSC frequency as observed here in EC neurons and reported in embryonic neuronal cultures [50]. Such increases of CC and sEPSC frequency could lead to a better network integration of DHA-treated neurons, enabling them to collect and process a larger flow of information.

Neuronal hyperactivity in the 3xTg-AD mouse model: complex action of DHA

In recent years, mounting evidence support the hypothesis that defective neuronal activity plays an important role in the development of symptoms in early AD [4,5,44]. Functional magnetic resonance was used to detect an hyperactivity of EC and hippocampus during the early stages of the disease, before full blown neurodegeneration occurs [10]. Hyperactive episodes akin to non-convulsive epileptic seizures were clearly demonstrated in a mouse model of AD [5,15]. Neuronal activity and the formation of Aβ peptides are part of a vicious circle evidenced in organotypic culture [62], whereas populations of hyperactive neurons surrounding senile plaques were evidenced with calcium imaging in animal models [63].

However, little is known about cellular mechanisms leading to such cerebral hyperactivity in AD. Our study highlights potential mechanisms deriving from intrinsic properties of neurons. Most importantly, an increase in firing activity was recorded in 3xTg-AD neurons, for which at least two explanations can be proposed. On one hand, the loss of surface area, as suggested by lower CC, is likely to have altered current dispersion throughout the membrane of 3xTg-AD neurons. Hence, a fixed depolarizing input produces a higher current density and voltage variation in smaller neurons, as schematized in Figure S2 and previously shown in modelized motoneurons [64]. On the other hand, the action potential modifications observed in 3xTg-AD neurons could contribute to increase their firing rate. Indeed, the lower amplitude of action potential recorded in 3xTg-AD EC neurons may be the results of an increase in the voltage undershoot. Such key modifications in action potential are known to reduce calcium-dependent potassium current and, consequently, enhance firing activity [65,66,67,68,69]. The reduction of post-spike hyperpolarization, a calcium-dependent current [68,70] observed in 3xTg-AD animals, support this idea.

The effect of DHA on neuronal activity was more complex. In NonTg animals, DHA induced hyperpolarization of EC neurons, consistent with reported antiarrhythmic and anticonvulsant effects of DHA [71,72,73,74,75]. Accordingly, DHA was also found to increase the rheobase in NonTg mice. Overall, these observations support the idea that DHA downregulates neuronal activity. However, the effect of DHA on resting potential or rheobase were not observed in 3xTg-AD mice. Similar alterations of resting potential were reported in neocortical cells/dentate granule cells of APP/PS1 mice model of AD [15] and in vitro Aβ application was known to depolarize neuron [76,77], suggesting a key role of Aβ pathology in alteration of resting potential. In addition, DHA did reduce firing activity following sustained activation (F-I curves), but not sufficiently to fully prevent the increase seen in 3xTg-AD mice. Here again, altered dispersion of injected current on larger DHA-enriched neurons is a compelling explanation for the decreasing effect of DHA on F-I curves [64]. The correlation between CG and F-I curves stands in agreement with this proposition. In summary, DHA may reduce neuronal activity through hyperpolarization of the cell membrane and by reducing the firing activity in NonTg mice. However, these effects of DHA
were partly blunted by Aβ/tau pathologies and not sufficient to fully prevent hyperactivity of EC neurons in 3xTg-AD mice.

At the synaptic level, we observed a rise of sEPSC frequency in 3xTg-AD mice, without any concurrent change in synaptic input indexed with synaptophysin. This indicates higher spontaneous activity of afferent excitatory synapses, consistent with EC hyperactivity. The lower rheobase and the steeper F-I curves in 3xTg-AD neurons suggest an involvement of neuronal activity in the observed synaptic hyperactivity. Reciprocally, synaptic hyperactivity is known to increase glutamate-dependent conductance, resulting in cell depolarization and an increase of F-I curves [78,79,80,81]. Such a synergy between synaptic and intrinsic properties may favor unrestrained brain activity and thus provides an interesting explanation for the hyperactivation of the entorhinal-hippocampal circuitry observed in the early stages of AD.

DHA modulates intrinsic properties of EC neurons: cognition enhancing and antiseizure activities?

It is well known that EC neurons transmit cerebral information to the hippocampus [29], a region strongly involved in memory...
DHA Prevents Cellular Dysfunction in AD

A. NonTg Control
   High DHA
   
B. 3xTg-AD Control
   High DHA
   
C. Graph showing control vs high DHA for frequency (Hz)
   - NonTg
   - 3xTg-AD
   
D. Graph showing control vs high DHA for amplitude (pA)
   - NonTg
   - 3xTg-AD
   
E. NonTg
   Cell capacitance (pF)
   Frequency (Hz)
   - NonTg
   - 3xTg-AD
   
F. 3xTg-AD
   Cell capacitance (pF)
   Frequency (Hz)
   - 3xTg-AD
   
G. Western blot analysis for TBS-PSD95, TBS-Actin, DS-PSD95, DS-Actin, DHA, 3xTg-AD
   - Control
   - High DHA
   
H. Western blot analysis for TBS-Synaptopo, TBS-Actin, DS-Synaptopo, DS-Actin, DHA, 3xTg-AD
   - Control
   - High DHA

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and cognition. The defects in novel object recognition of 13-month-old 3xTg-AD mice observed here is consistent with at least two previous reports [82,83]. Chronic dietary treatment with DHA improved object recognition and 3xTg-AD mice treated performed as well as NonTg animals on control diet. Such protective action of DHA against deterioration of cognition is in agreement with previous studies in APP mouse models of AD, which develop only Aβ pathology [20,21], and with clinical trials on cognitive impairment associated with aging.

Indeed, recent clinical assays report improvement of cognitive performance following n-3 PUFA intake, in cognitively normal or individuals with mild AD or MCI [27,84,85,86], but not in moderate or advanced AD [84,85,86,87,88,89]. In addition, our data is consistent with most case-control and longitudinal observational studies based on reported food consumption or blood fatty acid measurements, which support an association between consumption of fish and a lower risk of developing dementia [90,91,92], although no such association was detected in two large cohorts [93,94].

One of the most interesting observations reported here are the frequent episodes of low activity recorded specifically in 3xTg-AD mice. These short sequences of low activity likely had a central origin not caused by motor deficits, as 3xTg-AD and NonTg mice performed similarly in grip strength test. Seizure-like activity has been previously evidenced in animal models of AD, mainly based on EEG readouts and calbindin-D28k immunoreactivity [5,15]. These anomalies were specifically associated with abnormal network excitability and remodeling of inhibitory circuits in the hippocampus-cortical loop [5]. Reminiscent of the present observations, Palop and al. reported that during EEG-confirmed seizures, mice were immobile and subsequently resumed their exploratory behavior [5]. The higher spontaneous activity of excitatory synapses found in the entorhinal cortex of 3xTg-AD mice is consistent with the contention that cerebral hyperactivity coincides with the observed freezing behavior. Most interestingly, chronic DHA treatment significantly reduced the occurrence of these episodes of low activity in 3xTg-AD mice, consistent with previous evidence of anti-seizure action of DHA [71,72,73,74,75]. Since remodeling of neuronal circuits in the hippocampus may be instrumental in the neuronal network abnormalities seen in APP mice [5], the regulating effect of DHA on CC and firing activity in EC neurons may underlie its potential preventive effect of DHA on cognitive decline and akinetic episodes observed here in the 3xTg-AD mouse.

Conclusion

In summary, our data show that 3xTg-AD mouse exhibited poor performance in object recognition task and frequent episodes of low activity akin to a seizure-like freezing behavior. These cognitive defects were associated with series of alterations in passive, active and synaptic properties of EC neurons, including (i) reduced CC, (ii) increased firing activity and (iii) enhanced sEPSC frequencies. Chronic treatment with DHA exerted a full, or at least partial, preventive action against these electrophysiological and behavioral defects, except on the basal hyperactivity of EC neurons. Overall, our results show that chronic DHA treatment has a direct effect on neuronal function, which is highly relevant to its cognition-enhancing properties and potential therapeutic effects against epilepsy and AD.
batch to batch variations. The dietary treatment started at 4 months of age and lasted until the mice were sacrificed between 12 and 14 months of age (13.2 ± 1.6 months) for biochemical analysis (up to 19 mice per group) and electrophysiological experiments (8 mice per group). This age range was chosen to avoid any interaction with developmental or maturation processes in younger ages and to gather endpoints at an age when Aβ/tau pathology and cognitive deficits are readily detectable. To perform biochemical analysis, animals were perfused with PBS buffer (pH 7.2) containing protease inhibitors (1 tablet/200 ml; Sigma, St.Louis, MO). Brain regions were dissected from left hemisphere. Frontal cortex (≈20 mg) was used to estimate brain content of fatty acids whereas the rest of the cortex (parietal, temporal and occipital cortex) was pooled for biochemical measurements.

**Preparation of tissue samples**

After adding 8 volumes of Tris-buffered Saline (TBS) containing Complete™ protease inhibitors cocktail (Roche, Indianapolis, IN), 10 µg/ml of pepstatin A, 0.1 mM EDTA and phosphatase inhibitors (1 mM each of sodium vanadate and sodium pyrophosphate, 50 mM sodium fluoride), frozen samples were sonicated briefly (3×10 s) and centrifuged at 100,000×g for 20 min at 4°C to generate a TBS-soluble intracellular and extracellular fraction (soluble fraction). The TBS-insoluble pellet was sonicated in 5
Lipid extraction and gas chromatography

Volumes of lysis buffer (150 mM NaCl, 10 mM NaH2PO4, 1% Triton X-100, 0.5% SDS, and 0.5% deoxycholate) containing the same protease and phosphatase inhibitor cocktail. The resulting homogenate was centrifuged at 100,000 g for 20 min at 4°C to produce a lysis buffer-soluble fraction (detergent-soluble fraction). The pellets (detergent-insoluble fractions) were homogenized in 175 μl of 90% formic acid followed by a short sonication (3 x 10 s). The resultant suspension was centrifuged (15,000 x g; 4°C; 20 min) and 20 μl of the supernatant was neutralized with 1:13 dilution of Tris-base 2 M (pH 10) to be used for ELISA (see below). The rest of the supernatant was dried out by SpeedVac (Thermo Savant, Waltham, MA), solubilized in Laemmli’s buffer and processed for Western immunoblotting [31].

ELISA

Insoluble Aβ40 and Aβ42 were measured by the Aβ [1–40] and [1–42] ELISA kits (Biosource, Camarillo, CA). Soluble Aβ40 (kit II) and Aβ42 (kit high-sensitive) were measured using Human Aβ ELISA kits from WAKO (Osaka, Japan). The two ELISAs were performed according to manufacturer’s recommendations and the plates were read at 450 nm using a Synergy™ HT multi-detection plate reader (Biotek, Winooski, VT) [31].

Western immunoblotting

For Western immunoblotting [31], protein concentration was determined using bicinchoninic acid assays (Pierce, Rockford, IL). Equal amounts of protein per sample (15 μg of total protein per lane) were added to Laemmli’s loading buffer, heated to 95°C for 5 min before loading, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted onto PVDF membranes (Millipore, Billerica, MA) before blocking in 5% nonfat dry milk and 1% bovine serum albumin (BSA) in PBS-Tween 20 for 1 h. Membranes were immunoblotted with appropriate primary and secondary antibodies followed by chemiluminescence reagents (Lumiglo Reserve, KPL, Gaithersburg, MD). Band intensities were quantified using a KODAK Image Station 4000 MM Digital Imaging System (Molecular Imaging Software version 4.0.57, Carestream Health, Rochester, NY). The following antibodies were used in this study: anti-actin (ABM, Richmond, Canada), anti-CAMKII (Stressgen, Victoria, Canada), anti-cofilin (Cell signaling technology, Boston), anti-Drebrin (MBL International, Woburn, MA), anti-group IV iPLA2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PAK1 (Invitrogen, Ontario, Canada), anti-PAK3 and anti-total PAK (Cell signaling technology, Danvers, MA), anti-phospho PAK: P141 (BioSource, Camarillo, CA), anti-PSD-95 (Millipore, Billerica, MA), anti-SNAP-25 (Covance, Princeton, NJ), anti-synaptophysin (Millipore, Billerica, MA), anti-synaptotagmin 3 (Sigma, St. Louis, MO), anti-tau-13 (Covance, Princeton, NJ), anti-tau CP13 (phosphorylated at serine 202/threonine 205, gift from Dr Peter Davies, Albert Einstein College of Medicine, New York, NY).

Lipid extraction and gas chromatography

Since the incorporation of dietary DHA is similar in different regions of the cortex [102,103,104,105], lipid profiles were determined only in the frontal cortex. Experiments were performed as previously described [31,32]. Approximately 20 mg of frozen frontal cortex from each mouse was used for lipid extraction. Weighed brain tissues were homogenized with BHT-Methanol (Sigma, St. Louis, MO) and two volumes of chloroform (J.T. Baker, Phillipsburg, NJ) and 0.5 volume of NaH2PO4 (0.2 M)-buffer solution were added to the resulting homogenate. After centrifugation at 3500 RPM, the lower layer (chloroform fraction) was collected [106]. Lipid extracts were transmethylated with BF3-MeOH (Alltech, State college, PA) at 100°C for 60 min. After cooling down, 2 ml of water and 2 ml of hexane [J.T. Baker] were added. After homogenization and centrifugation at 3500 RPM, the upper layer (hexane fraction) was collected, dried down to about 100 μl and transferred to a gas chromatography autosampler vial and capped under nitrogen. Fatty acid methyl esters in brain tissue were quantified on a model 6890 series gas chromatograph (Agilent Technologies, Palo Alto, CA) using a FAST-GC method. One microliter of each sample was injected at a 25:1 split ratio. Peak identification of fatty acid methyl esters was performed by comparison to the peak retention times of a 28-component methyl standard (Nu-Chek Prep, Elysian, MN) [107].

Slice preparation for electrophysiology recordings

Horizontal brain slices of NonTg (8 controls and 8 high DHA) and 3xTg-AD (8 controls and 8 high DHA) mice were prepared from the right hemisphere as previously described [108,109]. Briefly, mice were deeply anesthetized with ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip), and decapitated. The brain was removed quickly (<60 s) and placed in an ice-cold solution containing (mM): 210 sucrose, 3.0 KCl, 1.0 CaCl2, 3.0 MgSO4, 1.0 Na2HPO4, 26 NaHCO3 and 10 glucose, saturated with 95% O2 and 5% CO2. Horizontal slices of 300 μm were cut from inferior to superior brain with a vibrating tissue slicer (VT 1000 s, Leica, Wetzlar, Germany), and kept in artificial cerebral spinal fluid (ACSF) containing (mM): 124 NaCl, 3.0 KCl, 1.5 CaCl2, 1.3 MgCl2, 1.0 Na2HPO4, 26 NaHCO3 and 20 glucose, saturated with 95% O2 and 5% CO2 at room temperature (21–23°C). Slices were allowed to recover for at least 1 h before recording.

Patch-clamp recording

For recording, a slice was transferred to a submerged-type chamber and continuously exposed to ACSF heated to 30–32°C, saturated with 95% O2 and 5% CO2 and flowing at a rate of 2.0±0.2 ml/min. When mentioned, DHA (Cayman Chemical Compagny, Michigan City, IN) at a concentration of 2 μM was added to the extracellular solution. This concentration of DHA is under its critical concentration for micelle formation (CMC) [110,111,112]. DHA in solution achieves equilibriun into the membranes phospholipid within 10 minutes [53,113] by an adsorption mechanism [114]. The slices were viewed first with a 4x objective and deep layer of EC was located beside hippocampus (Figure 1). In general, two slices could be recorded per hemisphere. Large deep layer neurons in the EC were then viewed under near-infrared illumination with a 40x water-immersion objective (Fuor, 40x, 0.80W, Nikon, Mississauga, ON, Canada) and a CCD camera (IR-1000, MTI, Michigan City, IN).

Experiments were conducted at 30–32°C. Patch pipettes were pulled from thick wall borosilicate glass (1.5/0.84 mm, WPI, Sarasota, FL) on a horizontal puller (P-97, Sutter Instruments, Novato, CA). The pipette solution contained (mM): 100 KMeSO4, 15 KCl, 4 ATP-Mg, 10 creatine phosphate, 10 Hepes, 0.5 EGTA [pH 7.2 with KOH]. Electrodes had resistances between 2 and 5 MΩ. Liquid junction potential, estimated to be +5 mV, was not corrected. The seal resistance was greater than 2 GΩ. Whole-cell recordings were made at the soma with a Multiclamp 700A amplifier (Molecular devices, Sunnyvale, CA). The series resistance, usually between 7 and 20 MΩ, was compensated using the bridge balance in current-clamp and was not compensated in voltage-clamp. Experiments were conducted using the Axograph 4.9 program (Molecular devices). Data were digitized at 8 or

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were rejected.

**Selection of neurons and data analysis for electrophysiology experiments**

Among 186 recorded cells (49 NonTg/control, 34 NonTg/high-DHA, 52 3xTg-AD/control, 51 3xTg-AD/high-DHA), 167 neurons had sustained spiking (41 NonTg/control, 32 NonTg/high-DHA, 47 3xTg-AD/control, 47 3xTg-AD/high-DHA) whereas the rest of neurons had non-sustained spiking. To study neurons with the same electrophysiological properties, we analyzed only neurons having a sustained spiking. AxoGraph 4.9 and Origin 7 (OriginLab, Nothampton, MA) software programs were used for analysis. Passive and active properties were tested in I-clamp, whereas spontaneous excitatory postsynaptic currents (sEPSC) were quantified in V-clamp with a voltage clamp to -60 mV. The firing rate was estimated by counting the number of spikes during the step and the result was plotted versus the amplitude of the injected current (F-I graph). The slope (F-I curve) was calculated for the firing frequencies included between 0 and 15 Hz by using linear regression analyses. The rheobase was estimated graphically on the F-I graph. Action potentials were detected using the event detection package of the AxoGraph and the results were analyzed with Origin 7. The action potential was characterized in the last 1.5 second and the threshold was measured using a cursor, by inspecting a 5 ms segment around the rising phase of the action potential. The input resistance (R) was estimated from the graph slope of voltage variation (V) versus hyperpolarizing current injection (I). The calculation was derived from the formula \( V = R \cdot I \). The injected current duration was 400 ms and hyperpolarized current amplitudes were 50, 100, 150 and 200 pA. The cell capacitance (CC) was estimated by the formula \( t \cdot T = CC \cdot V \) (i.e. 1st order RC circuit), where \( T \) is the time constant of voltage variation (measured by fitting a single exponential function), CC corresponds to the linear slope of the graph displaying the relationship between \( t \) and \( V \). We measured CC in I-clamp because this recording mode generated more accurate values than V-clamp [41].

The membrane conductance (Gm) was calculated from the resistance and CC of the neuron using the formula \( Gm = R^{-1} \cdot CC^{-1} \). The sEPSC were automatically detected using the event detection package of axograph. This package uses a pre-established template (amplitude: 5 pA, rise-time: 0.6 ms, decay time: 3.5 ms, baseline: 1.5 ms, length: 4 ms) for detecting synaptic events. Events with amplitude below 4 pA were rejected.

**Locomotor activity**

Horizontal and vertical activities were monitored for 10 min at night (complete darkness) in an automated Omitech Digiscan apparatus (AccuScan Instruments, Columbus, OH). The box had a dimension of 20 cm × 20 cm. Horizontal activities were detected by a set of infrared sensors ranged in XY axes while vertical activities were detected only in the X axis. The distance between each detector was 2.5 cm. Recordings and data processing were made using the software VersaMax 4.20 (Molecular Devices). Horizontal and vertical activities were expressed by the number of beam interruptions, split in 1.5-s intervals. For instance, we evaluated the number of beam interruptions for a few simple movements: 2–4 for a head movement (left to right), 4–5 for a 90-degree rotation and 3–6 for a 5-cm movement.

**Exploration/recognition test**

The object recognition task is based on the spontaneous tendency of rodents to explore a novel object longer than a familiar one. We opted for a non-stressful cognitive test because 3xTg-AD mice display a high anxiety level [83,115], which could confuse the interpretation of their behavioral response in more stressful cognitive tasks (such as the Morris water maze). During the conditioning phase, animals were placed individually in a standard mouse cage (29.2 cm × 19 cm × 12.7 cm) containing two objects for a period of 10 min. The objects were comparable in size, texture and shape complexity. The objects were either rectangular (4 cm × 4 cm × 6 cm), cylindrical (4 cm diameter × 6 cm high), conical (3.8 cm in diameter at the base and 1.8 cm in diameter in height, 4.5 cm high) or triangular (2.5 cm per side × 6.5 cm high). The test was repeated one hour later with a known object and a new one. Object recognition was investigated after one hour because the recognition index of our control mice was significantly decreased after this time, thus reducing the sensitivity of the test. The time spent exploring and sniffing each object was recorded. The ratio of time spent exploring the new versus the known object was used as an indicator of recognition and memory [116,117,118,119]. Animals whose exploration was not considered sufficient to allow recognition (20 s per item) were rejected from the analysis.

**Physical and muscular evaluation**

Animals were suspended by the forelimbs to a stretched cable of 2 mm diameter, and the time mice remained clinging to the cable was measured to assess muscular tone. Each animal underwent two tests and the best score was kept.

**Statistical Analysis**

Values were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using a two-way ANOVA for the study of two variables simultaneously. When variable interaction was detected, statistical comparisons were performed using a one-way ANOVA followed by Tukey-Kramer post-hoc test. When only 2 groups were compared, statistical comparisons were performed using an unpaired Student’s t-test. The DHA application protocol was analyzed using paired Student’s t-test. Correlation analyses were performed using linear regressions. The relationship between molecular parameters (fatty acid content, synaptic and pathologic markers) and cellular electrophysiological parameters was calculated by attributing one electrophysiological value par animal (corresponding to the average of a minimum of two recording cells). Correlations between electrophysiological parameters were carried out using values from single neurons.

**Supporting Information**

**Figure S1 Tissue preparation for electrophysiological recordings.** (A) Side-view of the mouse brain. The black line represents the 300-μm horizontal section used in this study. (B) Horizontal mouse brain section stained with haematoxylin nuclear counterstain. Whole-cell recordings (REC) were made in deep layer of EC. Abbreviations: CPu, caudate putamen (striatum); Hipp, hippocampus. (TIFF)

**Figure S2 Cellular and membranous models to explain the modulation of CC on current density and voltage variation.** [A, B] The injected current is dispersed throughout the membrane surface. Thus, a neuron with a larger membrane surface area will have a smaller current density, since membrane conductance is kept constant. Consequently, this smaller current density produces a smaller voltage variation. Mathematical
formulas to explain modulation of CC on resting potential at cellular and membranous level were given in panels C and D. 

Formula abbreviations: IR: input resistance (cellular resistance); CC: cell capacitance, \( G_{\text{c}} \): cell conductance, \( G_m \): membrane conductance, \( I_{\text{inj}} \): injected current, \( I_m \): membrane current or current density, AV: voltage variation, \( \% \): proportional.

(TIF)

**Table S1** Summary of the effects of DHA dietary treatment and transgene expression effects on proteins, animal weight and lipid content in the frontal cortex. Values for synaptic markers were normalized with actin. Values are expressed as mean ± SEM. Statistical comparisons were performed using a two-way ANOVA for the study of two variables simultaneously. When variable interaction was detected, statistical comparisons were performed using a one-way ANOVA followed by Tukey-Kramer post-hoc test. Abbreviations: AA: arachidonic acid; CAMKII: calcium/camodulin-dependent protein kinase II; DHA, docosahexaenoic acid; DS: detergent-soluble (membrane fraction); TBS: Tris buffer saline (cytosolic fraction); PAK: p21-activated kinase; PSD95: postsynaptic density-95. *P<0.05; **P<0.01 (significantly different of NonTg mice fed with control diet). #P<0.05 (significantly different of 3xTg-AD mice fed with high-DHA diet).

(TIF)

**Table S2** Summary of DHA dietary treatment and transgene expression effects on pathologic markers in 3xTg-AD mice. Values of tau in TBS-soluble fractions were expressed as a ratio over actin quantified on the same blots, TBS-soluble A\(\beta\)40 and A\(\beta\)42 values were expressed as picograms per milligram of protein while FAE-A\(\beta\) values were expressed as picograms per milligram of tissue. Tau in FAE fractions was quantified by relative optical density. Values are expressed as mean ± SEM and number of mice analysis is indicated between brackets. Statistical comparisons were performed using an unpaired Student’s t-test. Abbreviations: TBS: Tris buffer saline; FAE: formic-acid extract (detergent-insoluble fraction).

(TIF)

**Table S3** Summary of electrophysiologic properties of deep layer pyramidal neurons from NonTg and 3xTg-AD mice fed with control or high-DHA diet. Electrophysiologic data were obtained from 8 mice per groups. Values are expressed as mean ± SEM and number of recorded neurons is indicated between brackets. Statistical comparisons were performed using a two-way ANOVA and P value was given in the right column. When variable interaction was detected, statistical comparisons were performed using a one-way ANOVA followed by Tukey-Kramer posthoc test. ***P<0.001 (significantly different of other groups).

(TIF)

**Table S4** Summary of behavioral outcomes. Values are expressed as mean ± SEM. Statistical comparisons were performed using a two-way ANOVA for the study of two variables simultaneously. When variable interaction was detected, statistical comparisons were performed using a one-way ANOVA followed by Tukey-Kramer post-hoc test. *P<0.05 (significantly different of 3xTg-AD mice fed with high-DHA diet).

(TIF)

**Table S5** Summary of the intrinsic properties of entorhinal cortex neurons before, during and after an application of 2 \( \mu \)M DHA. Electrophysiologic data were obtained from 5 mice. Values are expressed as mean ± SEM. Statistical comparisons were performed using paired Student’s t-test. Abbreviations: F-I, firing rate versus injected current.

(TIF)

**Table S6** Number of spontaneously active neurons in each group. Electrophysiologic data were obtained from 8 mice per groups.

(TIF)

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**Author Contributions**

Conceived and designed the experiments: FC DA. Performed the experiments: DA CJ CT. Analyzed the data: DA FC. Contributed reagents/materials/analysis tools. Wrote the manuscript: DA FC.

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