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Neuroprotective Role of Dietary Supplementation with Omega-3 Fatty cids in the Presence of Basal Forebrain Cholinergic Neurons Degeneration in ged Mice

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bstract: As major components of neuronal membranes, omega-3 polyunsaturated fatty acids (n-3 PUFA) exhibit a wide range of regulatory functions. Recent human and animal studies indicate that n-3 PUFA may exert beneficial e ects on aging processes. Here we analyzed the neuroprotective influence of n-3 PUFA supplementation on behavioral deficits, hippocampal neurogenesis, volume loss, and astrogliosis in aged mice that underwent a selective depletion of basal forebrain cholinergic neurons. Such a lesion represents a valid model to mimic a key component of the cognitive deficits associated with dementia. Aged mice were supplemented with n-3 PUFA or olive oil (as isocaloric control) for 8 weeks and then cholinergically depleted with mu-p75-saporin immunotoxin. Two weeks after lesioning, mice were behaviorally tested to assess anxious, motivational, social, mnesic, and depressive-like behaviors. Subsequently, morphological and biochemical analyses were performed. In lesioned aged mice the n-3 PUFA pre-treatment preserved explorative skills and associative retention memory, enhanced neurogenesis in the dentate gyrus, and reduced volume and VAChT levels loss as well as astrogliosis in hippocampus. The present findings demonstrating that n-3 PUFA supplementation before cholinergic depletion can counteract behavioral deficits and hippocampal neurodegeneration in aged mice advance a low-cost, non-invasive preventive tool to enhance life quality during aging.

Keywords: aging; cholinergic system; omega-3 fatty acids; prevention; cognitive deficits; neuroprotection

1. Introduction

Given dementia is a major cause of death and disability in older population and no effective pharmacological treatment has been identified to date, there is considerable interest in identifying lifestyle approaches, such as diet, able to prevent cognitive decline during aging [1–4]. Among the different forms of dementia, Alzheimer's disease (AD) is the most common (60–70% of cases) and currently affects 47 million people worldwide [5–7]. Its prevalence rises exponentially with age and, due to increasing lifespan, it has been predicted to double every 20 years, causing a huge burden on healthcare costs [5,8]. AD is characterized by irreversible and progressive brain atrophy, loss of memory, and cognition. Specifically, basal forebrain cholinergic neurons degeneration and the subsequent loss of cholinergic neurotransmission in the cerebral cortex and limbic system are retained pathophysiological events crucial in triggering the cognitive deterioration observed in patients with AD dementia [9,10].

In the past three decades, the availability of saporin immunotoxins allowed studying the role of basal forebrain cholinergic system in several cognitive functions and its implications in aging and dementia [11–13]. In fact, saporin immunotoxins selectively cause death of cholinergic cells by inhibiting ribosomal protein synthesis when it is taken up into cells expressing the low-affinity p75 neurotrophin receptor [11,14,15]. The resulting permanent and selective saporin-dependent massive loss of cholinergic basal forebrain neurons mimics neuropathological features and cognitive symptoms associated with mild cognitive impairment (MCI) and mild AD.

In the present study we used the mu-p75-saporin (sap) immunotoxin intracerebroventricularly injected in aged mice to elicit the basal forebrain cholinergic depletion. In the experimental model of first stages of AD so obtained we analyzed the neuroprotective properties of pre-lesional treatment with omega-3 polyunsaturated fatty acids (n-3 PUFA).

n-3 PUFA are one the major components of neuronal membranes and key modulators of neuroinflammation, oxidative stress, and neurogenesis [16,17]. They include eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and alpha-linolenic acid (ALA) [16,18]. Longer-chain n-3 fatty acids (EPA and DHA) are synthetized by shorter-chain n-3 fatty acids (ALA) [19,20]. However, biological conversion is inefficient, especially during aging [19,21–24]. In addition, shorter-chain fatty acids cannot be synthesized by humans [21,25]. Therefore, diet is the most important source of these fatty acids. Their daily intake could be from plant-derived ALA and from fish and marine EPA and DHA, and their supplements [26]. Unfortunately, nutritional research indicates that the "Western pattern diet" does not provide the aged brain with an optimal supply of n-3 PUFA, and aging *per se* is associated with a decrease in cerebral n-3 PUFA [27].

Notably, n-3 PUFA are reported to exert beneficial and neuroprotective effects on the aging brain [23], when deterioration in neuronal function and decline in cognitive performance, mainly those hippocampal-dependent, have been consistently reported. These age-related impairments are reflective of synaptic loss, decreased neurogenesis, synaptic plasticity, neuronal density, and gray matter volume, particularly in the hippocampal circuits [28–33]. Other studies indicate that deficits in hippocampal functions are associated with neuroinflammation and oxidative stress [33–36]. Interestingly, experimental studies in rodents have shown on one hand that n-3 PUFA supplementation improves neurogenesis and synaptogenesis, as well as executive functions and learning abilities, and on the other hand that n-3 PUFA deficiency is associated with memory deficits and impaired hippocampal plasticity [2,3,16,37,38]. Preclinical evidence from our laboratory confirmed that age-related alterations may lead to irreversible neuronal loss of gray matter volume in the hippocampus and prefrontal lobes [39,40], in line with previous studies in humans [41–43]. Specifically, we demonstrated that 8-week n-3 PUFA supplementation in aged mice robustly ameliorates mnesic functions and coping skills via increased neurogenesis and reduced hippocampal neurodegenerative processes [39], in association with foci of greater gray matter volume in fronto-hippocampal areas [39,40].

Human longitudinal studies based on direct or indirect indices of n-3 PUFA consumption correlate with better cognitive functioning and reduced risk of dementia, higher total brain and regional gray

matter volumes [44–49] and reduced white matter hyperintensity [50,51]. Some interventional studies reported that n-3 PUFA supplementation improves cognition in healthy elderly subjects [52–54] and in subjects with MCI [55–58]. Many reports have also demonstrated the benefits of a diet rich in n-3 PUFA, as the Mediterranean diet, against age-related cognitive decline in MCI subjects and AD patients [59–65]. Anyway, still little is known about the brain mechanisms and correlates of the preserved cognitive functions in relation to the preventive e ects of n-3 PUFA dietary intake during aging.

To this end, here we focused on the neuroprotective action of n-3 PUFA by investigating the influence of an 8-week oral pre-lesional treatment with a mixture of EPA, DHA, and DPA on the behavioral deficits and hippocampal degeneration induced by immunotoxic forebrain cholinergic lesions during aging. To this aim, emotional, motivational, social and mnesic performance as well as hippocampal morphological and biochemical correlates of cholinergically depleted aged mice pre-treated with n-3 PUFA or olive oil (used as isocaloric control) were compared with those of pre-treated with n-3 PUFA or olive oil sham-lesioned animals (Figure 1). After behavioral testing, neurodegeneration of hippocampal networks was analyzed by measuring neurogenesis levels in the dentate gyrus (DG) as well as volumes and astrogliosis in the hippocampus, which is one of the main projection areas of the lesioned cholinergic projections from medial septum/diagonal band.

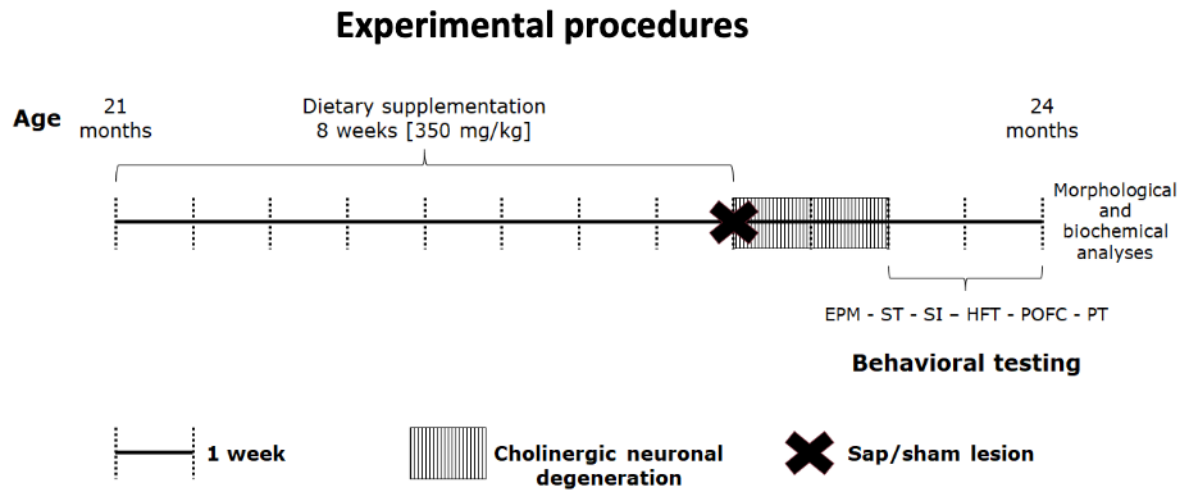

Figure 1. Experimental procedures. After 8-week oral supplementation with n-3 PUFA, 21-month old aged mice have been subjected to intracerebroventricular (i.c.v.) injections of mu-p75-saporin or saline (sham lesion) to selectively deplete the forebrain cholinergic system. Two weeks after the lesion, the animals were behaviorally tested by means of validated tasks (Elevated Plus Maze, EPM; Splash Test, ST; Social Interactions, SI; Hidden Food Test, HFT; Predator Odor Fear Conditioning, POFC; Porsolt Test, PT). At the end of testing battery, mice were sacrificed, and brains collected for morphological and biochemical analyses.

2. Results

2.1. Behavioral Testing

2.1.1. Elevated Plus Maze (EPM)

Since anxiety is reported to increase in aging rodents [66] and cholinergic manipulations are known to influence anxiety levels [67,68], in the present study we used the EPM as a validated test to measure anxiety in rodents based on their natural aversion for heights and open spaces [39,40,69,70].

After a square root transformation (to adjust for normality), duration and frequency EPM data were analyzed by three-way analyses of variance (ANOVA) (diet x lesion x arm). Defecations were not normally distributed.

As for the duration of time spent in the closed vs. open arms, the ANOVA revealed a significant arm e ect ($F_{1,38}$ = 51.56, p < 0.000001), while diet ($F_{1,38}$ = 0.06, p = 0.81) and lesion ($F_{1,38}$ = 1.11, p = 0.30) e ects were not significant. The interactions diet x lesion ($F_{1,38}$ = 0.13, p = 0.72), arm x diet ($F_{1,38}$ = 0.12, p = 0.74), arm x lesion ($F_{1,38}$ = 0.39, p = 0.54), arm x diet x lesion ($F_{1,38}$ = 0.22, p = 0.64) were not significant.

The ANOVA performed on frequency of entries in the closed vs. open arms showed significant diet ($F_{1,38}$ = 5.76, p = 0.02) and arm ($F_{1,38}$ = 40.96, p < 0.000001) e ects as well as a significant first level diet x lesion interaction ($F_{1,38}$ = 4.29, p = 0.04). Lesion e ect ($F_{1,38}$ = 1.81, p = 0.19) and interactions arm x diet ($F_{1,38}$ = 0.68, p = 0.41), arm x lesion ($F_{1,38}$ = 1.31, p = 0.26) arm x diet x lesion ($F_{1,38}$ = 0.53, p = 0.47) were not significant. Post-hoc comparisons calculated on the significant interaction demonstrated that the oil sap group explored the arms less frequently than the remaining groups (oil sap vs. oil sham, p = 0.02; oil sap vs. n-3 PUFA sham, p = 0.03; oil sap vs. n-3 PUFA sap, p = 0.02; Figure 2).

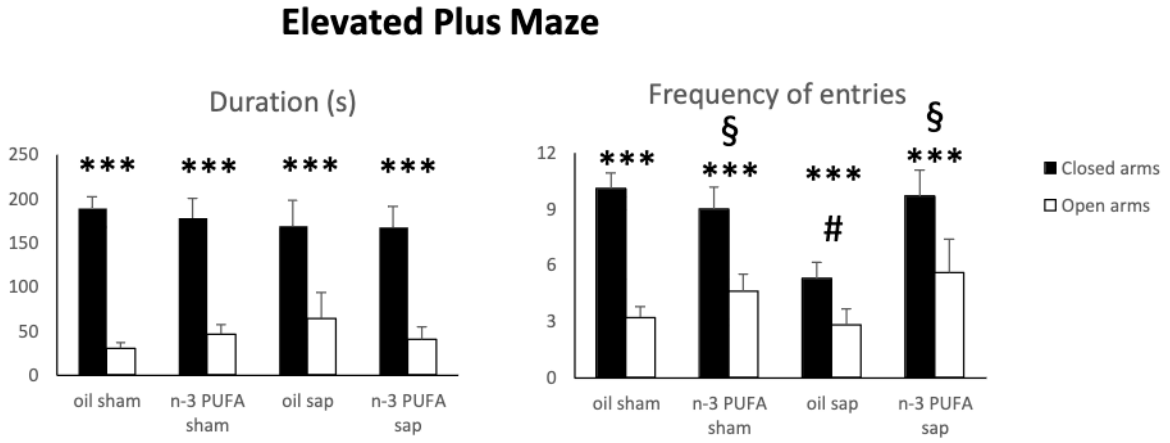

Figure 2. Elevated Plus Maze data. Duration and frequency of exploration of the closed and open arms in the four experimental groups. Data are reported as mean and SEM (oil sham, n = 12; n-3 PUFA sham, n = 10; oil sap, n = 10; n-3 PUFA sap, n = 10). Arm e ect: *** p < 0.000001; Diet e ect: § p < 0.05; Diet x lesion e ect: # p < 0.05.

No di erences were found in the number of defecations (Kruskal–Wallis test; H = 3.08, p = 0.38).

These findings indicate that n-3 PUFA pre-treatment was able to increase explorative behavior (frequency of entries) and to prevent its lesion-induced reduction, while the expected preference for the closed arms was kept unaltered by both dietary and lesional treatments.

2.1.2. Splash Test (ST)

The ST is based on the assessment of grooming behavior considered to be a form of self-care/motivational behavior that parallels with some symptoms of depression, such as apathetic behavior [71], and it is associated with hedonic reactivity in the sucrose preference test and increased immobility in the forced swim test [72,73].

No di erences were found in self-care and motivational behaviors among groups (Supplementary Materials Figure S1). In particular, as demonstrated by Kruskal–Wallis analysis, mice belonging to all experimental groups showed not significantly di erent duration (H = 2.39, p = 0.49) and frequency (H = 5.52, p = 0.14) of grooming. The number of defecations was also similar in all groups (H = 2.35, p = 0.50).

2.1.3. Social Interactions (SI)

The SI test is used to investigate social behaviors, which are known to decrease with age in rodents [66,74].

Since most behaviors displayed in SI test were sporadic and not normally distributed, we analyzed social interaction data by means of non-parametric analysis. Sexual behaviors were not observed in any group of mice.

No di erences were found among groups in total duration (H = 1.67, $p = 0.64$) and frequency (H = 3.15, $p = 0.37$) of social behaviors, as well as in total duration (H = 0.89, $p = 0.83$) and frequency (H = 6.92, $p = 0.07$) of non-social behaviors (Supplementary Materials Figure S2A,B). No significant di erences were observed when single social or non-social behaviors were analyzed (Supplementary Materials Table S1). No di erences were evident in the number of defecations of the four experimental groups (H = 0.68, $p = 0.88$).

2.1.4. Hidden Food Test (HFT)

The HFT checks whether food-deprived mice can find the pleasant food pellet hidden beneath the cage's bedding to uncover eventual deficits in olfactory abilities [75]. Impairments in the sense of smell are common during aging and may be due to the deterioration of the peripheral sensory epithelium or central olfactory relays as well as to degenerative processes a ecting cognitive processing of odors in the brain [76]. Age-related neurodegenerative diseases, such as AD, seem to involve selective pathology in specific brain structures linked to olfactory processing [77]. In addition, in the present study we verified the olfactory capabilities within experimental mice groups because the sensitivity to odors was relevant to perform the subsequent POFC test.

No di erences between latency to dig out and latency to eat the palatable food pellet during the HFT were found in all experimental groups (Mann-Whitney U test; U = 0, $p = 1$), thus we collapsed the two parameters (linked to sensory odor perception and motivation, respectively) by analyzing the average time to dig out and eat the palatable food among groups. As demonstrated by Kruskal–Wallis analysis, no di erences in mean latency to dig out and eat the palatable food pellet (H = 2.97, $p = 0.39$) were found among the four experimental groups (Supplementary Materials Figure S3).

These findings indicate both the preserved ability to smell volatile odors and the comparable motivation levels in lesioned and sham animals regardless of their pre-treatment.

2.1.5. Predator Odor Fear Conditioning (POFC)

The POFC involves the use of the predator odor as a natural unconditioned fear stimulus (instead of an aversive electric foot-shock) to assess the integrity of hippocampal networks associated with associative learning and memory [78,79]. It has been previously reported that in mice the exposure to predator odor (e.g., coyote urine) is able to activate place cells in CA1 and modify their firing patterns to establish a spatial representation of the fearful experience [80]. Also, research on AD patients showing early atrophy of medial temporal lobe structures indicated marked impairments in fear conditioning [81,82].

Since one oil sham mouse died before testing, the number of animals belonging to oil sham group performing this test was 11. As expressed by percentages, the retention index was analyzed by a two-way ANOVA (diet x lesion) after angular transformation {arcsine [square root(percentage of freezing/100)]}. Freezing time during exposure session was analyzed by non-parametric analyses due to the lack of normality distribution of the data.

The two-way ANOVA (diet x lesion) revealed a significant diet x lesion interaction ($F_{1,37} = 4.84$, $p = 0.03$), while diet ($F_{1,37} = 4.06$, $p = 0.05$) and lesion ($F_{1,37} = 0.76$, $p = 0.39$) e ects were not significant. Post-hoc comparisons demonstrated that n-3 PUFA pre-treatment ameliorated the retention of aversive contextual memory in cholinergically depleted aged mice (Figure 3). In fact, lesioned aged mice pre-treated with omega-3 (n-3 PUFA sap) displayed similar retention index values in comparison to both sham groups (n-3 PUFA sap vs. oil sham, $p = 0.42$; n-3 PUFA sap vs. n-3 PUFA sham, $p = 0.62$). On the contrary, lesioned aged mice pre-treated with olive oil (oil sap) showed inferior retention index values either when compared to lesioned aged mice pre-treated with n-3 PUFA ($p = 0.008$) and