



Transgenerational reduction of dietary polyunsaturated fatty acids | Research and cognition in Wistar rats

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Abstract: Polyunsaturated fatty acids (PUFAs) have an important impact on the development of the brain, especially during the prenatal and the early postnatal phases, and on the functioning of the adult brain. Deficiencies and imbalances of PUFAs can have significant effects on cognitive functions. Decreased levels of omega-3 (n-3) PUFAs have been suggested to be associated with symptoms of attention deficit/hyperactivity disorder (ADHD). The main symptoms of ADHD are hyperactivity, inattention and impulsivity. The aim of the present study was to elucidate the effects of n-3 PUFA deficiency on attention functions, impulsivity and activity by assessing the behavior of the fourth generation of n-3 PUFA depleted Wistar rats. Four generations of Wistar rats were outbred, and throughout the whole breeding period, dams and their offspring were fed with an n-3 PUFA-deficient diet. The study was performed in male offspring of the fourth generation of n-3 PUFA deficient dams. Twelve males from the deficient group and twelve males fed both prenatally and throughout the entire experiment with an n-3 fatty acid-sufficient diet (control group) were tested. Behavioral testing was performed using the three-choice-serial-reaction-time task (3CSRTT). The present data showed no relevant effects of a transgenerational n-3 PUFA deficiency on attention, impulsivity and activity. A longer period of n-3 PUFA deprivation may be needed to arrive at reduced n-3 PUFA concentrations in the brain which could produce behavioral effects. It may therefore be necessary to examine the effects of transgenerational n-3 PUFA deprivation on rat cognitive functioning over more than four generations.

Key words: Attention deficit/hyperactivity disorder; attention; impulsivity; activity; omega-3 polyunsaturated fatty acids; rat.

1. Introduction

There are numerous indications that decreased levels of polyunsaturated fatty acids (PUFAs) are associated with mental illness. PUFAs encompass omega-6 (n-6) PUFAs, e.g. arachidonic acid (AA, C20:4 n-6), and omega-3 (n-3) PUFAs, e.g. eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). Among the various kinds of PUFAs, n-6 and n-3 PUFAs have an especially important impact on both brain development and functioning [1]. These essential nutrients cannot be synthesized by mammals and hence need to be supplied through dietary intake [2,3]. PUFAs are involved in various neuronal activities – from influences on the

membrane fluidity and neurotransmission processes to the regulation of gene formulations [1,4]. Deficiencies and imbalances of PUFAs have significant effects on cognitive functions and may be associated with disorders such as attention deficit/hyperactivity disorder (ADHD) [1,5,6].

ADHD is one of the most common psychiatric disorders of childhood and adolescence. The cardinal symptoms of ADHD are hyperactivity, attention deficits and increased impulsivity [7–9]. A dysfunction of dopaminergic neurotransmission appears to be important in this disorder [10]. However, the exact cause of ADHD and the underlying neurobiology are still

unknown [11–15]. Apart from biological, genetic and environmental factors, the importance of PUFA deficiency as a cause of symptoms of ADHD is often considered [1,16,17].

Neurodevelopmental studies showed that an adequate amount of PUFAs is important during prenatal (particularly in the last trimester of gestation) and early postnatal development [18–23]. After birth, the growth of the central nervous system (CNS) progresses rapidly, increasing the vulnerability of the brain to organismic deficits in PUFA levels [1,24]. PUFAs such as AA and DHA are present in the milk of humans and mammals, supporting normal growth and development of infants [25–27]. Moreover, the amount of n-3 and n-6 PUFAs in milk is strongly dependent on the mother's fat intake [28,29]. Several studies demonstrated that a lack of DHA in preterm infants through the maternal diet reduced the DHA concentration in the cerebral cortex and in phospholipids of red blood cells [20,30–32]. Additionally, it has been shown that infants fed with a diet low in DHA are at increased risk for neurological and neurocognitive deficits, e.g. lower IQ scores and visual impairments [33–40]. These results show that the infant brain is not protected from harmful effects resulting from an insufficiency of PUFAs [29,41].

Empirical studies have shown that plasma concentrations of n-3 PUFAs are decreased in children and adults with symptoms of ADHD [16,42]. These reduced concentrations of n-3 PUFAs are also related to behavioral problems and learning disabilities [43,44]. In ADHD research, several studies reported lowered levels of AA and DHA in red blood cells of children and adolescents with ADHD [6,16,42,45,46]. Other studies demonstrated elevated ratios of n-6/n-3 PUFAs (e.g. AA/EPA) in the plasma of ADHD-affected children and adults [16,42,47,48]. Moreover, Colquhoun et al. [17] and Mitchell et al. [49] described symptoms such as extreme thirst (polydipsia), increased desire to urinate (polyuria) and dry hair or skin in children with ADHD. These manifestations are associated with a reduced amount of n-3 PUFAs in the blood [16].

Taken together, these research findings indicate possible links between deficiencies in organismic levels of n-3 PUFAs and both the presence of ADHD symptoms and neurocognitive deficits. However, the relevant studies differed widely in their methodology, the types and sizes of employed samples as well as the exact parameters measured. Moreover, the number of investigations is small. Furthermore, the difficulty of controlling and quantifying dietary intake of PUFAs in

human studies is an important consideration, given the fact that dietary intake is the only source of PUFAs in humans. In the future, more experimental and clinical studies should be conducted [5,50] in order to consolidate these findings.

It is still unknown whether a deficiency in PUFAs induces ADHD symptoms. PUFAs cannot be synthesized by the human body but are necessary for normal metabolism [2,3,51]. PUFAs are a main element of phospholipids, which are one component of the brain's neuronal cell membranes. Therefore the amount of PUFAs influences the property of the neuronal membrane and the function of the related receptors and transporters [52–54]. It is possible that an PUFA deficiency affects the cellular signal processes, synapse function and neurotransmission, e.g. in the dopaminergic and serotonergic systems [3,52,53,55–57]. These neurotransmitters are involved in the regulation of attention [58–61]. Furthermore, PUFAs modulate the brain gene transcriptions [1]. Collectively, these findings suggest that normal intercellular communication, as well as physiological membrane status, are critically dependent on n-6 and n-3 PUFA levels [1,62,63].

In preclinical studies using rodent models, experimental inductions of n-3 PUFA deficiency led to deficits in the regulation of cognitive functions, locomotor and exploratory activity as well as the emotional status [64–68]. In order to decrease CNS levels of DHA in rodent offspring, the diet of dams needs to be devoid of all n-3 PUFAs throughout the gestation and lactation periods [65]. Several studies demonstrated that an n-3 PUFA deficiency in the brain of rodents obtained over generations is associated with an increase in activity [27,66,69], impairments in spatial learning [64,70,71], working memory [72] and olfactory discrimination learning [73]. Such n-3 PUFA deficient animals have also been shown to display increased rates of anxiety [74], aggression and depression [75]. Moreover, it has been reported that low dietary levels of n-3 PUFAs during gestation and throughout the postnatal period resulted in a decreased level of DHA in the retina and cerebral cortex in nonhuman primates [76]. These changes have been associated with motor and cognitive impairments (e.g. visual performance) [77,78].

As previously noted, a lack of nutritional essential fatty acids has been linked with symptoms of ADHD [43,44]. The biological basis for this assumption lies in the significant biological changes directly resulting from a dietary lack of such fatty acids [59]. Unfortunately, most human studies regarding the effect of n-3 PUFAs in ADHD

are inconsistent with one another; for details see [1,50]. The present state of preclinical animal evidence shows an association between n-3 PUFAs and altered motor activity [27,66]. However, there is currently an absence of animal studies investigating the effects of n-3 PUFA deficiency in the developing brain on other ADHD-related symptom domains, i.e. attention and impulsivity. Preclinical animal studies can make an extremely valuable contribution to the better understanding of the role of PUFAs in the symptoms of ADHD.

In the present experiment, the aim was to examine the effects of an n-3 PUFA deficiency on attention, impulsivity and activity by investigating the fourth generation of n-3 PUFA depleted Wistar rats. The three-choice-serial-reaction-time task (3CSRTT) was used for this purpose.

2. Methods

2.1. Animals and experimental diet

The present experiment was performed in accordance with the national laws (German law on Protection of Animals) and the principles of laboratory animal care (NIH publication No. 86- 23, revised 1996). The rats were handled according to the guidelines of the Federation for European Laboratory Animal Science Associations (FELASA). Four generations of Wistar rats were bred in our laboratory and fed an experimental n-3 fatty acid-deficient (n-3 Def) diet. The first generation of female Wistar rats was delivered by Charles River Laboratories (Sulzbach, Germany). The study was started with three-month-old male rats of the fourth generation of PUFA-deficient dams. Twelve males from the deficient group served as the experimental treatment group and were kept on n-3 Def diet throughout the study period. The control group was comprised of twelve males which were fed an n-3 fatty acid-sufficient (n-3 Suf) diet both prenatally and throughout the study period. The feeding conditions had been employed in pregnant dams and were kept constant during gestation, lactation, post-weaning and behavioral test phases. Access to food was restricted, since the behavioral paradigm used in this study (3CSRTT) is based on food reinforcement. Water was provided ad libitum. The rats' weight was carefully controlled, and a weight reduction of more than 5% was avoided in order to prevent stress [79,80] and subsequent changes in the dopaminergic system [81]. The rats were housed in standard cages under standard animal laboratory conditions (12:12 h light/dark cycle, room temperature 22 °C, humidity 50%) in the animal laboratories of the University of Regensburg. All

treatments, trainings and tests were performed during the light phase between 9 a.m. and 4 p.m. After the experiments, rats were sacrificed using carbon dioxide.

The experimental diets (n-3 Def and n-3 Suf) were prepared by Ssniff Spezialdiäten GmbH (Soest, Germany). Both diets are based on the American Institute of Nutrition-93 G (AIN93G) and meet all the current nutrition standards for rat growth [82]. The fatty acid composition of the diets is shown in Table 1. The diets were stored at -20 °C and provided fresh daily.

Table 1. Fatty acid composition of the experimental diets (Ssniff Spezialdiäten, Soest, Germany)

	n-3 fatty acid-deficient diet	n-3 fatty acid-sufficient diet
Energy (Atwater), MJ/kg*	17.1	17.1
kJ% protein	18	18
kJ% carbohydrates	60	60
kJ% fat	22	22
Fatty acids, % of diet		
C 6:0	0.05	0.05
C 8:0	0.62	0.59
C 10:0	0.49	0.47
C 12:0	3.64	3.48
C 14:0	1.41	1.35
C 16:0	0.85	0.84
C 18:0	0.28	0.29
C 20:0	0.01	0.01
C 18:1	0.77	0.82
C 18:2 n-6	1.58	1.54
C 18:3 n-3	0.01	0.27

* Physiological fuel value

2.2. Three-choice-serial-reaction-time task (3CSRTT)

The task is based on the five-choice-serial-reaction-time task [83,84]. In the present study, a three-choice variant of the paradigm was used (holes no. 3, 5, 7). The remaining unused holes were covered. The experiment was performed using four ventilated wooden chambers (Campden Instruments, Loughborough, Leicestershire, England) containing a stainless steel chamber (26 cm ×

26 cm × 30 cm height). The steel chambers were lighted by 3-Watt light bulbs. Each chamber was equipped with three holes, which were arranged horizontally in the curved rear wall (see Figure 1). The holes were 2 cm above the chamber floor (stainless steel grid), each hole had a diameter of 2 cm and adjacent holes were 6 cm apart. In each hole, an infrared photocell was installed in order to detect a nose poke response of the rat to the hole. In addition, each hole was equipped with a standard light bulb (3 W). The animals were required to

respond correctly to a stimulus by a nose poke into one of the three holes. A stimulus was defined as the illumination of a hole by the light bulb, and only one hole at a time could be illuminated. A correct response was rewarded with a food pellet (45 mg dustless sucrose pellets, Bio-Serv, Frenchtown, New Jersey, USA) which was dispensed into a food tray at the front wall (opposite the holes). False responses, premature responses or omissions were punished with a 5-s period of darkness.

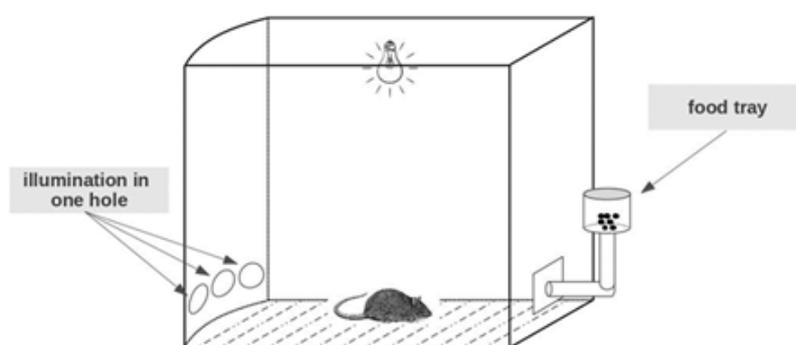


Figure 1. Three-choice-serial-reaction-time task (3CSRTT)

The behavioral paradigm consisted of three phases. In the habituation phase, the ambient light was permanently turned on, 10 pellets were baited in the food tray and one pellet was placed in each illuminated hole. The rats were required to habituate to the boxes for 30 min a day. The habituation phase was finished when all pellets were found and collected, which was accomplished within two consecutive days. In the training phase, the rats were required to learn to respond correctly to the stimulus (i.e. random illumination of a hole, once per trial) in order to obtain a food pellet. The animals were trained on five consecutive days per week for 6 weeks (30 sessions). The stimulus duration (SD) was gradually reduced when a rat responded correctly within one training session of 30 min in at least 80% of the trials (number of correct trials/total correct and false responses, expressed as percent), and the omission rate was less than 20% (number of trials missed/total trials completed, expressed as percent). The

SD lasted from 60 s (training level 1) to 1.5 s (final training level). All other parameters were kept constant during the training phase (inter-trial interval ITI of 5 s). In the final (testing) phase, the stimulus duration was 1.5 s and the test sessions were similar to the training sessions except that the ITIs varied randomly between 1.5 s, 2.5 s, 3.5 s, 4.5 s, 5.5 s, 6.5 s, 7.5 s and 8.5 s. The order in which the rats were tested was randomized in all phases. See Figure 2 for the possible response trials in 3CSRTT.

2.3. Statistical analysis

The following parameters regarding attention were analyzed: (1) number of correct responses, (2) number of false responses (commission errors), (3) number of missed responses (omission errors), (4) percentage of correct responses (i.e. number of correct responses/total correct and false responses, expressed as percent) and (5) percentage of missed responses (i.e. total number of missed responses/total trials completed, expressed as

percent). In addition, the following parameters concerning impulsive behavior were compared between groups: (6) number of premature responses, (7) number of panel pushes during ITI, (8) number of time-out responses and (9) number of perseverative responses. Moreover, the following parameters concerning activity were examined: (10) number of trials completed, (11) correct response latency, (12) incorrect response latency and (13) reward collection latency.

A mean value of performance for each group was calculated. All findings concerning group differences are expressed as means \pm standard errors ($M \pm SE$). Statistical analyses were performed using the nonparametric Mann-Whitney U-Test (between subjects design), and an α -level of 0.05 was applied. All statistical analyses were performed using the statistical package IBM SPSS Statistics 23 for Windows.

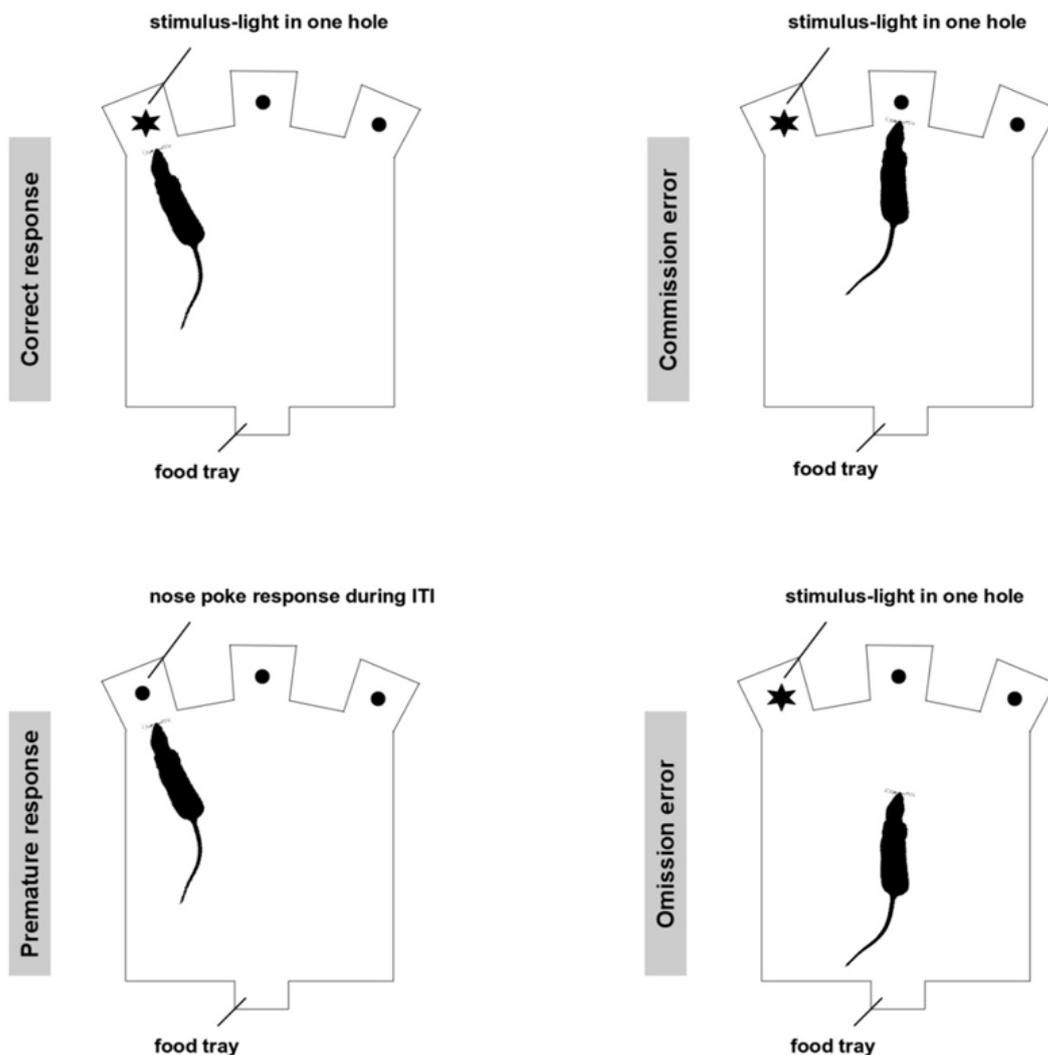


Figure 2. Possible reactions of the rats in the 3CSRTT

3. Results

3.1. Comparison between the n-3 fatty acid-deficient (n-3 Def) and n-3 fatty acid-sufficient (n-3 Suf) groups

All results are presented as means \pm standard errors.

3.1.1. Training phase

About 30 sessions were needed until the rats were able to fulfill the criteria. In the training phase, no significant differences between the two groups were found (data not shown).

3.1.2. Test phase

The results in regard to the performance-related parameters are given in Table 2. The comparisons between the experimental conditions revealed a statistically significant difference with respect to the number of panel pushes during ITI. The n-3 Def group made significantly more panel pushes at the food-tray than the n-3 Suf group. None of the remaining comparisons reached statistical significance.

Table 2. Performance of the diet groups n-3 Def and n-3 Suf on attention, impulsivity and activity as measured by 3CSRRT (means \pm standard errors)

	n-3 Def group (n=12)	n-3 Suf group (n=12)	p-value	Z-value
Attention parameters				
No. of correct responses	34.33 \pm 4.66	40.42 \pm 5.23	.298	1.040
No. of commission errors	2.50 \pm 0.71	2.83 \pm 0.67	.660	.440
No. of omission errors	24.50 \pm 3.16	16.58 \pm 2.85	.068	-1.822
% of correct responses	92.95 \pm 1.76	92.91 \pm 1.71	.884	-.145
% of omissions	41.75 \pm 5.19	30.15 \pm 5.65	.157	-1.415
Impulsivity parameters				
No. of premature responses	5.67 \pm 1.47	7.58 \pm 3.16	.704	-.379
No. of panel pushes during ITI	73.75 \pm 14.15 ^A	51.17 \pm 16.89	0.043	-2.022
No. of time-out responses	9.75 \pm 1.74	12.75 \pm 3.82	.908	-.116
No. of perseverative responses	2.08 \pm 0.96	1.67 \pm 0.51	.880	.151
Activity parameters				
No. of trials completed	61.33 \pm 4.05	59.83 \pm 3.78	.834	-.210
Correct response latency (s)	1.01 \pm 0.07	0.92 \pm 0.05	.356	-.924
Incorrect response latency (s)	1.81 \pm 0.31	2.05 \pm 0.32	.757	.309
Reward collection latency (s)	1.35 \pm 0.15	1.12 \pm 0.04	.564	-.577

n-3 Def group = n-3 fatty acid-deficient group; n-3 Suf group = n-3 fatty acid-sufficient group

^A p < 0.05 compared with n-3 Suf group

4. Discussion

The effect of PUFA deficiency on attention, impulsivity and activity in rats has not been sufficiently investigated. The aim of the present study was therefore to examine the role of n-3 PUFA depletion over four generations by measuring attentional processes in the fourth generation of these depleted rats using the 3CSRTT.

At the descriptive level, the n-3 Def group made fewer correct responses and missed more signals than the n-3 Suf animals. These results might be taken to suggest that attentional processes could be negatively affected by n-3 PUFA depletion in rats. However, neither of these between-group differences was statistically significant. Seen in this light, the present findings do not support previous observations of depletion-induced cognitive deficits, e.g. impairments in spatial learning, working memory and olfactory discrimination learning [64,70–73]. Moreover, the present study results run counter to previous findings in human studies showing an association between a lack of DHA in the diet of preterm infants and neurocognitive deficits, e.g. lower IQ scores [33–40]. Our outcomes also conflict with experimental findings obtained in nonhuman primates, where a deficiency in n-3 PUFAs was associated with a decrease in visual acuity [77] and performance [78].

However, CSRTT employs more parameters in the rating of attention, such as the percentage of correct responses and missed reactions, as well the number of false responses. This outcome also failed to be statistically significant. These findings are inconsistent with the premise that a PUFA deficiency exerts an influence on dopaminergic and serotonergic neurotransmission [3,52–56], since it is known that these neurotransmitters are involved in the modulation of attention [58–61]. However, the n-3 PUFA deprivation in rats over generations may produce other developmental changes or induce an unnaturally high inflammatory level, which may have an impact on cognition [85]. Some studies, conducted in different animal models, show that diet-induced reductions of DHA levels in the brain are associated with changes in neuronal plasticity as well as memory deficits [86–88].

Nevertheless, it is important to note that attention parameters cannot be considered as a single value. Instead, they should be viewed in the context of more generalized activity. Therefore, the second aim of the present experiment was to investigate activity-related effects of a diet deficient in n-3 PUFAs. The CSRTT provides no valid parameters for mobility. However, the total number of trials completed can indicate a general

response-activity. No statistically significant differences between groups could be observed in this regard. However, at the descriptive level, the n-3 Def rats completed slightly more trials compared to the n-3 Suf group. These findings might indicate some increase in number of responses in the n-3 Def group. Interestingly, the n-3 Def rats are also marginally slower in correct response and reward collection. These differences in behavior between groups may be due to an influence on the motivational state [65,89]. However, given the inconsistent pattern of results at the descriptive level and the complete lack of statistically significant differences, the present study was unable to find activity-related effects of a diet deficient in n-3 PUFAs.

The third aim of the present experiment was to investigate impulsivity-related effects of a diet-induced n-3 PUFA deficiency. The n-3 Def diet significantly increased the number of panel pushes in the food-tray during ITI compared to the n-3 Suf rats. Therefore, these data may suggest that an n-3 PUFA deficient diet produced negative effects on behavioral control. This result is in accordance with several previous studies, which have investigated the effect of n-3 PUFAs on impulsivity [90] and general activity of rodents [59,91]. However, an important consideration is that only one in four between-group comparisons regarding impulsivity-related parameters reached statistical significance. Since Dervola and colleagues [90] studied the effects of n-3 PUFA supplementation on reinforcer-controlled behavioral performance, one obvious – and potentially critical – difference that might account for differences in study results is the employed direction of n-3 PUFA modulation, i.e. supplementation versus depletion. Nevertheless, the presently employed CSRTT offers more parameters to assess impulsive behavior: the number of premature responses, the number of time-out responses and the number of perseverative responses. Moreover, the variable ITI period implemented in the present testing procedure is more suited to assess behavioral control, since the variation of this parameter directly taps the ability to control a behavioral response to an anticipated stimulus event.

In conclusion, the present data, derived from the measuring of attention, impulsivity and activity in the fourth generation of n-3 PUFA-deficient Wistar rats, revealed no relevant effects of an n-3 PUFA deficiency over four generations. Our profile of results is consistent with a previous study, which demonstrated a significant decrease in n-3 PUFA levels in the brain of mice (53%) and no concomitant impairments of either olfactory

function or spatial learning in the Morris water maze [92]. In contrast, other studies have reported conflicting results. The significant reduction of DHA phospholipids in the brain (50% to 80%) affected cognitive processes [70–73,93]. A plausible explanation for the discrepancy between the previous and the present findings could be provided by the different models of rodents and the more complex learning paradigm that we used. It would therefore be of interest to examine and compare the different tests in one animal model regarding cognition and the deficit in n-3 PUFAs.

As the present study showed no marked effects of n-3 PUFA depletion on attention, impulsivity and activity parameters in rats, it may be interesting to perform further investigations regarding PUFA depletion over a longer time period. The biosynthesis of DHA from ALA in rodents is more effective than in nonhuman primates [76]. Thus, a longer period of n-3 PUFA deprivation is needed to achieve a reduced DHA concentration in the rat brain, allowing a meaningful comparison with humans [85,94]. Thus, it would be necessary to examine the impact of a prenatal n-3 PUFA deprivation on the cognitive functions in rats over more than four generations [95].

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Conflict of interest

The authors declare no conflict of interest.

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