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n-3 Polyunsaturated fatty acid supplementation restored impaired memory and GABAergic synaptic efficacy in the hippocampus of stressed rats

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While chronic stress induces dendritic atrophy in the hippocampus and impairs learning and memory, supplementation with n-3 polyunsaturated fatty acids (n-3 PUFA) is known to improve learning and memory of control rats. Whether n-3 PUFA supplementation improves dendritic morphology, synaptic transmission, and memory of chronically stressed rats remains unknown. In this work, we randomly assigned male Sprague-Dawley rats in four experimental groups: two unsupplemented groups, control and stress, and two supplemented groups with n-3 PUFA (DHA and EPA mix), control + n-3 PUFA and stress + n-3 PUFA. Dendritic morphology and synaptic transmission in the hippocampus were evaluated by Golgi stain and patch-clamp tools, respectively. The Y-maze and Morris water maze were used to analyze the effects of chronic stress on memory. Supplementation with n-3 PUFA improved dendritic architecture and restored the frequency of inhibitory post-synaptic currents of hippocampal pyramidal neurons of rats from stress group. In addition, n-3 PUFA supplementation improved spatial memory. Our results demonstrate that n-3 PUFA supplementation had three beneficial effects on stressed rats: prevented or compensated dendritic atrophy in CA3; restored the probability of GABA release in CA1; and improved spatial memory. We argue that n-3 PUFA supplementation can be used in treating stress-related psychiatric disorders such as depression and anxiety.

Keywords: Stress, n-3 PUFA, Memory, Hippocampus, Synaptic transmission, GABA

Introduction
Stress is a nonspecific biological response of an organism to any demand that allows it to restore homeostasis and adapt to environmental pressures (i.e. stressors). Stress response is mediated by the activation of the hypothalamus–pituitary–adrenal (HPA) axis, the main neuroendocrine arm of stress responses provoking glucocorticoid secretion. The orchestration of stress responses and modulation of synaptic transmission are strongly mediated by glucocorticoids, corticosterone (CORT) in rodents, and cortisol in humans.

There is abundant evidence that both stress and diet affect brain physiology. However, the interaction between the two remains unclear. Docosahexaenoic acid (22:6 n-3 PUFA; DHA) and eicosapentaenoic acid (22:5 n-3 PUFA; EPA) are the predominant dietary n-3 polyunsaturated fatty acids (n-3 PUFAs). It has been shown that DHA is one of the major components of the phospholipid membrane in neurons. DHA has a key role in neuronal physiology such as neurotransmitter release modulation, synaptic membrane fluidity, and long-term potentiation (LTP). Thus, adequate dietary availability of DHA and EPA is fundamental to brain function. Supplementation with n-3 PUFA improves cognitive functions like executive function, learning, and memory in both humans and animal models while rats subjected to an n-3 PUFA-deficient diet show memory impairments and atrophy of hippocampal neurons. On the other hand, chronic stress increases the n-6/n-3 ratio, suggesting lower n-3 PUFA status in stressed animals that affects the sensitivity to stress. Chronic stress in mice
is associated with changes in brain PUFA composition and serotonin levels in the hippocampus.\textsuperscript{22}

It has been reported that chronic stress impairs learning and memory in several animal models.\textsuperscript{23–26} These cognitive alterations are associated with dendritic atrophy in CA3 pyramidal neurons in the rat hippocampus.\textsuperscript{27} In this way, nutritional $n$-3 PUFA deficiency in mice produces a chronic stress state reflected by HPA axis hyperactivity, dendritic atrophy in the prefrontal cortex, and emotional alterations, whereas $n$-3 PUFA supplementation induces resilience to these alterations.\textsuperscript{28} We demonstrated that $n$-3 PUFA supplementation does not affect locomotor activity, improves learning, and has strong anti-stress and anxiolytic effects on stressed rats.\textsuperscript{16}

GABAergic signaling in the hippocampus has an important regulatory influence on the HPA axis. There are direct excitatory outputs from ventral hippocampus to bed nucleus of the stria terminals GABAergic cells, which in turn inhibits the HPA axis by the hypothalamic paraventricular nucleus.\textsuperscript{4,29–31}

It has been shown that changes in dendritic architecture strongly affect synaptic plasticity and neuronal firing properties.\textsuperscript{32–34} There is no consensus about the effects of chronic stress on synaptic inhibitory transmission. On the one hand, chronic stress and glucocorticoids induce dendritic atrophy in CA3 pyramidal neurons and increase GABAergic synaptic transmission in the hippocampus.\textsuperscript{35–37} On the other hand, rats subjected to unpredictable chronic stress show reduced GABAergic synapti
c transmission.\textsuperscript{38} In addition, mild chronic stress results in lower hippocampal levels of GABA\textsuperscript{39} and parvalbumin-positive GABAergic interneurons, which may affect brain oscillations in stressed rats.\textsuperscript{36,40}

There is little evidence about the effects of $n$-3 PUFA supplementation on hippocampal neuronal morphology and GABAergic transmission in the hippocampus of stressed rats. Therefore, in this study we hypothesize that $n$-3 PUFA supplementation restores the harmful effects induced by chronic stress on the hippocampus. Our results indicate that $n$-3 PUFA restores the dendritic architecture of hippocampal pyramidal neurons and the frequency of inhibitory post-synaptic currents (IPSCs), as well as improving long-term memory.

**Material and methods**

**Animals**

Male Sprague–Dawley rats (80–100 g, 21 days old at the start of the experiment) (Charles River, Wilmington, MA, USA) were housed (three animals per cage), under a 12/12 light/dark cycle (lights on at 8:00 am). The animals were maintained in a temperature and humidity-controlled room (20 ± 1°C, 60%) and weighed every day. All procedures relating to animal experimentation were in strict accordance with animal care standards outlined in the National Institute of Health (USA) guidelines and approved by the Animal Ethics Committee of the Universidad de Valparaíso. Efforts were made to minimize the number of animals used and their suffering.

**Experimental design**

Table 1 shows the number of animals used in each experiment. Scheme 1 shows the experimental design used in this study. Rats were maintained with ad libitum access to food (Prolab RMH 3000, LabDiet\textsuperscript{40}, St. Louis, MO, USA) and water during all experiments. The fatty acid composition of the food was fat (ether extract) 5.8%, fat (acid hydrolysis) 7.1%, cholesterol 199 ppm, linoleic acid 1.63%, linoleic acid 0.19%, arachidonic acid 0.02%, total saturated fatty acids 1.71%, total monounsaturated fatty acids 1.57%, $n$-3 PUFA 0.37%, $n$-6 PUFAs 1.72%, 4.6 ratio of $n$-6 PUFAs/$n$-3 PUFAs. Animals were fed 15–30 g of rat chow per day. The control diet used in this investigation was comparable with the diet of previous studies.\textsuperscript{16,21,41}

Rats were randomly assigned to four experimental groups: two unsupplemented groups, control and stress, and two supplemented groups with $n$-3 PUFA, control + $n$-3 PUFA and stress + $n$-3 PUFA. Rats of stress group were subjected to the restraint stress procedure. Animals of control + $n$-3 PUFA and stress + $n$-3 PUFA groups were supplemented daily with 1.0 ml of fish oil (Knop Laboratories S.A. Santiago, Chile), a mix of 100 mg of DHA and 25 mg of EPA per kg animal weight by oral administration.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Number of animals used in each experiment</th>
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<tr>
<td>Experiment</td>
<td>Control No supplementation</td>
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<tr>
<td>Morphological study (Golgi staining)</td>
<td>$n = 6$</td>
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<tr>
<td>Electrophysiological analyses: sIPSC and mIPSC</td>
<td>$n = 6$</td>
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<tr>
<td>sEPSC</td>
<td>$n = 6$</td>
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<tr>
<td>Y-maze</td>
<td>$n = 9$</td>
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<tr>
<td>Morris water maze</td>
<td>$n = 9$</td>
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<td>Total</td>
<td>36</td>
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Animals received n-3 PUFA fatty acids between post-natal days (PND) 21 and 59 (Scheme 1). We used a DHA/EPA ratio of 4, which is close to the fatty acid composition of phosphatidylserine and choline in the developing human brain and breast milk. Supplementation was applied once per day; the rat was picked up from its home cage and gently held in the hand of the experimenter for oral n-3 PUFA administration. Rats of all experimental groups were equally manipulated.

We did not use PUFA placebo (e.g. frial or olive oils) in this study as the neuronal excitability in CA1 could have been affected in the electrophysiological experiments. The main n-6 PUFA is arachidonic acid which causes a decrease in GABAergic transmission in the hippocampus. On the other hand, n-9 PUFA (oleic acid) increases the post-synaptic potential, firing rate, and the excitatory sharp waves in CA1.

Rats were randomly selected from 8 litters after weaning. They were kept three to a cage to reduce the number of siblings in each experimental group. Rats from control group rats were never exposed to distress and repeated restraint stress was applied in a room other than the one where rats were kept.

Handling procedure and restraint stress
Rats were removed every day by hand and transferred to another cage on the pan of a balance to be weighed. Different investigators did this procedure from those that applied the chronic stress. The same procedures were applied to all rats. Animals were placed in acrylic restrainers (6 cm wide × 12 cm long and then 6 cm wide × 20 long as the rats grew) in their home cages. They were subject to restriction for 6 h every day, beginning at 10 am, from the 36 to 57 PND (21 days of chronic stress). Restainers were perforated at each end for ventilation to avoid overheating the animals. During the stress protocol, animals could breathe without difficulty and urinate and defecate without being in constant contact with their waste.

Morphological data analysis
Immediately after completion of the stress protocol, each rat was killed under deep anesthesia with isoflurane (Novafarma Service S. A., Reg. I.S.P. Nº F-9189, Quilicura, Santiago, Chile). The brain was removed quickly and processed using the FD Rapid GolgiStain kit (FD Neuro Technologies, Inc., Ellicott City, MD, USA). Coronal sections were cut at 150 µm on a sliding cryostat (Microm®, Walldorf, Model H525, Germany). Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and coverslipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed.

To compare the results of the present study to those of previous reports that have shown that repeated restraint stress produces significant dendritic remodeling in the hippocampus, we analyzed the effects of n-3 PUFA supplementation on short-shaft pyramidal neurons in the c area. These neurons are characterized by short apical shafts and densely branched apical and basilar trees. Our morphometric analysis was restricted to the dorsal portion of the hippocampal CA3 (interaural 540 mm and bregma −3.60 mm).

One experimenter did the morphological analysis independently, randomly selecting 10 hippocampal pyramidal neurons per animal that met the following selection criteria: (1) the presence of untruncated dendrites; (2) consistent and dark impregnation along the entire dendritic field; and (3) relative isolation from neighboring impregnated neurons to avoid overlap. In order to reduce error in data acquisition and self-deception by the experimenter, the latter had no knowledge of whether the sample analyzed was from the unsupplemented or n-3 PUFA experimental group. Camera lucida tracings (500X, BH-2, Olympus Co., Tokyo, Japan) were obtained from selected neurons and then scanned (eight-bit grayscale TIFF images with 1200 dpi resolution; EPSON ES-1000C), along with a calibrated scale for

Scheme 1 Experimental design. Experimental design (not to scale) from day 21 to 59. Rats were handled, supplemented, and weighed daily from weaning (post-natal day 21) to the end of the stress protocol. Rats of the stressed group were subject to 6 h of restraint stress beginning on day 21 to PND 57. Immediately after completion of stress protocol, a set of rats was used for Golgi staining (Experiment-1). At PND 58, rats were subjected to electrophysiological recording (Experiment-2). Two new sets of rats were used to evaluate short-term and long-term spatial memory 1 day after the stress protocol ended using the Y-maze test and Morris water maze, respectively, (Experiment-3).
subsequent computerized image analysis. Custom-designed macros were used in Scion image 1.6 software (Scion Corporation, Frederick, MD, USA) for morphometric analysis of digitized images. The dendritic lengths of neurons selected from all rats were determined. Sixteen selected neurons were averaged to obtain a single value of dendritic length from each rat, and group means were obtained from each subject.

Electrophysiology
In addition to its effects on the CA3, chronic stress induces volume atrophy of the rat CA1 hippocampus. Therefore, stress-induced morphological changes on CA3 neurons may affect glutamate release from the terminals of Schaeffer collateral projections to CA1 neurons. Schaeffer collateral projections to CA1 may affect glutamate release from the stratum radiatum and close to recorded pyramidal neuron synapses (PNs). We, therefore, evaluated the effects of chronic stress and n-3 PUFA supplementation on both GABAergic and glutamatergic synaptic transmission in the CA1 area. A new set of rats was used to study the effects of n-3 PUFA supplementation on the efficacy of glutamatergic and GABAergic transmission in the hippocampi of rats from stress group. After completion of the stress protocol, rats were decapitated under deep anesthesia with isoflurane. Brains were quickly removed and submerged in cold (−4°C) artificial cerebrospinal fluid (ACSF) (in mM: 124.00 NaCl, 2.69 KCl, 1.25 KH2PO4, 2.00 MgSO4, 26.00 NaHCO3, 2.00 CaCl2, 10.00 glucose). The pH of the ACSF was stabilized at 7.4 by bubbling carbogen (95% O2, 5% CO2). Coronal brain slices (300−350 μm) were cut with a Vibratome (Campden Instruments, model MA752, Loughborough, England) and maintained in the ACSF (≥1 h, at room temperature: 20−22°C). Slices were transferred to a 2 ml chamber fixed to an upright microscope stage (NIKON, model Eclipse FN1, Tokyo, Japan) equipped with infrared differential interference contrast video microscopy and 40× water immersion objectives. Slices were superfused with carbogen-bubbled ACSF (2 ml/min) and maintained at room temperature (22−24°C). As needed, 2-amino-5-phosphonopentanoic acid (D-AP5; 50 μM) and 7-nitro-2,3-dioxo-1,4-dihydroquinazoline-6-carbonitrile (CNQX; 20 μM) were added to the ACSF.

Whole-cell recordings were made from the soma of CA1 pyramidal neurons with patch pipettes (4−8 MΩ) filled with an internal solution that contained (in mM): 100 Cs-Gluconate, 10 HEPES, 10 EGTA, 4 Na2-ATP, 10 TEA-Cl, and 1 MgCl2-6H2O, buffered to pH 7.2−7.3 with CsOH. Recordings were made in voltage-clamp modes using an EPC-7 patch-clamp amplifier (HEKA Instruments, Massachusetts, MA USA). In voltage-clamp experiments, the Vh was adjusted to −50 or 0 mV to record excitatory post-synaptic currents (EPSCs) or IPSCs, respectively. Series resistance in the voltage-clamp configuration was compensated to ~70% and neurons were accepted only when seal resistance was >1 GΩ and series resistance (7−14 MΩ) did not change by more than 10% during the experiment. The liquid junction potential was measured (−6 mV) but was not corrected. Voltage-clamp data were low-pass filtered at 3.0 kHz and sampled at rates between 6.0 and 10.0 kHz using an A/D converter (ITC-16, InstruTech, Massachusetts, MA USA) and stored with Pulse FIT software (HEKA Instruments, Massachusetts, MA USA). The Pulse Fit program was used to generate stimulus timing signals and transmembrane current pulses. The recording analysis was made off-line with the pClamp software (Clamp-fit, Molecular Devices Corporation, Chicago, IL, USA). EPSCs and IPSCs were evoked with a concentric bipolar electrode (60 μm diameter, tip separation ~100 μm (FHC Inc., Maine, ME, USA), placed at the stratum radiatum and close to recorded pyramidal neurons (~100 μm). An average of EPSC (n = 10, 6 rats) and IPSC (n = 10, 6 rats) were obtained under voltage clamp by repeated stimulation at 0.3 Hz.

Chemicals were purchased from Sigma-Aldrich Chemistry (Santiago, Chile), and Tocris (Bioscience, Pittsburgh, PA, USA). To establish the pre- or postsynaptic locus expression of these synaptic changes, we analyzed the paired-pulse ratio (PPR), which was quantified by (R2/R1)100, where R1 and R2 are the amplitude of the first and second IPSC or EPSC, respectively. To determine whether chronic stress simultaneously affects glutamatergic and GABAergic pyramidal neuron synapses (PNs), we voltage-clamped CA1 PNs at the reversal potential for evoked EPSCs or IPSCs, respectively. Values of the reversal potential of EPSCs and IPSCs were estimated from current−voltage relationships of EPSCs (0.3 ± 0.5 mV; n = 10) and IPSCs (−64.2 ± 2.3 mV; n = 10), respectively. In some experiments, excitatory or inhibitory synaptic transmissions were isolated after blocking GABA receptors with picrotoxin (10 μM) or NMDA and AMPA receptors with D-AP5 (50 μM) and CNQX (20 μM).

Calculation of the multiplicity factor
The multiplicity index was calculated to estimate the degree of connectivity between interneurons and pyramidal neurons in the CA1. The spontaneous and miniature GABAergic post-synaptic currents, spontaneous inhibitory post-synaptic currents (sIPSC) and miniature inhibitory post-synaptic current (mIPSC) were compared in CA1 neurons of rats from control and stress groups. To determine the multiplicity index, we first obtained the mean amplitude and frequency values of sIPSC and mIPSC separately, recorded before and after adding tetrodotoxin (TTX 500 nM). Multiplicity was calculated as the mean...
The amplitude of action potential-driven events (‘a’) divided by mean quantal size (‘q’: mean amplitude of mIPSC recorded in TTX). The ‘a’ value was determined for each cell by subtracting the contribution of mIPSC to the pool of events collected in absence of TTX, using the expression for ‘a’

\[
a = \frac{f_b - f_q}{f_b - f_q}
\]

where ‘fb’ and ‘fq’ denote the mean frequency values of events recorded before and after the addition of TTX to the perfusion media, respectively, and ‘b’ is the mean amplitude of both sIPSC and mIPSC.

The sIPSCs and sEPSCs and mIPSCs were analyzed off-line using the Minianalysis software (MiniAnalysis; Synaptosoft, New Jersey NJ, USA), which allowed visual detection of events and selection for the analysis of currents that exceeded an arbitrary threshold.

**Behavioral procedures**

Short-term memory was measured by Y-maze tests which were conducted on PND 58; a day after the completion of the stress protocol (Scheme 1). All animals were naive to the test situations. The behavioral tests were conducted in the test room from 10:00 am to 4:00 pm, with recording of the rats’ activities by IP cameras fixed above the behavioral apparatus and connected to a computer in another room. Videos were acquired by the Nuuo software (Nuuo, Taipei, Taiwan) and analyzed using the ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA). All mazes were thoroughly cleaned with 5% ethanol solution after each trial. In all experiments, animals from control and stress groups were evaluated at the same time.

**Y-maze**

The test consisted of three equilaterally intersecting black Plexiglas arms (58 cm long × 19 cm wide × 38 cm high) and several extra-maze cues on the surrounding walls. The three arms were assigned as Novel, Start, and Other, and were counterbalanced among rats. Rats from control and stress groups were tested at the same time and in separate Y-mazes. In the training, one arm (Novel) was blocked and the animals were placed in the Start arm to explore both the Start and Other arms for 15 min. In the tests following the training, the Novel arm was unblocked and rats were returned to their home cages and room. Four hours later, rats were returned to the same start location used during training and were allowed to freely explore all arms for 5 min. Rats tended to explore novel environments, consequently an intact spatial memory if the rats showed a preference for the Novel arm. Entry into an arm was defined when the animal placed all limbs onto the arm. Behavior was videotaped and entries were converted into percentages. To analyze the stress effect on spatial memory ability, the percentage of entries into Novel arm was measured after training.

**Water maze**

The Morris water maze was used to determine the effects of n-3 PUFA supplementation on long-term memory. We choose the 19°C version of the Morris water maze, because in this protocol chronically stressed rats showed impairments in memory acquisition compared to the respective rats from control group.53 The maze consisted of a blue circular tank (183 cm in diameter and 51 cm in height) placed in a sound-attenuated room with controlled temperature. Posters with geometric shapes were attached to the walls of the room for use as distal visual cues by animals when placed in the maze. The tank contained water colored with nontoxic black tempera paint at 19°C and 30 cm deep. The water maze was conceptually divided into four equal quadrants: east (E), west (W), north (N), and south (S). A circular Plexiglass platform (diameter, 13 cm; height, 28 cm) was submerged 2 cm in one quadrant (e.g. N–W). The platform provided the only escape from the water.

We used a new set of rats for this experiment. Training was done during the light period, approximately between 9:00 am and 2:00 pm, and was run for three consecutive days (learning). Four acquisition trials were applied per day to all rats. Each trial consisted of releasing the animal from the wall of each three quadrant of the pool (e.g. N–E, S–E, S–W), which differed for every trial. The rat then had 60 s to swim and reach the hidden platform. The rat was allowed to stay on the platform for 15 s before being dried off and transferred to a heated holding cage. If the rat did not reach the hidden platform before 60 s, it was guided there by the experimenter. There was a 10-min inter-trial interval and at the end of every training day of training, all animals were towel- and fan-dried and returned to their home cages. Twenty-four hours after the final training trial on day 3, the platform was removed and the rats were given a 60 s probe trial to analyze memory consolidation. The quadrant where the platform was previously located was termed the target quadrant, and the quadrant directly across from the target quadrant was termed the opposite quadrant. All tests were recorded by a video camera located in the roof of the test enclosure and analyzed using the ANY-maze video tracking system. Spatial memory consolidation in the probe trial was assessed by comparing the time taken and the distance traveled between the target and opposite quadrants.

**Statistical analysis**

Results from the Y-maze tests and dendritic morphology were analyzed by two-way analysis of
variance (ANOVA) for stress (control or stress) and diet (no supplementation, n-3 PUFA), followed by a Bonferroni post hoc comparison test.

For the electrophysiological studies, data analysis and statistical evaluations were made with the pClamp (Molecular Devices Corporation) and with the Origen 7.0 (Originlab Corporation, Northampton, MA, USA) softwares. Results are given as mean ± SEM. (n = number of cells). Statistical analyses were performed using the Kolmogorov–Smirnov (cumulative probability) and two-way ANOVA test (GraphPad).

Results

Effects of n-3 PUFA supplementation on hippocampal dendritic morphology

There is abundant evidence that chronic stress induces dendritic atrophy in CA3 pyramidal neurons in the rat hippocampus, as well as memory impairment.21,24 To determine whether n-3 PUFA supplementation prevents stress-induced dendritic atrophy, we compared apical dendritic length using Golgi stain. Camera lucida drawings and photomicrographs of representative CA3 pyramidal neurons of rats from stress and control groups are shown in Fig. 1A.

The total apical dendritic length was substantially less in the CA3 pyramidal neurons of rats from stress group (1130 ± 99 µm) than in those of rats from control group (1516 ± 116 µm, 25.5% difference; P < 0.05). The statistical analysis did not show difference between rats from control + n-3 PUFA and stress + n-3 PUFA groups, while animals from stress + n-3 PUFA group had significantly greater total apical dendritic length than did rats from stress group (stress + n-3 PUFA group = 1974 ± 284 µm, stress group = 1130 ± 99 µm; P < 0.05) (Fig. 1A and B).

All these results suggest that n-3 PUFA supplementation prevented or compensated dendritic atrophy induced by chronic stress in CA3 neurons.

Effects of n-3 PUFA supplementation on glutamatergic and GABAergic synaptic transmission

We analyzed the effect of chronic stress and n-3 PUFA supplementation on the efficacy of glutamatergic synapses and did not observe any significant effects (Fig. 2D–E). To determine whether chronic stress and n-3 PUFA supplementation modify the efficacy of GABAergic synapses on CA1 PNs, we voltage-clamped CA1 PNs and recorded the corresponding evoked and spontaneous inhibitory synaptic currents, eIPSC and sIPSC, respectively. The PPR of the rats from control group was characterized by depression, with R2 smaller than R1, indicating that the group of stimulated inhibitory synapses had a higher...
control + n-3 PUFA (65.86 ± 4.46%) increased the PPR compared to the rats of control group (47.44 ± 3.40%, $P < 0.05$), while GABA release was higher in the rats of stress + n-3 PUFA group (51.53 ± 5.80%) than in the rats of stress group (72.14 ± 3.80%, $P < 0.05$) (Fig. 2B and C).

Taken together, these results suggest that chronic stress only affects GABAergic synaptic efficacy, without altering glutamatergic synaptic transmission.

**Effects of n-3 PUFA supplementation on spontaneous GABAergic neurotransmission in the hippocampus**

We found that only GABAergic synapses were affected by chronic stress in the CA1. Consequently, we studied the effects of n-3 PUFA supplementation on inhibitory GABA neurotransmission in CA1 PNs (Fig. 3A–C).

The sIPSC frequencies of rats from stress group (1.49 ± 0.14 Hz) were significantly lower compared to rats from control group (2.62 ± 0.11 Hz, $P < 0.01$). On the other hand, rats from stress + n-3 PUFA group (2.22 ± 0.24 Hz) had significantly higher sIPSC than animals from control + n-3 PUFA (1.33 ± 0.45 Hz, $P < 0.05$; $n = 8$ cells/4 rats; Fig. 3B left). Animals from control + n-3 PUFA group (1.33 ± 0.45 Hz) had significantly sIPSC lower frequencies compared to rats from control group (2.62 ± 0.11 Hz, $P < 0.01$) (Fig. 3B), while animals from stress + n-3 PUFA (2.22 ± 0.12 Hz) had higher frequency of sIPSC than rats from stress group (1.49 ± 0.14 Hz, $P < 0.05$) (Fig. 3B).

Chronic stress and n-3 PUFA supplementation did not affect sIPSC and mIPSC amplitudes, respectively (rats from stress group: 8.76 ± 8.1 pA/44.39 ± 7.0 pA; rats from control group: 73.28 ± 8.7 pA/52.49 ± 2.2 pA, $P > 0.05$) (Fig. 3B and D right). To determine whether alterations in inhibitory synaptic transmission were due to the changes in the interneurons excitability, we measured mIPSC and observed that the mIPSC frequencies of rats from both control + n-3 PUFA and stress groups were 0.14 ± 0.02 Hz and 0.36 ± 0.06 Hz, $n = 5$ cells/4 rats, respectively, whereas the frequency in rats from control group was 0.52 ± 0.02 Hz ($P < 0.05$; $n = 8$ cells/4 rats; Fig. 3C left). Animals from control + n-3 PUFA group (0.14 ± 0.02 Hz) had significantly mIPSC lower frequencies compared to respective rats from the stress + n-3 PUFA group (0.63 ± 0.05 Hz, $P < 0.01$) (Fig. 3C).

Regarding presynaptic loci, chronic stress decreased sIPSC and mIPSC frequencies (Fig. 3B and C left), suggesting that stress-induced reduction of GABA release occurs presynaptically as a result of decreased probability of release. Moreover, chronic stress had no effect on post-synaptic GABA efficacy (Fig. 3C, right). Thus, we observed that chronic stress

![Figure 2](image_url)
significantly decreased sIPSC and mIPSC frequencies, whereas amplitude was not affected.

To determine whether chronic stress had reduced the number of synapses, we estimated synaptic network connectivity of excitatory and inhibitory synapses on the basis of the multiplicity index. For each cell, we recorded the sIPSC and mIPSC during 500 nM TTX perfusion. We did not find changes on multiplicity indices of GABAergic interneurons and CA1 pyramidal neurons by chronic stress or n-3 PUFA supplementation, suggesting that chronic stress (stress group: 1.60 ± 0.08; control group: 1.74 ± 0.11, t-test, \( P > 0.05 \)) and n-3 PUFA supplementation (stress + n-3 PUFA: 1.73 ± 0.21; control + n-3 PUFA: 1.37 ± 0.09, t-test, \( P > 0.05 \)) did not affect the degree of connectivity between inhibitory interneurons and CA1 pyramidal neurons.

**Effects of n-3 PUFA supplementation on spatial memory**

**Short-term memory**

Chronic stress impaired the rats’ performance in the Y-maze. This finding was supported by the 4-h delay
version of the Y-maze. Figure 4A and B shows the effect of both stress and diet on spatial memory.

The post hoc test showed that chronic stress significantly decreased the percentage of entries into the Novel arm (rats from stress group: $22.82 \pm 1.88$; from control group $33.04 \pm 3.3$; $P < 0.05$) (Fig. 4A and B). This effect was prevented with $n$-3 PUFA supplementation (rats from control + $n$-3 PUFA group = $40.74 \pm 2.6$; rats from control + $n$-3 PUFA group = $21.22 \pm 3.6$; $P < 0.05$) (Fig. 4A and B). An unexpected result was that rats from control + $n$-3 PUFA group had a lower percentage of entries into the Novel arm than rats from control group (rats from control + $n$-3 PUFA group = $21.22 \pm 3.6$, rats from control group: $33.04 \pm 3.3$; $P < 0.05$) (Fig. 4A).

Our results indicate that $n$-3 PUFA and chronic stress impaired short-term memory, while $n$-3 PUFA supplementation prevented the effect of chronic stress on short-term memory of rats from control group.

**Long-term memory**

Afterward, we analyzed the effect of chronic stress and $n$-3 PUFA on long-term memory using Morris water maze. The two-way repeated measures ANOVA did not show a significant effect of treatment on the time and distance to reach the platform on the third day of training (Fig. 5A and C).

During the probe trial on day 4, there were no significant differences for swim time in the target versus opposite quadrant in the rats from stress group (Fig. 5B, right side). Conversely, rats from control group spent more time in the target versus the opposite quadrant (target quadrant $= 19.50 \pm 1.03$ s, opposite quadrant $= 13.99 \pm 1.54$ s, $P < 0.05$). Interestingly, animals from stress + $n$-3 PUFA group swam longer in the target versus the opposite quadrant (target quadrant $= 24.11 \pm 0.58$ s, opposite quadrant $= 14.84 \pm 0.38$ s, $P < 0.001$) (Fig. 5B, right side). Likewise, rats from control and control + $n$-3 PUFA groups spent more time in the target versus the opposite quadrant (rats from control group: target quadrant $= 19.50 \pm 1.03$ s, opposite quadrant $= 13.99 \pm 1.54$ s, $P < 0.05$; control + $n$-3 PUFA group: target quadrant $= 19.30 \pm 1.49$ s, opposite quadrant $= 12.92 \pm 1.86$ s, $P < 0.01$) (Fig. 5B, left side).

Distance data were consistent with the latency analysis. There were no significant differences between rats from stress group for swim distance in the target versus opposite quadrant (Fig. 5D, right side). Rats from control and control + $n$-3 PUFA groups swam greater distance in the target quadrant compared to the opposite quadrant (control group: target quadrant $= 0.28 \pm 0.44$, opposite quadrant $= 0.31 \pm 0.47$ m, $P < 0.05$; control + $n$-3 PUFA group: target quadrant $= 0.37 \pm 0.37$, opposite quadrant $= 0.44 \pm 0.37$ m, $P < 0.05$) (Fig. 5D, left side).

Supplementation with $n$-3 PUFA increased the swim distance in the target versus the opposite quadrant (rats from stress + $n$-3 PUFA: target quadrant $= 5.34 \pm 0.09$ m, opposite quadrant $= 3.44 \pm 0.2$ m, $P < 0.01$) (Fig. 5D and E).

Taken together, these results suggest that chronic stress impaired long-term memory, while $n$-3 PUFA supplementation prevented it.

**Discussion**

The present study demonstrates that $n$-3 PUFA supplementation had three beneficial effects on rats exposed to repeated restraint stress: prevented or compensated dendritic atrophy in the CA3; restored the probability of GABA release in the CA1; and improved spatial memory.

First, we analyzed whether restraint stress and $n$-3 PUFA affect the morphology of CA3 pyramidal neurons. Figure 2 shows that supplementation with $n$-3 PUFA prevented or compensated dendritic atrophy induced by chronic stress in CA3 neurons. It has been proposed that stress-induced hippocampal atrophy is produced by an increase in glucocorticoid
Figure 5 Learning and memory in the Morris water maze. (A) Time acquisition: rats in all groups showed reduced times to the platform across training days. Rats from stress group took longer to locate the hidden platform during training days compared to the respective rats from control group. (B) Time probe: rats from control, control + n-3 PUFA, and stress + n-3 PUFA groups spent more time in the target quadrant than the opposite quadrant. Moreover, there were no significant differences for swim time in the target versus opposite quadrant in the rats from stress group. (C) Distance acquisition: animals of all groups showed significant reduction in distance to reach the platform location across training days. (D) Distance probe: animals from control, control + n-3 PUFA, and stress + n-3 PUFA groups performed similarly on the probe trial, covering more distance in the target quadrant than the opposite quadrant. Rats from stress + n-3 PUFA showed intact long-term memory. (E) Representative tracking plots of control and stressed rats in the Morris water maze. Error bars indicate the means ± SEM. An asterisk (*) indicates significant differences.
receptors expressed in CA3. Chronic stress may have decreased proplastic protein levels (e.g., BDNF and PSA-NCAM) in the hippocampus that induces dendritic atrophy. Proplastic proteins are implicated in neurite extension, cell survival, and synaptic plasticity. On the other hand, n-3 PUFA supplementation may increase the level of the proplastic proteins that prevent dendritic atrophy in the CA3 of rats from stress group.

Figures 2 and 3 show that restraint stress decreased the GABAergic synaptic efficacy in the CA1. Morphological changes induced by restraint stress in CA3 neurons could affect the excitability of these neurons and decrease glutamate release from the presynaptic terminal of Schaeffer collateral projections to CA1 neurons. Thus, the excitatory/inhibitory balance in the CA1 field may be modified, as shown in Figs. 2 and 3, in which restraint stress modifies GABAergic but not glutamatergic synaptic transmission in the CA1. In line with this, reduction of commissural/associational input to CA3 LTP in chronically stressed rats has been shown. This suggests that changes in the dendritic architecture of CA3 pyramidal neurons affect the excitability of the CA3-CA1 circuit and induce deficits in hippocampal-dependent memory tasks.

Unexpectedly, results showed that rats from control + n-3 PUFA group had significantly decreased the probability of GABA release in the CA1 significantly as compared to animals from the control group (Figs. 2 and 3). This result suggests that the method used to supplement n-3 PUFA (repeated oral administration) was also stressful for rats. Accordingly, we found that oral administration was stressful for the rats, because this method resulted in higher CORT levels after acute swimming in rats from control + n-3 PUFA group than the rats from control group (Fig. 3B, right side). Therefore, it is possible that in our experiments chronic stress induced by repeated oral administration had increased the PPR index and decreased the mIPSC frequency in rats from control + n-3 PUFA (Figs. 2 and 3). On the other hand, animals from both control + n-3 PUFA and stress + n-3 PUFA groups were equally manipulated during oral supplementation; therefore, when the results of both groups are compared, it is possible to see the specific effects of n-3 PUFA on chronically stressed rats. Figures 2 and 3 show that PPR index and mIPSC frequency were lower and higher, respectively, in rats from stress + n-3 group compared to control + n-3 PUFA, and thereby n-3 PUFA improved the probability of GABA release in the CA1 of chronically stressed rats. However, n-3 PUFA did not improve GABAergic neurotransmission in the rats from control + n-3 PUFA group compared to animals from control group (Figs. 2 and 3). Consequently, we cannot explain this controversy with the results obtained in this research; so further experiments will be required.

Figures 4 and 5 show that restraint stress impaired short- and long-term memory. There is considerable evidence that highly synchronized theta activity in the entorhinal cortex and hippocampus plays a critical role in learning and memory. For example, suppression of hippocampal theta activity in rodents impairs spatial memory. The CA1 area of the hippocampus is a theta rhythm generator and GABAergic interneurons located in the CA1 are key in generating theta oscillations. Chronic stress attenuates theta activity in the hippocampus and induces CA1 volume atrophy. Interestingly, we found that chronic stress reduced the frequency of sIPSC and mIPSC in the CA1. Consequently, it is possible that inhibition synaptic efficacy over pyramidal cells increased the excitability of hippocampal CA1 circuits. As a result, stress-induced impairment in short- and long-term spatial memory could be due to the imbalance between inhibitory and excitatory activity in CA1 area. On the other hand, both frequency of mIPSC in CA1 and dendritic morphology in CA3 were recovered after n-3 PUFA supplementation, which in turn may improve the CA3-CA1 circuit and spatial memory of rats from stress group. Increase of paired-pulse index plus the reduction of mIPSC frequency indicates that the restraint stress affects mainly the presynaptic mechanism of GABA release, which was prevented by n-3 PUFA supplementation (Fig. 2).

An earlier report that showed the opposite results, namely that restraint stress increases the sIPSCs in CA1. In that study, the rats were kept under an inverse light cycle (lights off from 7:00 am to 7:00 pm, active period), while in our study the rats were subjected to a normal light cycle (lights on from 8:00 am to 8:00 pm, rest period). Restraint stress has a stronger impact when the rats are immobilized during their active period than the rest period. For example, 7 days of restraint stress only in the active period of the rats induces adrenal hypertrophy and decreases body weight. Therefore, we suggest that in the rats used in the Hu study, plasma CORT levels were higher than in the animals used in our experiments. Acute restraint stress, which induces higher plasma CORT levels and dexamethasone treatment, a synthetic CORT, both increase the sIPSC in CA1 of rats. Conversely, restraint stress does not affect plasma CORT levels at baseline during rest periods.

Another explanation of the effects of n-3 PUFA supplementation on the spatial memory of rats from stress group found in this study could be modulation of the immune system. It has been reported that chronic
stress induces activation of central macrophages, which increases pro-inflammatory mediators in the brain and decreases the release of anti-inflammatory cytokines like IL-4 and IL-10. Fat-1 mice are genetically modified to display endogenously elevated n-3 PUFA tissue contents and exhibit lower n-6/n-3 PUFA ratios. A recent study shows that fat-1 mice are resilient to LPS-induced pro-inflammatory cytokine production and spatial memory impairments.

It is generally accepted that n-3 PUFA improves the cognition of subjects, whether human or experimental animals. Our results indicate the opposite that n-3 PUFA treatment impairs short-term memory and GABAergic transmission in the CA1 in control animals (Figs. 2 and 4). A possible explanation for this controversy is related to the effect of n-3 PUFA on GABAergic transmission in the CA1 of rats from control group. In these animals, the treatment decreased the presynaptic GABA release mechanism (Figs. 2 and 3). In this sense, the CA1 regulates memory in the Y-maze, so that the decrease in the inhibitory tone in the CA1 could have affected the generation of the theta rhythm, which is necessary to synchronize the hippocampus with the prefrontal cortex during learning, resulting in diminished memory in these animals. It is important to note that the locomotor activity of these animals was comparable to that of the rats from control group, which indicates that the effects of n-3 PUFA were specific to the memory.

Another unexpected result was that the rats from control + n-3 PUFA did not present alterations in the consolidation of long-term memory in the Morris water maze (Fig. 5). The question is how a rat that presented impaired short-term memory could consolidate long-term memory. A possible answer is that the CA3-CA1 circuit regulates long-term memory in the Morris water maze.

Supplementation with n-3 PUFA did not suppress all GABAergic activity in the CA1 of rats from control + n-3 PUFA group but rather only reduced it (Figs. 2 and 3). As well, the n-3 PUFA treatment did not affect the morphology of pyramidal neurons in the CA3 of these animals (Fig. 1). This evidence indicates that the effect of n-3 PUFA on the CA3-CA1 circuit of rats from control group was slight and it is possible that during the three days of learning in the Morris water maze, the CA3-CA1 circuit was over-stimulated, attenuating the effect of n-3 PUFA on the CA3-CA1 circuit of rats from control group. On the other hand, in the Y maze the rats were submitted to only two trials in one day and perhaps the CA3-CA1 circuit was less stimulated than during the Morris water maze, with a decrease observed in the short-term memory of the rats that were supplemented with n-3 PUFA. The effects of chronic stress on the CA3-CA1 circuit were stronger than the n-3 PUFA supplementation. Besides decreasing GABAergic activity in the CA1, restraint stress induced dendritic atrophy in the CA3 (Figs. 1–3). This may explain why rats exposed to repeated restraint stress presented impaired short- and long-term memory.

In conclusion, our data demonstrate that n-3 PUFA improved memory and prevented hippocampal dendritic atrophy in rats from stress group. Furthermore, n-3 PUFA supplementation prevented stress-induced decreases in GABAergic synaptic efficacy in the CA1. Our study proposes the hippocampus as a target for the positive effects of n-3 PUFA supplementation to improve memory in an animal model of stress-related psychiatric disorders.

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