

# **Docosahexaenoic acid restores learning and memory functions altered by acute manganese intoxication in mice**

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## **Abstract:**

Manganese (Mn) is a trace essential element and neurotoxic at higher levels, provokes cognitive impairment via oxidative stress, neuroinflammation, and neurotransmitter disarrangement. Therefore, this study aimed to investigate whether DHA, a major omega-3 polyunsaturated fatty acid that is known to have potent neuroprotective effects, rescues deficits in learning and memory induced by Mn in mice. DHA was given daily for 6 days at the beginning of acute manganese intoxication to adult Swiss mice. Spatial learning, short-term memory long-term memory and executive function was assessed with the Morris Water Maze test and Puzzle Box tests. Mnexposure caused a significant impairment in all the neuropsychological areas, spatial memory function, and inhibitory avoidance retention and prolonged escape latency time (ELT) than problem-solving ability. Most importantly, DHA co-treatment completely ameliorated the cognitive deficiency, including changes in learning curves and memory retention. These results reinforce the idea that DHA protects against Mn-induced neurotoxicity, at least in part due to its antioxidative, anti-inflammatory and synaptoprotective effects. In conclusion, DHA might be a potential nutritional intervention for preventing or reversing heavy-metal induced learning and memory deficits.

**Keywords: Manganese neurotoxicity; Docosahexaenoic acid (DHA); Cognitive impairment; Spatial memory; Neuroprotection; Oxidative stress**

## **1. Introduction**

Manganese (Mn) is an important trace element, involved in enzymatic systems, synthesis of neurotransmitters, antioxidant protection and energy metabolism. Despite its role in normal physiologic processes, overexposure to Mn is neurotoxic and contributory to cognitive, motor, and affective deficits collectively known as manganism. Mn-induced neurotoxicity occurs via mechanisms by oxidative stress, neuroinflammation, mitochondrial dysfunction and changes in dopaminergic and cholinergic systems resulted in some neurobehavioural deficits [1,2]. In humans, Mn exposure is also due to contaminated food, air and drinking water, as well as occupational (e.g. welding, mining or battery manufacture) and industrial sources including steel manufacturing for

instance. The cognitive impairment caused by Mn is particularly heavy in newborns and young children.

Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid highly concentrated in the central nervous system (CNS), is vital to maintain normal neuronal structure, enhance synaptic activities and facilitate anti-inflammatory responses. Such effects of DHA may be related to its potential to act against neuroinflammation, oxidative stress and neurotransmission altered pathways; hence it has putatively ameliorative activity against the Mn-induced neurotoxicity [3,4]. In animal studies, DHA supplementation has been found to improve learning and memory and might be helpful against cognitive deficits in neurodegenerative diseases.

Despite accumulating data supporting Mn-induced cognitive deficits, only a few studies have directly examined if DHA can protect against or reverse Mn-mediated learning and memory impairments. By focusing on several domains of cognitive function, including short-assessment term, long-term, spatial and executive memory this study aims to evaluate the potential protective role of DHA against acute Mn intoxication in mice.

## **2. Materials and Methods**

### **2.1. Materials**

#### **2.1.1 Chemicals**

Manganese chloride ( $MnCl_2$ ) was obtained from Panreac Química S.A.U. (Lot No. 0000261118, Barcelona, Spain). Docosahexaenoic acid (DHA,  $C_{22}H_{32}O_2$ ) was purchased from AlgoFit Solutions (Fleurbaix, France).

#### **2.1.2 Animals**

Adult Swiss mice (adult) were supplied by the central animal house of Cadi Ayyad University, Marrakech, Morocco. The mice were housed under standard laboratory condition in specific pathogen free (SPF) facilities with food and water provided ad libitum. Temperature was  $22 \pm 1^\circ C$  with a light/dark cycle of 12/12 h. All experiments were carried out according to EC/Faculty of Science Semlalia rulings on animal care and approved by Cadi Ayyad University, Marrakech and Respect European guidelines for ethics regarding approval and accreditation of the use of animals (February 1st, 2013, Nor: AGRG1238767A). All efforts were made to minimize suffering and distress of the animals.

## 2.2 Methods

### 2.2.1 Experimental Design

The adult male and female Swiss mice, weighing 25–35 g, were rerandomized into the following four groups (n = 6 animals per group to minimize animal use) in order that both acute Mn intoxication and DHA treatment be fully evaluated: control; Mn; DHA; and DHA + Mn. Six days-treatment periods, combining oral gavage (Days 1, 3, 5) with intraperitoneal injections (Days 2, 4, 6), were performed:

- ✓ **Control (C):** Mice received 0.9% sodium chloride (NaCl) by gavage and intraperitoneally on alternating days.
- ✓ **Manganese (Mn):** Mice received 0.9% NaCl by gavage and MnCl<sub>2</sub> (20 mg/kg, dissolved in 0.9% NaCl) intraperitoneally.
- ✓ **Manganese + DHA (Mn+DHA):** Mice were treated with DHA (200 mg/kg, by gavage) and MnCl<sub>2</sub> (20 mg/kg, i.p.) on alternating days.
- ✓ **DHA:** Mice received DHA (200 mg/kg, by gavage) and 0.9% NaCl intraperitoneally.

## 3. Statistical analysis

Statistical analysis was carried out using Sigmaplot V12.0 software. The data were analyzed using one-way analysis of variance (ANOVA). Post-hoc testing was performed using the Holm-Sidak test. Data was reported as mean ± S.E.M. and a significance threshold of  $p < 0.05$  was taken to be significant.

## 4. Behavioural study: Memory Assessment

### 4.1 Morris Water Maze (MWM) Test

MWT is a widely used spatial learning, memory and visuomotor guidance behavioral test in rodents [49]. For spatial learning, we used the number of trials over days for an animal to reach criterion; for reference memory, this was extinction choice (heads at the platform location) after the platform has been removed in a probe test (Vorhees & Williams, 2006). 1A) [11] fixed with a circular column (100 cm diameter, 60 cm high including the gap; depth is 40cm), maintained at constant temperature of ~21 °C. Drop powdered milk into the water and, in the maze of cutouts, it clouds. Visual markers line the walls of a room to guide people.

a 11 cm diam circular escape platform is partially submerged in the water. For every trial, the mouse is introduced in the pool facing the wall at one of four corners. Mouse must find the platform within 60 s in a trial. The mouse is given an additional 20 s to be placed on the raised platform by the experimenter, should it fail to find it within that time. During this time, the mouse is provided with an opportunity to explore its environment and acquire both internal and external cues, which it will use in subsequent trials, to navigate its way to the platform.

The pool is divided up into four quadrants: Southwest, Southeast, Northwest and Northeast. Initial point and platform location are systematically varied according to a predetermined order over six days. Every trial is recorded using a camera connected to a computer, and the data are analysed with the software ANY-maze.

Test procedure [5]:

**Habituation:** On day one, mice were habituated to the testing environment and trained to swim as well as learning that the platform is an escape by being placed on it. The water is transparent, and the platform is slightly above the surface of the water (1 cm) and mobile.

**Learning trial:** From day 2 to 5, the water is clouded with powdered milk; position of the submerged platform beneath the water and fix it in the south west quadrant. Spatial memory is also evaluated by recording the escape latency to find the platform for mice.

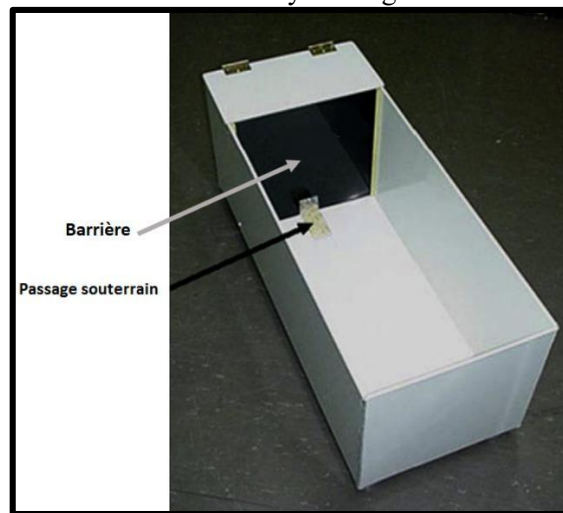
**Memory Assessment:** On the day following the final learning session, the platform is removed, and the mouse is placed in the north starting position. A single 60-second trial is conducted, and the time spent in the quadrant where the platform was previously located (SW; southwest) is recorded. Mice with intact memory are expected to spend more time in this target quadrant.



**Figure 1:** Morris water maze apparatus.

## 4.2 Puzzle Box Test

The Puzzle Box test is commonly used to measure problem-solving and executive function in rodents. The device is a white Plexiglas box, divided in two compartments by a partition. One chamber is an illuminated area (58 cm × 28 cm), the other a small, non-illuminated target area (15 cm × 28 cm) covered by its lid to form an enclosed space (Fig. 2). The two compartments are linked by a door in the barrier and an underpass which the animal can travel through to reach the dark chamber. Variables measured: The primary measure is the latency, which refers to how long it takes for the animal to reach the dark goal chamber. The mice conducted three trials for each day, making a total of nine trails (T1–T9) during 3 days. In these trials different degrees of difficulty were added to the runway leading to the dark chamber (see Table 1), [6].



**Figure 2** : Puzzle Box apparatus.

**Table 1.** Arrangement of the barrier and the underpass according to the trials

	Day 1			Day 2			Day 3		
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9
Task	The barrier is open	The door is closed	The door is closed	The door is closed	The door is closed	The door is closed	The door is closed	The door is closed	The door is closed
	Underground passage: Free	Underground passage: Free	Underground passage: Free	Underground passage: Free	Underground passage: Free	Underground passage: Free	Underground passage: Free	Underground passage: Free	Underground passage: Free

The mice must dig their way through the bedding to enter the dark chamber

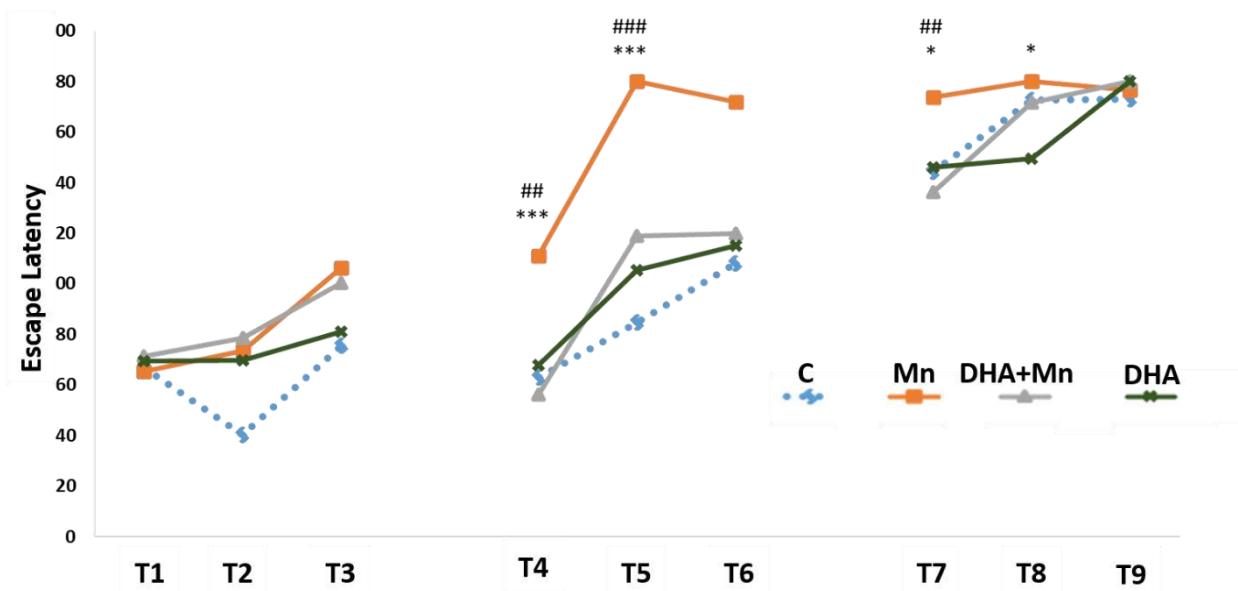
The mice must remove the cardboard to clear the

passage and enter the dark chamber

### 4.3 Neurobehavioral Study Memory Assessment

#### 4.3.1 Puzzle Box Test

The memory tests assess three legs of memory, short term, long term and executive as well as problem solving. Long-term memory was evaluated with trials 4 and 7, which are repetitions of trials 3 and 6 respectively. These tests are conducted at the start of days 2 and one day (24 h) later compared to the mere fatiguing trials. Short-term memory was assessed in VX3suffles 3, 6, and 9 by repeating them on the same day. Executive memory was tested in days 2, 5, and 8, with higher difficulty levels. We show here that manganese-intoxicated mice display robust long-term and executive memory deficits, although neither short-term nor motor memories are strikingly affected (Fig. 3). Supplemental DHA fully ameliorated the deficits in each of the memory domains measured by this assay.

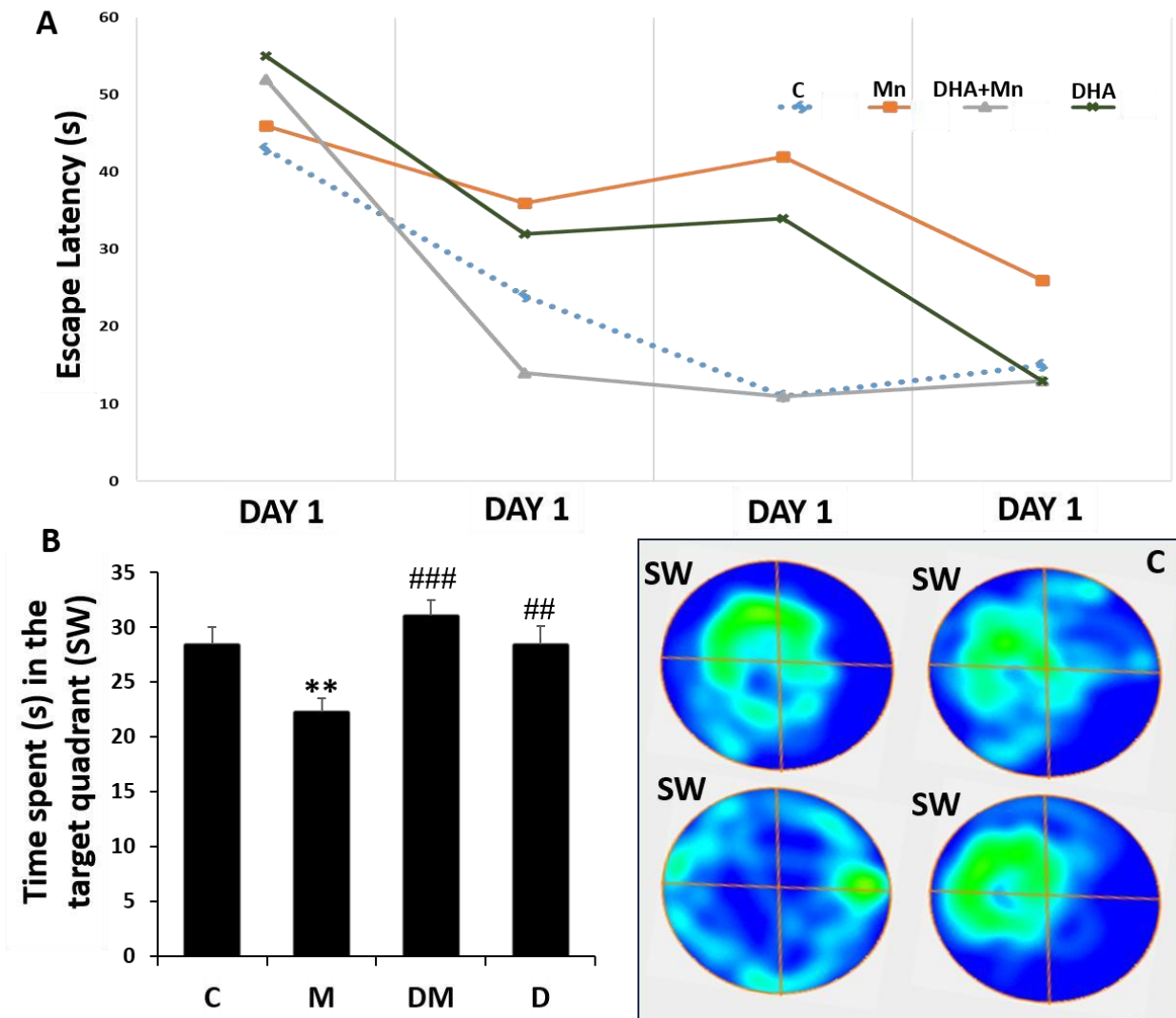


**Figure 3:** Graphical representation of Puzzle Box test results. C: Control; Mn: Manganese-intoxicated; DHA+Mn: Manganese-intoxicated + DHA-treated; DHA: DHA-treated.

Results are presented as mean values. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. C; ## $p < 0.01$ , ### $p < 0.001$  vs. Mn.

### 4.3.2 Morris Water Maze Test

We used the MWM test to test learning and long-term spatial memory in our mice. The results indicated that animals intoxicated with Mn exhibited impaired learning (Fig. 4A), evidenced by decreased time spent in the southwest (SW) quadrant, reflecting altered long-term spatial memory (Fig. 4B and 4C). Importantly, DHA treatment effectively ameliorated all deficits in learning and memory.



**Figure 4:** Graphical representation of the Morris Water Maze test results. A: Escape latency; B: Time spent in the SW quadrant; C: Swimming trajectories.

Groups. C: Control; Mn: Manganese-intoxicated; DHA+Mn: Manganese-intoxicated + DHA-treated; DHA: DHA-treated. Data are presented as Mean  $\pm$  SEM. \*\*p < 0.01 vs. C; ## p < 0.01, ### p < 0.001 vs. Mn.

## 5. Discussion

In the present study, we also found acute Mn exposure caused mice memory significantly impairments in short-term memory, long-term memory and executive memory tested by Puzzle Box test and there were long-term spatial learning and memory deficits in MWM water maze tests. Treatments with DHA co-treatment did restore memory performance in all dimensions explored, evidencing an important neuroprotective role of DHA. While the earlier studies demonstrated that DHA can alleviate the oxidative stress or inflammation induced by Mn exposure, few studies have shown a complete restoration of memory performance in all memories across memory paradigms examined. The present study is an extension to earlier biochemical and mechanistic studies that finds functional consequences of DHA's neuroprotection.

Several paths for toxicity of Mn can be described. Mn is able to enter the brain through the blood brain barrier (BBB) [7,8]. Once it enters the brain tissue, Mn accumulates in brain regions associated with cognitive function including frontal cortex hippocampus and basal ganglia. Oxidative stress and neuroinflammation as well as the disruption of neurotransmission systems, in these brain areas could affect memory and learning.

Conversely, astrocytes, the most abundant cell type of the brain and known to play a key role in both health and disease [9], are also an important target from Mn toxicity as they accumulate Mn at intracellular levels 50-60 folds higher than that of neurons particularly in mitochondria [10]. Mn disrupts neuronal homeostasis by (1) influencing astrocytic glutamine-glutamate cycle (Glu/Gln cycle, a which metabolite of Glu can be transported to neuron and then synthesized into GABA in neuron, has been showed showing Mn loses uptake by high-affinity component), and (2) producing OS etrogen and NOS. Mn also stimulates an increase in proinflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in the hippocampus, and causes reactive astrocytosis which may result in memory impairment [11,12]. The long-term Mn exposure ultimately results in disrupted neurotransmission and monoaminergic function, such as diminished 5-hydroxytryptamine (5-HT) and norepinephrine within striatum, accompanied by dopamine metabolism/deposition

interference [13]. Mn can also affect the glutamatergic signaling through inhibition of astrocytic excitatory amino acid transporters (EAAT1/2), resulting in a decrease of glutamate uptake and an increase of extracellular glutamate levels [14]. This leads to various associated effects including oxidative stress, NMDA receptor over-activation, calcium overload and at last neuronal dysfunction in hippocampus which probably is in relationship with memory and spatial learning deficiencies.

Co-treatment with DHA also recovers Mn -induced memory deficits via all its anti-inflammatory and anti-oxidative effects, thereby mitigate the increased oxidative stress and protect hippocampus neurons against excitotoxicity. Furthermore, DHA rescues memory and reverses the Mn-induced alteration of neurotransmission by increasing astrocyte defense response through Nrf2-mediated responses.

In conclusion, acute manganese exposure causes significant memory impairment that is due to changes in neurotransmission, increased oxidative stress and astrocyte dysfunction in the hippocampus. The memory functions return to that of control rats when DHA was co-treated, possibly via neuroprotection. All of these data support DHA as a potential therapeutic agent for the treatment of Mn-induced cognitive deficits.

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