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Deuterium-depleted water has stimulating effects on long-term memory in rats

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HIGHLIGHTS

- Deuterium-depleted water resulted in no significant changes in Y-maze.
- Reference memory errors were decreased in the DDW group in the radial 8 arms-maze.
- Time to finish the radial was reduced in DDW, compared to distilled water-controls.

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ABSTRACT

Deuterium-depleted water (DDW) is a water which has a 6–7-fold less concentration of the naturally occurring deuterium (20–25 ppm vs. 150 ppm). While administrated for a longer period, it may reduce the concentration of deuterium throughout the body, thus activating cellular mechanisms which are depending on protons (channels, pumps, enzyme proteins). The aim of the present work was to study, for the first time in our knowledge, the possible influence of deuterium-depleted water (DDW) chronic administration in normal Wistar rats, as compared to a control group which received distilled water, on spatial working memory and the locomotor activity (as studied through Y-maze) or both short-term and long-term spatial memory (assessed in radial 8 arms-maze task). Our results presented here showed no significant modifications in terms of spatial working memory (assessed through spontaneous alternation percentage) and locomotor activity (expressed through the number of arm entries) in Y-maze, as a result of DDW ingestion. Also, no significant differences between the DDW and control group were found in terms of the number of working memory errors in the eight-arm radial maze, as a parameter of short-term memory. Still, we observed a significant decrease for the number of reference memory errors in the DDW rats. In this way, we could speculate that the administration of DDW may generate an improvement of the reference memory, as an index of long-term memory. Thus, we can reach the conclusion that the change between the deuterium/hydrogen balance may have important consequences for the mechanisms that govern long-term memory, as showed here especially in the behavioral parameters from the eight-arm radial maze task.

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1. Introduction

Water (H₂O), the most common compound on Earth and the absolute necessity for life, does not have an identical isotopic composition throughout all liquid bodies on Earth. Differences may be minute, but nevertheless important from the biological standpoint. Its inhomogeneous isotopic composition is due to a

phenomenon called kinetic isotope fractionation [33]. This phenomenon represents the separation of molecules during physical processes like evaporation or liquefaction according to their molecular mass. Heavier molecules, like in the case of water, heavy water (D₂O) or semiheavy water (HDO) will accumulate in the lower part atmosphere, while the lighter H₂O molecules will be the first to evaporate. This phenomenon is widely used in separating D₂O from its lighter counterparts HDO and H₂O, for industrial purposes.

It is known that the natural levels of deuterium in terrestrial waters varies from 14 to 150 ppm [14], which means, grosso modo,

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that one of each 6500 atoms of hydrogen composing the biological structures is a deuterium one.

When the heavier isotope of hydrogen, deuterium, was discovered, the investigation of its combinations with hydrogen to form deuterated water had begun [25]. The research for establishing the biological effects of heavy (deuterated) water was extensive. Many studies were made for establishing its biological actions in animals and plants [13]. It has been long-time observed that while the small amounts (around 145–150 parts per million-ppm) are normal in all surface waters, increased amounts of deuterium, exceeding 15% determine structural, metabolic and functional alterations in various degrees [15].

One of the until recently unstudied byproducts of the separation process for obtaining heavy water is the so-called deuterium-depleted water (DDW). Initially considered a waste product, the water that left the distillation columns after the extraction process was discarded, until studies demonstrated that it contained a deuterium content which was way below the natural levels. Further extraction lead to obtain water which is composed of almost only H₂O, with very small amounts of deuterium (around 20 ppm).

Compared with the biologic changes determined by the excess deuterium in the water, the effects of reducing its concentration under the normal values are less studied. There is only a small amount of data published in the literature concerning the inhibition both of the growth rhythm of the fibroblast cultures and of the development of a tumor transplanted in mice [26], and just a few other publications which claim antineoplastic [9], antiaging [3], cell-stimulating [5] and radiation-protecting effects [6].

As the deuterium concentration difference between the intracellular and extracellular environments seems to balance rather quickly, a decrease of the deuterium concentration in the cellular microenvironment should produce also a decrease of the intracellular deuterium [24].

In this context, the aim of the present work was to study the possible influence of DDW chronic administration in normal Wistar rats, as compared to a control group which received distilled water, on spatial working memory and the locomotor activity (as studied through Y-maze) or both short-term and long-term spatial memory (assessed in radial 8 arms-maze task).

2. Materials and methods

2.1. Animals

Adult male Wistar ($n = 40$) rats, weighing 200–250 g at the beginning of the experiment, were housed in groups of five animals per cage and kept in a room with controlled temperature (22 °C) and a 12:12-h light/dark cycle (starting at 08:00 h), with food and water (distilled or DDW, depending on the study group) ad libitum.

The animals were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). This study was approved by the local Ethics Committee and also efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Materials

As an instrument for reducing of deuterium concentration in the organism of the lab animals, the DDW was used (27–30 ppm deuterium, compared with ~ 145–150 ppm deuterium, found in surface fresh waters) [27]. Deuterium-depleted water was obtained by a Cooperation Agreement with the Institute of Criogeny and Isotopic Research Râmnicu-Vâlcea, Romania. The rats were obtained from

Cantacuzino Institute Bucharest, Romania, while the behavioral tasks which we used were represented by Y-maze and eight-arm radial maze (Coulbourn Instruments). The control groups received distilled water from the same source mentioned above (Institute of Criogeny and Isotopic Research Râmnicu-Vâlcea). Distilled water was used as a control according to several other studies that used it on behavioral evaluations, in durations varying from 7 days to several months [7,28].

2.3. Experimental design

2.3.1. Treatment

The animals were randomly divided in two groups: 20 animals for the control group and 20 for the DDW treatment.

The treatment began 21 days before the behavioral testing. Memory functions were assessed through Y-maze and eight-arm radial maze tasks and performed during the days 22–37 of treatment (Y-maze on the 22th day and radial maze in the last 2 weeks). Thus, only one set of animals was used for both behavioral tasks.

The aforementioned duration of treatment was selected considering that a minimum of 21 days DDW treatment was deemed necessary in order to replace all the water in the organism and to change the hydrogen/deuterium ratio within the tissues of the test animals [20].

Additionally, the investigator was blind to the type of water ingested by the rats during the behavioral tasks.

2.4. Evaluation of memory function

2.4.1. Y-maze task

Short-term memory was assessed by spontaneous alternation behavior during a single session in the Y-maze task. The Y-maze used in the present study consisted of three arms (35 cm long, 25 cm high, and 10 cm wide) and an equilateral triangular central area. The maze was placed in exactly the same place during the procedure for all rats. In this way, the rat was placed at the end of one arm and allowed to move freely through the maze for 8 min. An arm entry was counted when the hind paws of the rat were completely within the arm. Also, the maze was cleaned with alcohol-free disinfectant wipes between each trial. Spontaneous alternation behavior was defined as the entry into all three arms on consecutive choices. The number of maximum spontaneous alternation behaviors was calculated as the total number of arms entered minus 2 and percent spontaneous alternation was calculated as (actual alternations/maximum alternations) × 100. As an example, the following sequence of arm entries (ABACBACBACB) will generate a percent spontaneous alternation behavior of 88.8% (as in [8 alternations/(11–2) arms entered] × 100). Also, the random selection of goal arms shows a 50% spontaneous alternation.

Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short-term memory. Additionally, the total number of arms entries was used as an index of the locomotor activity [4].

2.4.2. Eight-arm radial maze task

The eight-arm radial maze used in the present study consisted of eight arms, numbered from 1 to 8 (48 cm × 12 cm), extending radially from a central area (32 cm in diameter). The apparatus was placed 40 cm above the floor and surrounded by various extramaze visual cues placed at the same position during the study. At the end of each arm, there was a food cup that had a single 50 mg food pellet. Prior to the performance of the maze task, the animals were kept on restricted diet and body weight was maintained at 85% of their prefeeding weight over a week period, with water (distilled or DDW) being available ad libitum.

Before the actual training began, five rats were simultaneously pretrained in the radial maze and allowed to explore for 5 min and take food freely. The food was initially available throughout the maze but was gradually restricted to the food cup. The animals were pretrained for 4 days to run to the end of the arms and consume the baits. The pretraining trial continued until all the baits had been consumed or until 5 min had elapsed.

After pretraining, all rats were trained with one trial per day, for 7 days. Briefly, each animal was placed individually in the center of the maze and subjected to working and reference memory tasks, in which same five arms (nos. 1, 2, 4, 5, and 7) were baited for each daily training trial. The other three arms (nos. 3, 6, and 8) were never baited.

An arm entry was counted when all four limbs of the rat were within an arm. Measures were made for the number of working memory errors (entering an arm containing food, but previously entered) and reference memory errors (entering an arm that was not baited). Also, the time taken to consume all five baits was also recorded.

Reference memory is regarded as a long-term memory for information that remains constant over repeated trials (memory for the positions of baited arms), whereas working memory is considered a short time memory in which the information to be remembered changes in every trial (memory for the positions of arms that had already been visited in each trial) [8].

2.5. Data analysis

The animal's behavior in Y-maze maze was statistically analyzed by using Student's *t*-test (two tailed, unpaired). The eight-arm radial was analyzed by two-way ANOVA repeated measures for treatment effect (control vs. DDW), time effect (days of training) and treatment \times time interaction. All results are expressed as mean \pm SEM. $p < 0.05$ was regarded as statistically significant. The analyses were performed using the SPSS program (version 17.0).

3. Results

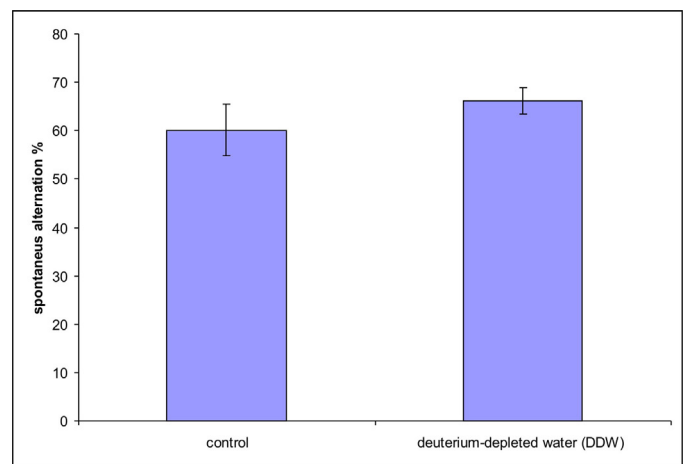
In this way, our *t*-test two tailed, unpaired analysis showed no significant differences ($p = 0.449$) regarding spatial working memory in the Y-maze task between the twenty rats from the DDW group and the control group of rats ($n = 20$) that received distilled water, suggesting no significant effects on spatial working memory (Fig. 1A).

Additionally, the locomotor activity in the Y-maze task, as expressed through the number of arm entries, was not significantly different ($p = 0.375$) in the group of rats which ingested DDW, when compared to the controls, by the means of two tailed, unpaired Student's *t*-test (Fig. 1B).

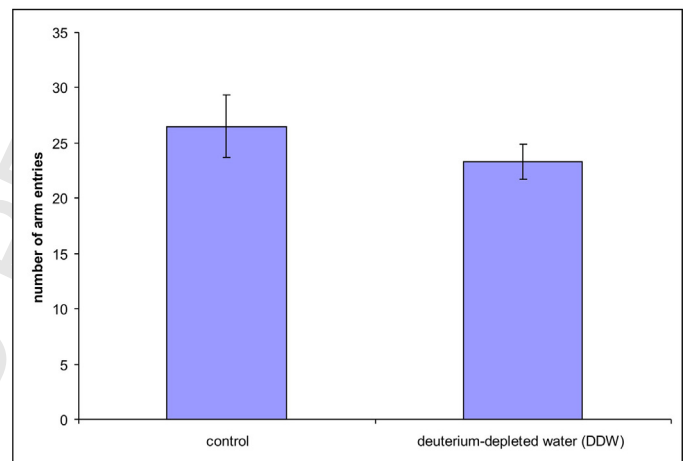
Regarding the results of the radial arm maze task, we also showed no significant differences between the group of rats which received DDW vs. the control rats (ANOVA, repeated measures; treatment effect: $F(1,19) = 1$, $p = 0.07$; days effect: $F(6,114) = 28$, $p < 0.0001$; treatment \times days interaction: $F(6,114) = 0.272$, $p = 0.949$), with regards to the number of working memory errors, as a specific parameter for the short-term memory (Fig. 2A).

Still, when it comes to the number of reference memory errors, our analysis showed a significant decrease (ANOVA, repeated measures; treatment effect: $F(1,19) = 58$, $p < 0.0001$; days effect: $F(6,114) = 14$, $p < 0.0001$, treatment \times days interaction: $F(6,114) = 2$, $p = 0.06$) in the DDW group, as compared to the controls, suggesting significant effects on the long-term memory (Fig. 2B).

Also, we report here a significant decrease (ANOVA, repeated measures; treatment effect: $F(1,19) = 72$, $p < 0.0001$; days effect: $F(6,114) = 50$, $p = 0.0001$; treatment \times days interaction: $F(6,114) =$



A



B

Fig. 1. The effects of DDW ingestion in the Y-maze task, as studied by two tailed, unpaired Student's *t*-test, showing no significant modifications in terms of spatial working memory (as seen by the spontaneous alternation percentage) and locomotor activity (as demonstrated by the number of arm entries). (A) Spontaneous alternation behavior. (B) The number of arm entries. The values are mean \pm S.E.M ($n = 20$ animals per control group and $n = 20$ for DDW group).

0.432, $p = 0.856$) of the time necessary to finish the eight-arm radial maze in the DDW group, when compared to control rats (Fig. 2C).

4. Discussion

In the present report we were interested, for the first time in our knowledge, in studying the effects of DDW ingestion in Wistar rats on Y-maze and eight-arm radial maze tasks. Our results showed no significant differences between the control group, which received distilled water and DDW group, in terms of spatial working memory (assessed through spontaneous alternation percentage) and locomotor activity (number of arm entries) in Y-maze. Additionally, no significant differences between the two aforementioned groups were found in terms of the number of working memory errors in the eight-arm radial maze, as a parameter of short-term memory.

However, significant statistical differences were found in the case of the reference memory errors in the radial arm maze, which were significantly decreased in the DDW group, as compared to controls. Additionally, the time necessary to finish the eight-arm radial maze was significantly reduced in the case of rats which received DDW, when compared to control-distilled water animals. Thus, it seems that the administration of DDW could have resulted

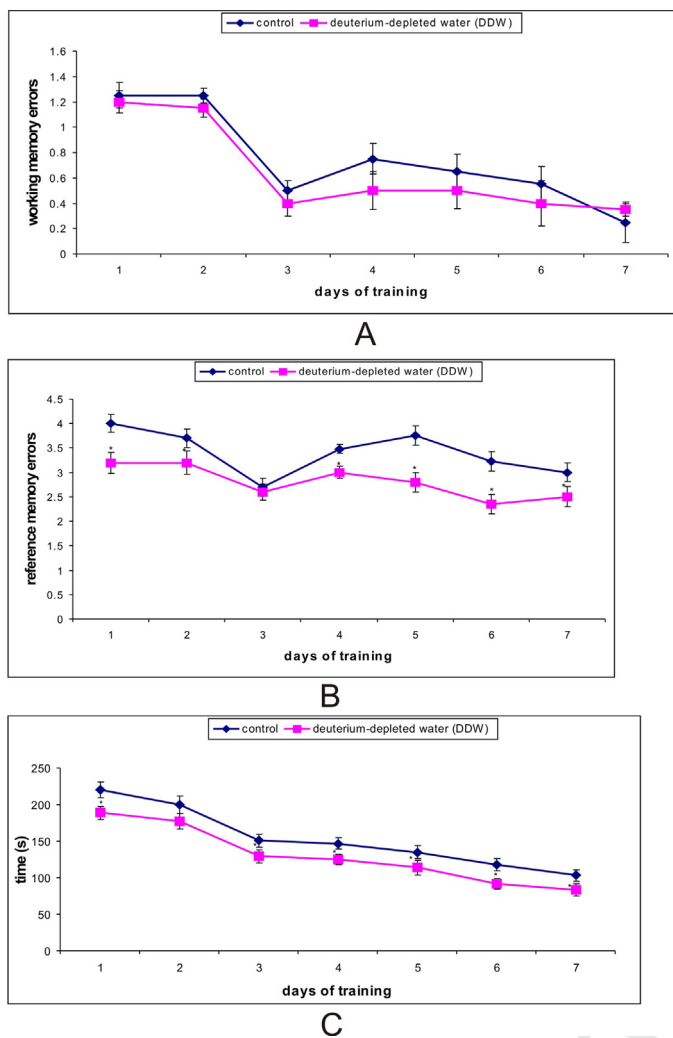


Fig. 2. The effects of DDW ingestion in the eight-arm radial maze task, as studied by two-way ANOVA repeated measures, demonstrating no modifications for the number of working memory errors, while a significant decrease in the number of reference memory errors and the time necessary to finish the task were also observed, suggesting an improvement of the reference memory, as a parameter for the long-term memory. (A) Working memory errors. (B) Reference memory errors. (C) Time taken to consume all five baits. The values are mean \pm S.E.M ($n = 20$ animals per control group and $n = 20$ for DDW group). * $p < 0.05$ vs. control group.

in a significant improvement of the reference memory, as an index of long-term memory.

In fact, the premise of the study begins from the idea that the replacement of deuterium present in normal amounts in water (~150 ppm) with hydrogen by DDW ingestion will have a stimulating effect on recognizable neural activity parameters. Thus, diminishing the deuterium concentration from the environmental water might lead to the activation of the proton transport through the membrane, with an increased efflux of protons toward the outside of the cell and a consecutive increase of intracellular (cytosolic) pH. It is common knowledge that intracellular pH is one of the triggers of cell division [21]. A very important aspect in the functionality of the Na^+/H^+ antiport is the detection of cytosolic pH. A common characteristic in several systems that regulate cell proliferation is their sensitivity to intracellular pH.

The replacement of H_2O with heavy (deuterated) water slows most biological and chemical reactions, including action potentials, sodium pump [17] and ion channel transit [10]. The most probable cause is the reduction of the flow speed through these channels by the deuterium oxide, either by the change of the proteins

themselves or of the environmental conditions in which they operate. It is known that aquaporins (water channels) – have a permeability at least 15% decreased for heavy water, when compared to normal water [19]. This in the conditions in which the environmental water exists already a load of 140–150 ppm deuterium. Experiments of whole-cell recording and patch-clamp using heavy water have demonstrated that deuterium can pass through proton channels only in a reduced proportion. Deuterium conductance was even lower than believed using only the mass solvent effects, which suggests that deuterium interacts in a specific manner with channels and pumps involved in proton transport [12].

There are several hypotheses that might explain the facilitation of long-term memory by DDW replacement of all water ingested. One of them might involve the Acid-Sensing Ion Channels (ASIC), which are a mainstay in the membrane transport systems of neurons. The peripheral nervous system has all the types of ASICs, while in the CNS only the ASIC 1a, 2a and 2b variants are to be found [30]. ASICs are involved in memory and learning [31] and are abundantly present in the hippocampus [32]. Smaller amounts can be found in the periaqueductal gray substance, amygdala, cingulum somato-sensory cortex and striated bodies [1,11].

In conditions in which the fluctuations of the extracellular pH are common in pathological conditions, it is to be expected the ASICs to have important roles in a large range of neurological impairments, as the pain, cerebral ischemia, convulsions or anxiety disorders. Moreover, recent studies have demonstrated that the ASICs and acidosis have regulating effects on the dendritic spines, one of the main sites of cerebral excitatory neurotransmission [16,34].

These data suggest that increased proton outflow, due to a 6–7-fold reduction in deuterium of body water, induces acidosis in the immediate vicinity of the neuronal membrane, while alkalizing the interior. These data suggest that influencing ASIC activity may regulate Ca^{2+} signaling in vivo. Also, using functional magnetic resonance imaging, one recent report shows that learning induces acidification in human brain [18]. These data may support or finding that long-term memory is potentiated by deuterium depletion in the brain tissue, which activates the ASICs and contributes to an increased synaptic efficacy.

These results of our study could also be explained by some of our previous unpublished observations that DDW ingestion results in modifications of the anxiety state and arousal levels, generally expressed as an increased in the exploratory behavior. Moreover, Sandstrom group demonstrated in 2005 that isolation stress, during the third postnatal week in male rats, affects only the working memory mechanism, but has no effect on the number of reference memory errors, in a 12-arm radial maze task [22,23].

Another aspect that could be important is the fact that we submitted the same set of animals to both Y-maze and eight-arm radial maze tasks. In this way, it is possible that one behavioral task could interfere with the other. However, the results we presented here do not suggest an interference between the two protocols used, since the working memory did not showed any significant modifications in both tasks, while only the number of reference errors and the time necessary to finish the test were significantly improved in the case of DDW rats.

Thus, further studies are warranted in order to determine the exact role of the DDW in various behavioral tasks. Our group has also undergo studies regarding the effects of DDW treated animals in other behavioral apparatus and for longer period of times, in order to fully understand how DDW affects behavioral functions, as well as experiments focusing on the administration of heavy (deuterated) water to animals, in order to see whether impairment on long-term memory will occur.

In this way, having available a tool for significantly reducing this ratio, we can reach to the *conclusion* that the change between the

deuterium/hydrogen balance may have important consequences for the mechanisms that govern long-term memory, as showed here especially in the behavioral parameters from the eight-arm radial maze task.

Uncited references

[2,29,35].

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