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DEUTERIUM DEPLETION INDUCES ANXIOLYTIC-LIKE EFFECTS IN RATS

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Abstract - Deuterium-depleted water (DDW) has a concentration of deuterium 6-7 times lower than naturally occurring water (20-25 ppm vs. 150 ppm). When administered for a longer period, it can reduce the concentration of deuterium throughout the body, activating cellular mechanisms that depend on protons. The aim of the present work was to investigate the influence of chronic DDW administration on anxiety-related processes in Wistar rats when compared to a control group that received distilled water, as studied in an elevated plus maze behavioral test. Our results describe a possible anxiolytic-like effect of DDW administration on rats, as shown by the increase in the percentage of time and number of entries in the open arms of the elevated plus maze. The administration of DDW also resulted in stimulated head-dipping behavior in the open arms, which is a behavioral change that characterizes the exploratory behavior and decreased inhibition/fear in an unfamiliar environment. We conclude that the change in this balance may have important consequences for many biological mechanisms. A deuterium desaturation treatment with DDW might have a use in anxiety disorders.

Key words: Deuterium-depleted water, anxiety, elevated plus maze.

INTRODUCTION

Deuterium is a ubiquitous isotope, with a steady presence of about 145-150 ppm in all waters. Its oxide is similar to water, but heavier. This difference in the molecular weight allows for its separation by isotopic fractionation (Young et al., 2002). The liquid obtained, "heavy water", is used as a neutron moderator in nuclear reactors, in nuclear magnetic resonance and organic chemistry. One of the byproducts of the separation process for obtaining heavy water is 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 that was far below natural levels. Through further extraction water that is composed of almost only H_2O, with very small amounts of deuterium (around 20 ppm) was obtained.

Compared to the biologic changes determined by excess deuterium in water, the effects of reducing its concentration were less studied. Published data describe the inhibition of fibroblast growth, development of tumor transplanted in mice (Somlyai et al., 1993), antineoplastic, anti-aging, cell-stimulating and radiation-protecting effects (Bild et al., 1999, 2004).

The aim of the present work was to investigate for the first time, the influence that chronic DDW administration could have on anxiety-related processes in normal Wistar rats when compared to a control group that received distilled water, as studied in an elevated plus maze behavioral task, a recognized behavioral tool for the study of anxiety-related states (Lister, 1990; Ciobica et al., 2011a).

MATERIALS AND METHODS

Animals

Adult male Wistar (n=40) rats, weighing 200-250 g at the beginning of the experiment, were kept in a room with controlled temperature (22°C) and a 12 h light/dark cycle (starting at 08:00 h), with food and distilled water or DDW provided *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. All procedures complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC). This study was approved by the local Ethics Committee and efforts were made to minimize animal suffering and to reduce the number of animals used.

Materials

As an instrument for reducing of deuterium concentration in the organism of the lab animals, deuterium-depleted water (DDW) was used (27-30 parts per million deuterium, compared with 145-150 parts per million deuterium, found in fresh surface waters) (Souchez, 1984). Deuterium-depleted water was obtained through a cooperation agreement with the Institute of Criogenic and Isotopic Research Râmnicu-Vâlcea, Romania. The rats were obtained from the Cantacuzino Institute, Bucharest, Romania. The behavioral assessment was performed using an elevated plus maze task (Coulbourn Instruments). The control groups received distilled water, from the same source mentioned above (Institute of Criogenic and Isotopic Research, Râmnicu-Vâlcea).

Experimental design

Treatment

The animals were randomly divided into two groups, 8 animals for the control group and 32 for DDW-treatment. The treatment began 21 days before the behavioral testing. Elevated plus maze was performed on the 22nd day of treatment. Rats were allowed to adapt to the experimental room conditions for 3 weeks before the beginning of the behavioral experiment. A minimum of 21 days DDW-treatment was deemed necessary to replace all the water in the organism and to change the hydrogen/deuterium ratio within the tissues of the test animals (O'Grady et al., 2012). The investigator was blind to the type of water ingested by the rats during the behavioral tasks.

Elevated plus maze

The elevated plus maze consisted of four arms, 49 cm long and 10 cm wide, elevated 50 cm off the ground. Two arms were enclosed by walls 30 cm high and the other two arms were exposed. The experiment began by transferring the rats to the test room, where they were allowed to rest for one hour. Each rat was gently placed in the central area with its nose facing one of the closed arms and allowed to freely explore the maze for 5 min. Each animal was used just once. All sessions were recorded using a camera placed above, in a room that was lit by a 60W bulb placed 180 cm above the maze (22 lux in central square of the maze). The time spent in each arm type was recorded, as well as the entries into either open or closed arms. An arm entry was counted when all four limbs of the rat were within an arm. Specific behavioral parameters such as head dipping in open-arms (sticking the head below the level of the maze and towards the floor), protected stretch-attend postures (stretching) in the closed arms (the animal stretches with the forepaws while maintaining the hind paws in the same place and then retracts to the original position) and number of grooming bouts/total time spent grooming (cleaning of any part of the body with the paws and/or the mouth), were measured.

The apparatus was cleaned with a 5%-ethanol solution and dried with a cloth before the next animal was tested. The percentage of time spent in the open arms (time spent in the open arms/time spent in all arms x 100) and the percentage frequency of entries into open arms (frequency of entries into open arms/ total entries into all arms x 100) was then calculated. This test is based on the natural aversion of rodents for open spaces. Consquently, both items described above (percentages of time spent into the open arms/ frequency of entries into open arms) are considered to reflect fear-induced inhibition from entering the open arms and can be related to the anxiety level experienced by the rat (Ciobica et al., 2011b). The measurement of other behavioral parameters increases the sensitivity of the elevated plus maze as an experimental model of anxiety, and their relevance will be detailed in the Discussion section. Additionally, the number of entries into closed arms is believed to reflect the locomotor activity (Lister, 1990; Bild et al., 2013).

Data analysis

The animal's behavior in elevated plus maze was statistically analyzed by using the Student's t-test (two-tailed, unpaired). All results are expressed as mean ± SEM. P <0.05 was regarded as statistically significant.

RESULTS

Regarding the percentage of time spent by the animals in the open arms of the elevated plus maze task, we observed a significant increase ($p = 0.014$) of this time in the DDW-treated rats compared to the control group (Fig. 1A). We also noticed a significant increase ($p = 0.027$) in the percentage frequency of entries into open arms in the rats that ingested DDW when compared to the controls (Fig. 1B). The decrease of both these parameters suggests a decrease in the anxiety-related behavior in rats, considering their natural aversion for open spaces.

This is also supported by the observation of an additional increase ($p = 0.041$) of the head-dipping behavior in the open arms (a parameter that is correlated with changes characterizing an exploratory behavior and decreased fear) of the elevated plus maze task in the rats that received DDW, as compared to control group (Fig. 1C). However, no significant differences were observed in the case of protected stretch-attend postures in the closed arms ($p = 0.798$), or in the number of grooming bouts ($p = 0.829$), between the DDW group and the control rats (Figs. 1 D and E, respectively).

A significant decrease ($p = 0.037$) in the total time spent grooming, as expressed in seconds, was noticed in the rats that received DDW compared to controls (Fig. 1F). No significant differences ($p = 0.072$) were recorded between the DDW group and controls for the number of closed arm entries (Fig. 1G), which is considered a parameter indicating the locomotor activity of the animals.

DISCUSSION

Our results describe a possible anxiolytic-like effect of DDW administration in rats, as shown by the increase in the percentage of time and number of entries into the open arms of the elevated plus maze. The ingestion of DDW also resulted in stimulated head-dipping behavior in the open arms, which is a behavioral change that characterizes exploratory behavior and decreased inhibition/fear in an unfamiliar environment (Lister, 1990; Bild et al., 2013).

Another behavioral parameter was the number of closed arms entries, which is considered an index that correlates with changes in locomotor activity (Wall et al., 2000). However, we did not see any significant differences in the number of closed arms entries when we compared the DDW-treated rats with the control group. This is an important point, since only when no significant modifications in general locomotor activity is observed do the aforementioned behavioral aspects indicate increased anxiety in the elevated plus maze. Regarding the protected stretch-attend postures in the closed arms and grooming bouts, we did not observe any significant differences. However, the total time

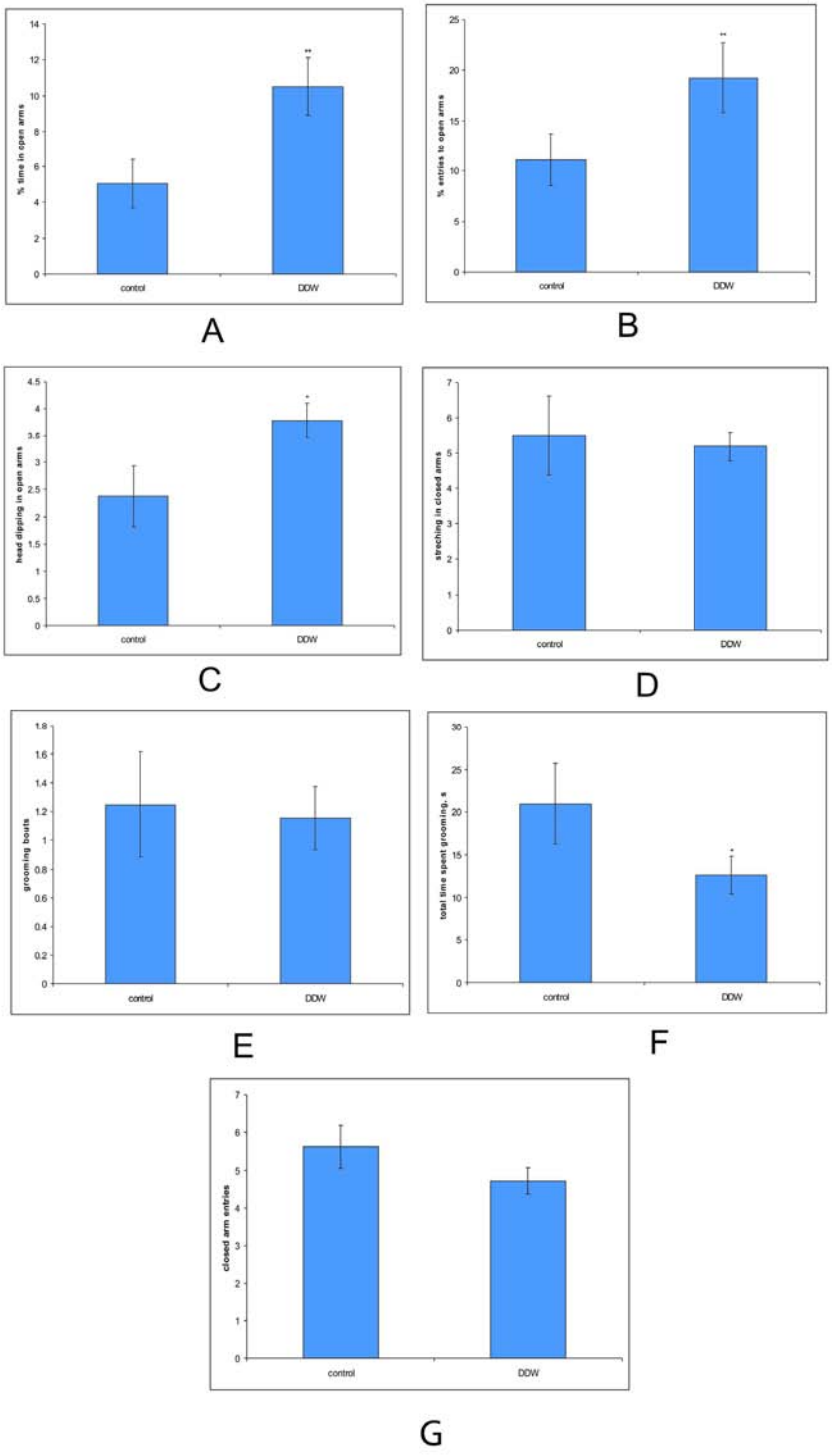

Fig. 1. Effects of DDW administration on the behavior of rats evaluated in the elevated plus maze task. A – percentage of time spent in open arms; B – percentage of entries in open arms; C – head-dipping in open arms; D – stretching in closed arms; E – grooming bouts; F – total time spent grooming; G – closed arm entries. The values are mean ± SEM (n = 8 animals per control group and n = 32 for DDW group). *p <0.05 vs. control group, **p <0.02 vs. control group.

spent grooming was increased in the DDW-treated rats.

These differences could be explained by reports stating that grooming behavior in an elevated plus maze is a displacement response, contradicting older theories that grooming, along with freezing, defecation and urination, are anxiety-related behaviors and an increased number of these events suggests a greater level of anxiety (Lister, 1990). A more recent reports has shown that rodents' grooming behavior can be increased in both high and low stress situations and there is a need for a more complex analysis of this kind of behavior (Kalueff et al., 2005).

The premise of the study was that the replacement of deuterium present in normal amounts in water (~150 ppm) with hydrogen by DDW ingestion would have a stimulating effect on recognizable neural activity parameters. With a lower diffusion coefficient than normal water, deuterated water has more difficulty entering the cells, produces a reduction in substrate diffusibility through membranes and reduces the speed of cellular enzymatic reactions. From here are derived the multitude of its inhibitory effects, including the vasorelaxation (Wang et al., 1993). The replacement of H_2O with heavy (deuterated) water slows down most biological and chemical reactions, including action potentials, sodium pump and ion channel transit. The most probable cause is the reduction in the flow-speed through these channels by deuterium oxide, either by the change of proteins themselves or from the environmental conditions in which they operate. It is known that aquaporins (water channels) – membrane proteins that allow water passage through the lipid bilayer of the cell membrane – have a permeability at least 15% lower for heavy water than for normal water (Mamonov et al., 2007).. The viscosity of deuterated water is higher than that of water, and the effects of reduction of the ion currents can also be reproduced using other non-electrolytic solutions (Kukita et al., 1997).

The effects of deuterium oxide seem to be more than those due to viscosity changes. 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 (Landowne et al., 2000).

It is obvious that even if we cannot postulate a complete reverse effect of the heavy water-like effects by the simple replacement of regular water in the environment, we can assume that a 6-7-fold reduction of the deuterium concentration (20-30 ppm compared with 145-150 ppm) can have an accelerating effect on the same phenomena, which are slowed in the case of deuteration. Reducing the deuterium concentration from the environmental water might lead to the activation of proton transport through the membrane, with an increased efflux of protons towards the outside of the cell and a consecutive increase of intracellular (cytosolic) pH. Kinetic isotopic effects can be observed also in other proton translocation systems, such as bacteriorhodopsin, cytochrome-C-oxidase or lactose-permease (le Coutre and Gerwert, 2006).

Ion and pH variations can modulate receptor function. Protons are very susceptible to oxygen or glucose changes in the CNS (Pasternack et al., 1996). Wang et al. (2005) concluded that extracellular protons have direct or allosteric interaction with the coupling site of GABA. GABAergic mediation is important for neural inhibition in mammals. GABA-induced inhibition is due to the activation of the $GABA_A$ ionotropic receptors, which mediate phasic and tonic chloride currents (Mohler, 2012). Synaptic and extracellular acidosis, a common presence in cerebral pathology (chronic ischemia, hypoglycemia) reduces $GABA_A$ coupling affinity for their physiological ligand. Wojtowicz et al. (2008), using micropotential miniature inhibitory postsynaptic current (mIPSC) recording and benzodiazepines on cultured neurons demonstrated that proton increase in the paracellular environment reduced tonic inhibition. Acid pH reduces A-type receptor affinity for GABA which, indicating that the anxiolytic action of

both benzodiazepines and protons are additive and affect the same receptor (Mozrzymas et al., 2003).

Several CLC (chloride channel) proteins in eukaryotes are not passive chloride channels but strictly coupled, electrogenic and secondarily activated Cl^-/H^+ antiports (Zifarelli et al., 2008). These antiports are pH-dependent, which stimulate their activation (Accardi et al., 2004). An increased chloride inflow is the direct consequence of such stimulation.
Of crucial importance in the control of the Cl^- electrochemical gradient are the CCC (chloride cation channels), necessary for the "typical" hyperpolarizing inhibition produced by ionotropic $GABA_A$ receptors (Blaesse et al., 2009).

Intense synaptic activity induces neuron swelling due to the intensification of ionic flow, mainly of Na^+ and Ca^{2+}, which are a major pro-osmotic driving force. As such, the neuronal volume has to be reestablished and an important part of this task is performed by the NKCC cotransporter. A recent study (Rybnikova et al., 2012) demonstrated that post-hypoxic brain swelling has important behavioral effects, mainly "post-hypoxic anxiety", demonstrated using the elevated plus maze test. Another study indicated that cellular hyperhydration is an effect of traumatic brain injury, with long-lasting behavioral consequences such as anxiety and memory impairment (Vink et al., 2010).

pH has been demonstrated to be involved in the regulation of neuronal activity, with internal alkalization having an inhibiting effect (Chesler,2003). The activation of proton extrusion could be also linked with an acceleration of cell volume recovery, with modulating effects on anxiety and memory that might explain our results.

Considering the possible antioxidant properties of DDW (Olariu et al., 2007), the aforementioned anxiolytic effects could also be explained by the correlations that might exist between the central oxidant status and anxiety mechanisms (Ciobica et al., 2009, 2010, Hogas et al., 2011). Bouayed et al. (2009) has described a linear correlation between some oxidative stress markers and different anxiety-related parameters in blood granulocytes, lymphocytes and monocytes, and neuronal and glial cells from the cerebellum, hippocampus and various cerebral areas.

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