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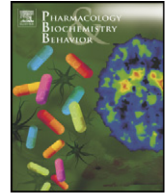

Chronic difluoromethylornithine treatment impairs spatial learning and memory in rats

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ABSTRACT

Recent evidence suggests that polyamines putrescine, spermidine and spermine are essential in maintaining normal cellular function. The present study investigated the effects of chronic treatment of difluoromethylornithine (DFMO, 3% in drinking water), a potent inhibitor of putrescine synthesis, for 54 consecutive days on animals'behavior and neurochemical levels in the CA1, CA2/3 and dentate gyrus sub-regions of the hippocampus and the prefrontal cortex. The DFMO group showed performance impairments in the place navigation and the probe test conducted 24 h after the training in the reference memory version of the water maze task, but not in the elevated plus maze, open field, object recognition, cued navigation and the working memory version of the water maze task when compared to the control group (drinking water only). DFMO treatment resulted in approximately 80–90% and 20% of reductions in the putrescine and spermidine levels, respectively, in the four brain regions examined, and a small reduction in agmatine level in the CA2/3, with no effects on spermine, glutamate and γ-aminobutyrate. The DFMO group showed decreased body weight relative to the control one. However, there were no significant differences between groups in the normalized brain, kidney and liver weights. The present study demonstrates that chronic treatment of DFMO depletes putrescine and decreases spermidine levels in the brain, inhibits growth, and impairs spatial learning and memory in the reference memory version of the water maze specifically. These findings merit further investigation to fully understand the functional role of endogenous polyamines in learning and memory.
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1. Introduction

Polyamines putrescine, spermidine and spermine are the downstream metabolites of L-arginine. In mammalian cells, putrescine is mainly derived from L-ornithine (the product of arginase) by ornithine decarboxylase (ODC). Putrescine combines with decarboxylated *S*-adenosylmethionine to produce spermidine via spermidine synthase, and spermine through a second aminopropyltransferase reaction involving spermine synthase. Spermidine/spermine N^1-acetyltransferase is the key enzyme involved in polyamine interconversion (Wallace et al., 2003). Agmatine, decarboxylated arginine, can be converted to putrescine by agmatinase (Halaris and Piletz, 2007; Wu and Morris, 1998), and hence is considered as a member of the polyamine family (Moinard et al., 2005).

The polyamines are widely distributed in mammalian cells, and their concentrations at physiological levels are important in maintaining normal cellular function (for reviews see Oredsson, 2003; Wallace, 2000; Williams, 1997). Difluoromethylornithine (DFMO) is a potent and irreversible inhibitor of ODC, and depletes putrescine mainly among the three polyamines (Gupta et al., 2009; Jänne et al., 1991; Malaterre et al., 2004; Metcalf et al., 1978; Slotkin et al., 1982; Sparapani et al., 1996). Because the polyamines are essential for cancer cell proliferation during tumorigenesis, DFMO has been used as a therapeutic and chemopreventive agent for cancers clinically (Babbar and Gerner, 2011; Meyskens et al., 2008; Simoneau et al., 2008). It has also been used as a pharmacological tool to investigate the effects of altered polyamine levels (Gupta et al., 2009; Malaterre et al., 2004).

The hippocampus is the key structure of the medial temporal lobe, and is important for certain types of learning and memory (Squire et al., 2004). There is a functional dissociation across its major subregions CA1, CA3 and dentate gyrus (DG). For example, the DG region creates a metric spatial representation and is involved in spatial pattern separation, whereas the CA3 and CA1 regions are important for pattern completion and sequence encoding, and temporal pattern association/completion and intermediate-term memory, respectively (Hoge and Kesner, 2007; Kesner, 2007; Kesner et al., 2004, 2008).

Abbreviations: DFMO, difluoromethylornithine; DG, dentate gyrus; GABA, γ-aminobutyric acid; HPLC, high performance liquid chromatography; i.c.v., intracerebroventricular; LC/MS, liquid chromatography/mass spectrometry; NO, nitric oxide; NOS, nitric oxide synthase; ODC, ornithine decarboxylase; PFC, prefrontal cortex; SVZ, subventricular zone.

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Neurogenesis is found in the DG in the adult brain, and these newborn cells can mature into functional neurons and play a role in maintaining hippocampal function (Aimone et al., 2011; Altman and Bayer, 1990; Clelland et al., 2009; Gage, 2002; Kempermann et al., 2004; Van Praag et al., 2002). Malaterre et al. (2004) reported that 3% DFMO in drinking water depleted putrescine mainly in the hippocampus, and significantly impaired adult neurogenesis in the DG in young rats. These findings suggest a novel role of endogenous putrescine in hippocampal neurogenesis.

Aging leads to cognitive decline and impairs neurogenesis in hippocampal DG (Driscoll et al., 2006; Wati et al., 2006). Interestingly, the putrescine level in the DG decreases with age (Liu et al., 2008c). Given the link between putrescine, hippocampal neurogenesis and hippocampal function (Aimone et al., 2011; Clelland et al., 2009; Malaterre et al., 2004), polyamine system dysfunction may contribute to cognitive decline during aging. Hence, it is important to understand how reduced level of putrescine affects animals' behavioral function, including learning and memory. Gupta et al. (2009) reported that acute depletion of putrescine (80–90% reduction in the hippocampus and prefrontal cortex) by DFMO administered intracerebroventricularly (i.c.v.) resulted in anxiety-like behavior and impaired memory for the object displacement in young adult rats, without affecting animals' locomotor and exploratory activities and spatial learning and memory. The present study was designed to investigate how chronic putrescine depletion affects animals' behavioral function in a number of commonly used behavioral tasks and the levels of polyamines, as well as glutamate and γ-aminobutyrate (GABA), the major excitatory and inhibitory neurotransmitters in the central nervous system. DFMO (3% in drinking water) decreased putrescine and spermindine levels in the sub-regions of the hippocampus and prefrontal cortex dramatically, and impaired spatial learning and memory in the reference memory version of the water maze task mainly, with mild or no effects on anxiety, exploration, locomotion and object recognition memory.

2. Methods

2.1. Subjects

Male Sprague–Dawley rats at age of 3 months, weighing between 320 and 380 g, were housed one per cage ($33 \times 21.5 \times 17.5$ cm^3), maintained on a 12-h light–dark cycle (lights on 8 a.m.) and provided *ad lib* access to food and water. Animal's body weight and water intake were monitored and recorded every day. Behavioral procedures were conducted during the light period of the light–dark cycle. All experimental procedures were carried out in accordance with the regulations of the University of Otago Committee on Ethics in the Care and Use of Laboratory Animals (Otago, New Zealand). Every attempt was made to limit the number of animals used and to minimize their suffering.

2.2. Drug and treatment

DFMO was a generous gift from Dr. Patrick Woster (Wayne State University, USA), who received it from Genzyme (Cambridge, MA). Rats were randomly divided into the control (drinking water only, n = 10) and DFMO (3% DFMO in drinking water, n = 10) groups. The dose of DFMO was based on Malaterre et al. (2004) and a pilot study. DFMO was freshly prepared every two days, and the animals were treated for 54 consecutive days. Animal's body weight and water intake were measured and recorded daily from 6 days prior to the treatment till the end of the study. The mean body weight and water intake across the first 6 days were used as baseline (shown as day 0 in Fig. 1, and block 0 in Fig. 2A and B). The behavioral tests started after 36 days of treatment and lasted for 18 days, and the animals were sacrificed on day 54 (Fig. 1). The same animals were used for all of the behavioral tests throughout the study.

2.3. Behavioral apparatus

All behavioral experiments were conducted in a windowless room with three clear and one red 75 W bulbs mounted on the ceiling. A video camera was mounted at ceiling height in the centre of the room used for recording the performance during the experimental period. A radio speaker was located adjacent to the video camera at ceiling height to provide background masking noise. The extramaze cues (the laboratory furniture, lights and several prominent visual features, as well as the location of the experimenter) were held constant throughout the experiments.

2.3.1. Elevated plus maze task

The elevated plus maze (to assess animal's anxiety level) was shaped like a plus sign in black-painted wood, with two unwalled (open) arms (50×13.5 cm^2), surrounded by a clear plexiglass of 4 cm and two walled (closed) arms ($50 \times 13.5 \times 29$ cm^2). The central area of the maze measured 13.5×13.5 cm^2. The maze was elevated 60 cm above the floor, and the arm locations were kept constant with north and south being the closed arms.

2.3.2. Open field

An experimental chamber, which consisted of a 60×60 cm^2 hardboard box with walls 20 cm high, was used for the exploration and object recognition experiments. All four of the chamber walls and the floor of the box were painted black. The floor of the box was divided into 36 equal-sized squares by white marking tape. The box was elevated approximately 1 m above the floor.

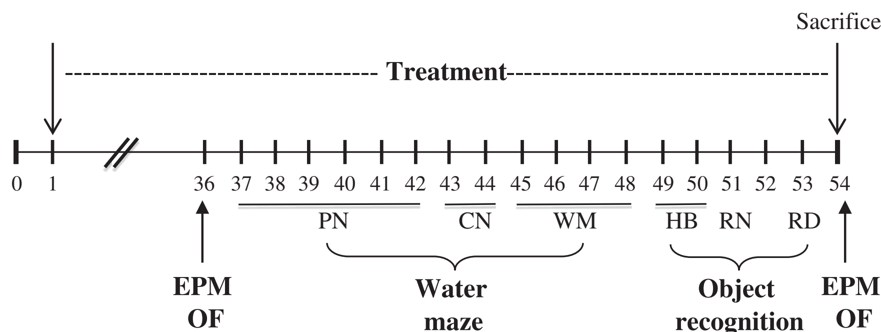

Fig. 1. Experimental timeline. Animals were given drinking water (Control, n = 10) or 3% DFMO in drinking water (DFMO, n = 10) for 54 consecutive days. Animals were tested in the elevated plus maze (EPM) and open field (OF) on days 36 and 54, in the place navigation (PN; days 37–42) and cued navigation (CN; days 43 and 44) of the reference memory version and the working memory version (WM; (days 45–48) of the water maze task, and the object recognition task (HB: habituation on days 49 and 50; RN: reaction to novel object on day 51; RD: reaction to displaced objects on day 53). All animals were sacrificed on day 54 after completion of the EPM and OF tests, and the brain tissues were harvested for the neurochemical analysis.

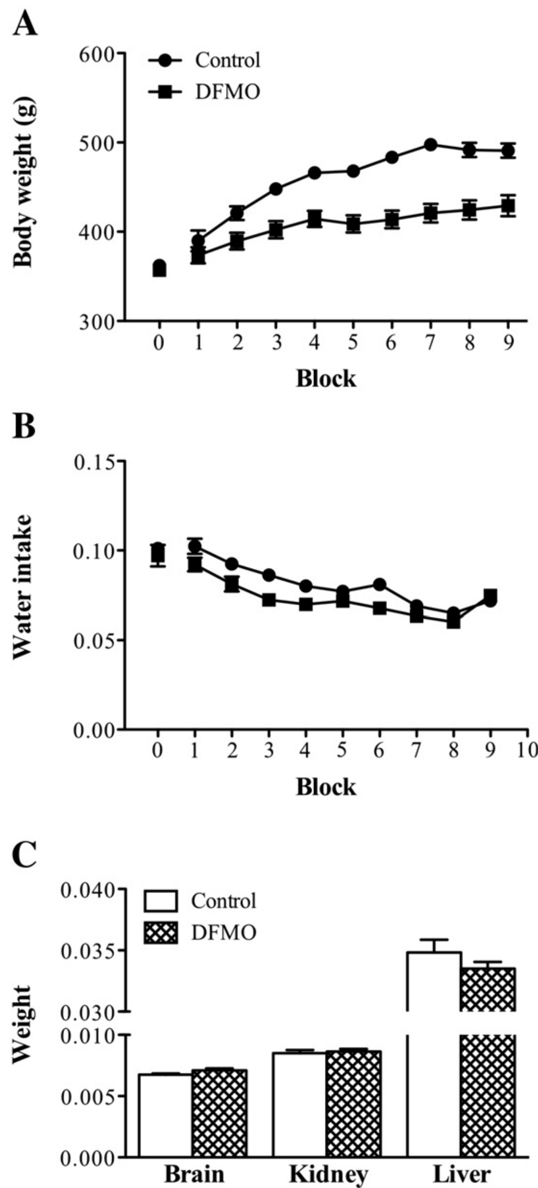

Fig. 2. Mean (±SEM) body weight (A; 6 days/block), normalized daily water intake by body weight (B; 6 days/block), and normalized post-mortem weights of brain, liver and kidney by body weight (C) in the control and DFMO groups (n = 10 in each group) during the experimental period. Although there was no significant difference between groups in body weight or water intake on block 0, the DFMO group had reduced body weight gain and water intake relative to the control group during the period of DFMO treatment. There were no significant differences between groups in the organ weights.

2.3.3. Morris water maze task

The water maze pool was a black circular tank measuring 150 cm in diameter and 45 cm in height. It was filled with water to a depth of approximate 25 cm and maintained at a temperature of 25 ± 1 °C. Four points around the edge of the pool were designated as north (N), south (S), east (E) and west (W), which allowed the apparatus to be divided into four corresponding quadrants (i.e. NE, NW, SE and SW).

2.4. Behavioral procedures

2.4.1. Elevated plus maze and open field (day 36 and day 54)

Animals were tested in both the elevated plus maze and open field on days 36 and 54 (Fig. 1). For each test, the order was counterbalanced between the two groups. The animals were placed at the centre of the plus maze facing one closed arm and left in the maze for a period of 5 min. The open field was set up immediately after completion of the elevated plus maze, and animals were placed into the chamber for 5 min. Animal's behavior was video taped and analyzed offline by a computerised tracking system (HVS 2020). For the elevated plus maze, the total time spent in the open or closed arms was analyzed. An entry was defined by placing all four paws into an arm, and no time was recorded when the animal was in the centre of the maze. For the open field, the duration of wall-supported rearings and the number of 10-cm wide grid squares traversed were analyzed.

2.4.2. Morris water maze task

2.4.2.1. Reference memory version (days 37–44)

2.4.2.1.1. Place navigation (days 37–42). During the place navigation, a black platform (10 cm in diameter) was located in the centre of the SE quadrant and submerged 2 cm below the water surface. There were six trials per day with 60-s intertrial intervals for six consecutive days. On each trial, the rat was placed into the pool facing toward the wall and was allowed to swim in search of the platform for a maximum of 90 s. The rat was permitted to remain on the located platform for 10 s before being removed and placed in a black high walled holding box. If the rat did not find the platform within 90 s of being placed into the pool, it was immediately placed on the platform for 10 s before being returned to the holding box. Starting locations (N, S, W, and E) were pseudo-randomly selected.

2.4.2.1.2. Probe tests (days 42 and 43). A probe test (Probe 1) was conducted 120 s after the final training trial of day 42. The platform was removed from the SE quadrant, and the rat was placed into the pool from a fixed starting point and was allowed to swim freely for a duration of 60 s. The second probe test (Probe 2) was conducted on day 43, which was approximately 24 h after completion of the final training trial of day 42.

2.4.2.1.3. Cued navigation (days 43 and 44). After completion of Probe 2, the platform was put back to the pool, and located in the centre of the NW quadrant for the cued navigation test. The platform was raised 2 cm above the water surface, and the edge of the platform was marked by yellow tape to make it more visible. There were six trials per day with 60-s intertrial intervals for two consecutive days, and the maximum searching time was 90 s. Starting locations were pseudorandomly selected on each day.

2.4.2.2. Working memory version (days 45–48). All rats were trained in the working memory version of the water maze task from day 45 for four consecutive days. There were two trials per day with the intertrial interval of approximately 120 s. Each trial consisted of a sample phase and a test phase. The starting location for each phase was different and there was no repetition across the two trials on each day. Between each trial, the location of the hidden platform was changed and the distance between the centre of the platform and the wall of the swimming pool was varied. A delay period of either 30 s or 180 s was inserted between the sample and test phases of each trial for each daily test, and the order of the delay was counterbalanced across trials and groups. The maximum time allowed for searching for the platform was 90 s and rats were allowed to remain on the platform for 10 s before being removed.

Following completion of the water maze experiments, several performance variables were analyzed from HVS 2020. Rat swimming speed was averaged across the 6 trials on each day across the 6 days of training during the place navigation. Path length (the distance the rats swam from the starting point to the platform) was measured for both the place and cued navigation (days 37–44), as well as the working memory version of the task (days 45–48). Thigmotaxic swimming (i.e., swimming close to the wall of the water maze) was quantified by dividing the maze into two circles and measuring the

time spent in the outer circle of the pool (15 cm wide) for the place and cued navigation. The absolute initial heading error was determined by the angle formed by a straight line from the start point to the platform and a line from the start point to the location of the animal after it had travelled 20 cm. For the probe tests, the percentage of path length in the target quadrant and the number of crossings over the previous platform location were measured (Bergin and Liu, 2010; Liu and Bergin, 2009; Liu and Collie, 2009).

2.4.3. Object recognition memory task (days 49–53)

The open field chamber was used for testing animals' object recognition memory (Gupta et al., 2009; Liu and Bilkey, 2001; Liu et al., 2004a). Each day's training consisted of two 5 min sessions. In the first session (phase 1), each rat was placed into the chamber with four objects, which were similar-sized items made of plastic or metal (4×5 cm^2), for 5 min. After the 5 min period had elapsed, each rat was removed from the apparatus to the holding box then returned to its home cage for 30 min. In the second session (phase 2), each rat was placed back into the apparatus for a further 5 min period. The objects were washed and dried, and the apparatus was wiped clean before the start of each session. The location of each object and the relationship between objects were counterbalanced within and between groups. For each rat, the pattern of the object locations was kept constant. The tests on days 49 and 50 were treated as habituation, and animal's reaction to a novel object or displaced objects was tested on day 51 and day 53, respectively (Fig. 1). During phase 2 of day 51, one of these ‘familiar’ objects was replaced by a novel object. During phase 2 of day 53, the locations of two of the familiar objects were swapped. To exclude the possibility of positional effects, the locations of the novel object (day 51) and the two familiar objects (day 53) were counterbalanced within and between groups.

Animal behavior was recorded, and the time spent in exploring each object during each phase on each day was analyzed offline. Exploration of an object was defined as the rat having its nose within 2 cm of the object and actively sniffing or touching the object. Turning around or sitting on the object was not considered an exploratory behavior (Gupta et al., 2009; Liu and Bilkey, 2001; Liu et al., 2004a).

2.5. Neurochemical procedures

On day 54, all rats were sacrificed by decapitation without anesthesia after completion of the elevated plus maze and open field tests (Fig. 1). The brains were rapidly removed and left in cold saline (4 °C) for at least 45 s. The sub-regions of the hippocampus (CA1, CA2/3 and DG), and the prefrontal cortex (PFC) were dissected freshly on ice (Liu et al., 2003, 2004b, 2005, 2008c; Gupta et al., 2009). All of the dissected tissue samples were then weighed, homogenized in ice-cold 10% perchloric acid (~50 mg wet weight per millilitre) and centrifuged at 12,000 *g* for 10 min to precipitate protein. The supernatants (the perchloric acid extracts) were frozen immediately and stored at −80 °C until analysis.

For each brain region, samples from both groups were assayed under the same experimental conditions. High purity agmatine, putrescine, spermidine, spermine, glutamate, GABA and internal standards (1, 7-diaminoheptane, and trazodone) were used (Sigma, Sydney, Australia). All other chemicals were of analytical grade.

Agmatine and putrescine concentrations in brain tissue samples were measured by a liquid chromatography/mass spectrometric (LC/MS/MS) method (Gupta et al., 2009; Liu et al., 2008a, 2008b, 2010). After adding internal standard to 20 μl of the perchloric acid extracts, the samples were alkalized with saturated sodium carbonate and derivatized with dansyl chloride. Agmatine, putrescine and internal standard were extracted with toluene. The toluene phase was evaporated to dryness, reconstituted and injected onto the LC/MS/MS system. The samples were analyzed by a reversed-phase C_{18} column (150×2.0 mm, 5 μm, Phenomenex) with 80% acetonitrile: 20% water containing 0.1% formic acid as mobile phase at a flow rate of 0.2 ml/min. The retention time of agmatine, putrescine and the internal standard were 1.7, 4.0 and 4.8 min, respectively. The total runtime was 15 min. Detection by MS/MS used an electrospray interface in positive ion mode. The standard curves for putrescine were linear up to 1000 ng/ml ($r^2>0.99$). The intra- and inter-day coefficients of variation were <15%. The concentrations of agmatine and putrescine in tissue were calculated with reference to the peak area of external standards and values were expressed as μg/g wet tissue.

Determination of spermidine, spermine, glutamate and GABA were carried out by using high performance liquid chromatography (HPLC) (Gupta et al., 2009; Liu et al., 2008b, 2009b, 2010). For spermidine and spermine, after adding internal standard (1,7-diaminoheptane) to 20 μl of the perchloric acid extracts, the samples were alkalized with saturated sodium carbonate and derivatized with dansyl chloride. Spermidine, spermine and internal standard were extracted with toluene. The toluene phase was evaporated to dryness, reconstituted and injected onto the HPLC system, which consisted of a programmed solvent delivery system at a flow rate of 1.5 ml/min, an autosampler, a reversed-phase C_{18} column, and a fluorescence detector set at the excitation wavelength of 252 nm and emission wavelength of 515 nm. Identifications of spermidine and spermine were accomplished by comparing the retention times of the samples with the known standards. Assay validation showed that the analytical method was sensitive and reliable with acceptable accuracy (88–112% of true values) and precision (intra- and inter-assay CV<15%). For glutamate and GABA, after adding internal standard (trazodone) to 30 μl of the perchloric acid extracts, the samples were alkalized with potassium hydrogen carbonate solution (pH 9.8) and derivatized with dansyl chloride in dark at 80 °C for 20 min. The reaction was stopped by adding 10 μl of acetic acid followed by centrifugation at 10,000 rpm for 10 min. Forty microliter of the supernatant was injected onto the HPLC system consisting of a programmed solvent delivery system at a flow rate of 1.0 ml/min, an autosampler, a reversed-phase C_{18} column, and a UV detector set at a wavelength of 218 nm. Identifications of glutamate and GABA were accomplished by comparing the retention times of samples with the known standards. Assay validation showed that the analytical method was sensitive and reliable with acceptable accuracy (92–107% of true values) and precision (intra- and inter-assay CV<15%).The concentrations of spermidine, spermine, glutamate and GABA in tissue were calculated with reference to the peak area of external standards and values were expressed as μg/g wet tissue.

2.6. Statistical analysis

All of the behavioral and neurochemical variables were analyzed using either Student *t*-test or two-way repeated measures of analysis of variance (ANOVA) followed by Bonferroni post-hoc tests (Zolman, 1993). The significance level was set at 0.05 for all comparisons.

3. Results

3.1. Physiological results

Fig. 2A presents the average body weight (6 days/block) in each group during the experimental period. There was no significant difference in the basal body weight between the two groups (block 0; $t(18)=0.54$, $p=0.60$). However, two-way repeated measures ANOVA revealed a significant group × block interaction ($F(8,144)=22.22$, $p<0.0001$) in the body weight across the 9 blocks, with significantly reduced body weight in the DFMO group relative to the control one on block 3 ($p<0.01$) and blocks 4–9 (all $p<0.001$).

Fig. 2B illustrates the average daily water intake normalized by body weight (6 days/block) during the experimental period. There was no significant difference between groups during block 0 ($t(18)=1.23$,

$p = 0.24$). However, ANOVA revealed a significant group × block interaction ($F(8,144) = 2.64$, $p = 0.01$), with significantly reduced water intake in the DFMO group relative to the control one on blocks 3 and 6 (all $p < 0.05$).

Fig. 2C presents the post-mortem brain, kidney and liver weights normalized by body weights in both groups. There was no significant difference between groups for the brain ($t(18) = 1.88$, $p = 0.08$), kidney ($t(18) = 0.41$, $p = 0.69$), or liver ($t(18) = 1.11$, $p = 0.28$).

3.2. Behavioral results

3.2.1. Elevated plus maze and open field

Fig. 3 illustrates animals' performance in the elevated plus maze on days 36 and 54. The control and DFMO groups spent similar amount of time in the closed arms on both days (day 36: $t(18) = 1.03$, $p = 0.32$; day 54: $t(18) = 0.46$, $p = 0.65$; Fig. 3A). The DFMO group spent significantly more time in the open arms relative to the control one on day 36 ($t(18) = 2.17$, $p < 0.05$), but not day 54 ($t(18) = 0.35$, $p = 0.73$) (Fig. 3B).

Animals' performance in the open field was presented in Fig. 4. There were no significant differences between the control and DFMO groups in terms of the duration of wall-supported rearings (day 36: $t(18) = 1.24$, $p = 0.23$; day 54: $t(18) = 0.50$, $p = 0.62$; Fig. 4A) and the number of grid squares traversed (day 36: $t(18) = 0.38$, $p = 0.70$; day 54: $t(18) = 1.10$, $p = 0.29$; Fig. 4B). However, the lower values for both measurements were noticed on day 54 relative to day 36.

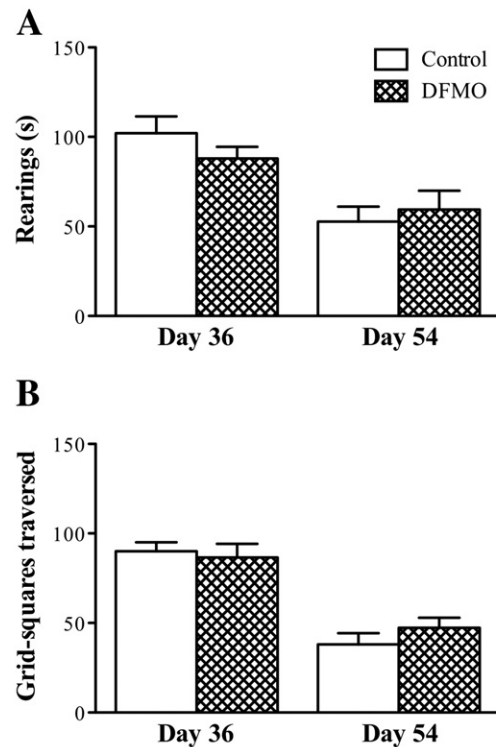

Fig. 4. Animals' performance in the open field. Mean (± SEM) duration of wall-supported rearings (A) and number of grid squares traversed (B) in the control and DFMO groups (n = 10 in each group). There were no significant differences between groups in these measurements on both days 36 and 54. Both groups displayed higher levels of exploratory and locomotor activity on day 36 as compared to day 54.

3.2.2. Morris water maze

3.2.2.1. Reference memory version

3.2.2.1.1. Place navigation (days 37–42). Animals were trained to find a hidden platform during days 37 to 42. There was no significant difference between the two groups in the swimming speed averaged across the 6 days of training (Control: 20.70 ± 1.22 cm/s; DFMO: 21.80 ± 1.24 cm/s; $t(18) = 0.63$, $p = 0.54$). For the path length measurement, two-way repeated measures ANOVA revealed significant effects of group ($F(1,18) = 6.37$, $P = 0.02$) and day ($F(5,90) = 45.52$, $p < 0.0001$), but not group × day interaction ($F < 1$), with greater path length in the DFMO group relative to the control one (Fig. 5A). When thigmotaxic swimming was analyzed, there was a significant group × day interaction ($F(5,90) = 4.83$, $p = 0.0006$), with the DFMO group generating markedly longer path length in the outer zone relative to the control one on days 37 and 38 (all $p < 0.05$; Fig. 5B). In terms of the heading error measurement, there was a significant effect of the day ($F(5,90) = 8.93$, $p < 0.0001$), but not group ($F < 1$) or group × day interaction ($F < 1$) (data not shown).

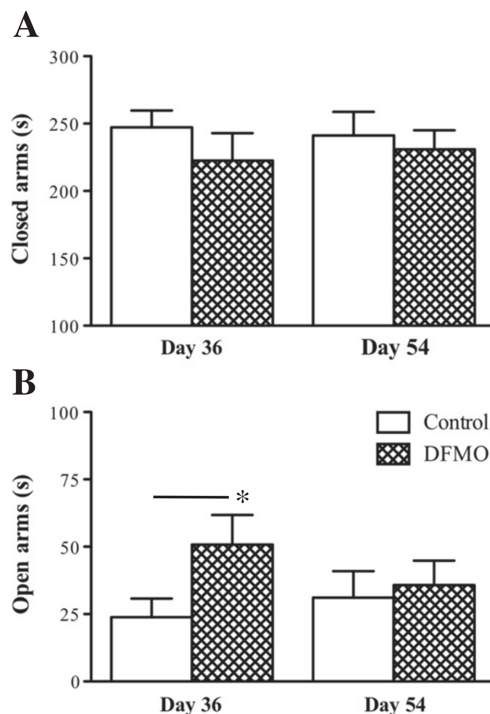

Fig. 3. Animals' performance in the elevated plus maze. Mean (± SEM) time spent into the closed (A) and open (B) arms in the control and DFMO groups (n = 10 in each group). The DFMO group spent more time into the open arms relative to the control one on day 36, but not day 54. There were no significant differences between groups in terms of the time spent into the closed arms on both days. * indicates a significant difference between groups at $p < 0.05$.

3.2.2.1.2. Probe tests (days 42 and 43). Animals were given a 60-s probe trial 120 s (Probe 1) or 24 h (Probe 2) after the final training trial on day 42. For Probe 1, there were no significant differences between groups in terms of the percentage of path length in the target quadrant ($t(18) = 0.30$, $p = 0.77$; Fig. 5C) and the number of platform crossings ($t(18) = 0.47$, $p = 0.64$; Fig. 5D). When animals′ performance during Probe 2 was analyzed, the DFMO group generated significantly shorter path length in the target quadrant ($t(18) = 2.59$, $p < 0.05$; Fig. 5C) and fewer number of platform crossings ($t(18) = 2.20$, $p < 0.05$; Fig. 5D) relative to the control one.

3.2.2.1.3. Cued navigation (days 43–44). Animals were trained to find a visible platform on days 43 and 44. When the path length was analyzed, ANOVA revealed a significant effect of day ($F(1,18) = 13.83$, $P = 0.0016$), but not group ($F < 1$) or group × day interaction ($F < 1$) (Fig. 5A). For thigmotaxic swimming, there was a significant effect of day ($F(1,18) = 33.56$, $p < 0.0001$), but not group ($F < 1$) or group × day interaction ($F < 1$) (Fig. 5B). In terms of the heading error measurement, there was a significant effect of the day ($F(1,18) = 4.71$, $p < 0.05$), but not group ($F < 1$) or group × day interaction ($F(1,18) = 1.18$, $p = 0.29$) (data not shown).

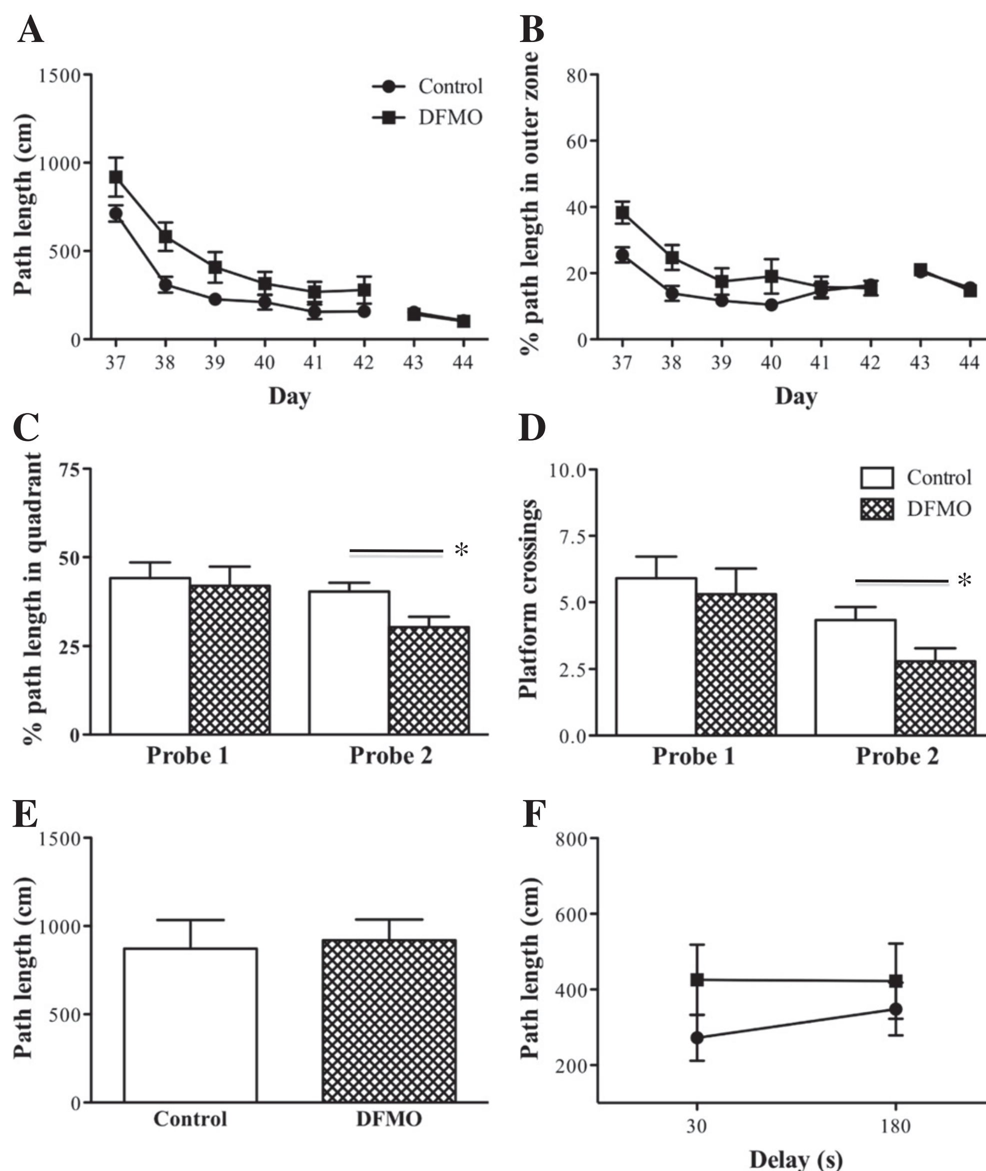

Fig. 5. Animals' performance in the water maze task. Mean (± SEM) path length to reach the platform (A) and percentage of path length spent in the outer zone of the apparatus (thigmotaxic swimming; B) during the place (days 37–42) and cued (days 43 and 44) navigation, and percentage of path length in the target quadrant (C) and number of platform crossings (D) during Probe 1 and Probe 2 conducted 120 s and 24 h after the final training trial of day 42, respectively) in the reference memory version of the water maze, and path length to escape to the platform during the sample (E; represents the mean of eight trials) and test (F; represents the mean of four trials) phases of the working memory version of the water maze in the control and DFMO groups (n = 10 in each group). The DFMO group generated significantly longer path length and displayed thigmotaxic swimming during the place navigation, and generated significantly shorter path length in the target quadrant and made fewer number of platform crossings during Probe 2 as compared to the control one. The DFMO group tended to generate longer path length during the test phase of the working memory version of the water maze. *Significant difference between groups at $p<0.05$.

3.2.2.2. Working memory version. Animals were tested in the working memory version of the water maze for 4 consecutive days (days 45–48). The path length during the sample phase was averaged across 8 trials over four days, and there was no significant difference between groups ($t(18)=0.24$, $p=0.81$; Fig. 5E). The path length during the test phase was averaged across 4 trials over four days for each delay. Two-way repeated measures ANOVA revealed no significant effect of group, delay or group × delay interaction (all $F<1$; Fig. 5 F), although the DFMO group tended to generate longer path length relative to the control one at both delays.

3.2.3. Object recognition task

The total time spent exploring all objects across the 4 phases on days 49 and 50 (habituation) in each treatment group was analyzed. A two-way ANOVA revealed a significant effect of phase ($F(3,54)=24.58$, $p<0.0001$), but not group ($F(1,18)=1.04$, $p=0.32$) or group × phase interaction ($F<1$), with reduced time in exploring objects across the 4 testing phases in both groups (data not shown).

During phase 2 of day 51, one of the familiar objects was replaced by a novel object. There was a significant effect of phase ($F(1,18)=11.13$, $p=0.005$), but not group ($F(1,18)=2.29$, $p=0.15$) or group × phase interaction ($F(1,18)=3.15$, $p=0.09$), with more time in exploring objects during phase 2 relative to phase 1 for both groups (data not shown). When the percentage of time spent in exploring the novel object during phase 2 of day 51 was analyzed, no significant difference was found between the control (48.13% ± 12.5) and DFMO (57.02% ± 10.53) groups ($t(18)=0.73$, $p=0.48$).

During phase 2 of day 53, the locations of two of the familiar objects were swapped. A two-way ANOVA revealed a significant effect of phase ($F(1,18)=5.36$, $p=0.033$), but not group ($F(1,18)=2.75$,

$p = 0.12$) or group × phase interaction ($F(1,18) = 3.04$, $p = 0.10$) in the total time spent exploring all objects across the two phases (data not shown). When the percentage of time spent in exploring the displaced objects during phase 2 of day 53 was analyzed, there was no significant difference between the control (38.46% ± 12.5) and DFMO (44.82% ± 10.65) groups ($t(18) = 0.39$, $p = 0.70$).

3.3. Neurochemical results

Fig. 6 illustrates DFMO-induced neurochemical changes in the sub-regions of hippocampus and the prefrontal cortex. For agmatine, there was a significant difference between groups in CA2/3 ($t(18) = 3.09$, $p = 0.006$), but not CA1 ($t(18) = 1.81$, $p = 0.08$), DG ($t(18) = 0.20$, $p = 0.85$) or PFC ($t(18) = 1.30$, $p = 0.21$), with decreased level of agmatine in CA2/3 in the DFMO group relative to the control one (Fig. 6A). The putrescine levels were dramatically decreased in the DFMO group relative to the control one in CA1 ($t(18) = 12.92$, $p < 0.0001$), CA2/3 ($t(18) = 8.63$, $p < 0.0001$), DG ($t(18) = 13.81$, $p < 0.0001$) and PFC ($t(18) = 11.74$, $p < 0.0001$) (Fig. 6B). There were also significantly decreased spermidine levels in the DFMO group relative to the control one in CA1 ($t(18) = 3.73$, $p = 0.0015$), CA2/3 ($t(18) = 5.53$, $p < 0.0001$), DG ($t(18) = 3.53$, $p = 0.0024$) and PFC ($t(18) = 6.75$, $p < 0.0001$) (Fig. 6 C). No significant difference was found between the two groups in spermine (CA1: $t(18) = 0.29$, $p = 0.77$; CA2/3: $t(18) = 0.67$, $p = 0.51$; DG: $t(18) = 0.06$, $p = 0.96$; PFC: $t(18) = 0.94$, $p = 0.36$; Fig. 6D), glutamate (CA1: $t(18) = 0.02$, $p = 0.99$; CA2/3: $t(18) = 0.15$, $p = 0.88$; DG: $t(18) = 1.06$, $p = 0.30$; PFC: $t(18) = 0.11$, $p = 0.92$; Fig. 6E), or GABA (CA1: $t(18) = 0.33$, $p = 0.75$; CA2/3: $t(18) = 0.44$, $p = 0.66$; DG: $t(18) = 1.43$, $p = 0.17$; PFC: $t(18) = 1.2{,}4$ $p = 0.92$; Fig. 6 F).

4. Discussion

The major route of the polyamine production in mammalian cells is *de novo* synthesis from ornithine by ODC. DFMO inhibits ODC and depletes putrescine mainly among the three polyamines (Gupta et al., 2009; Malaterre et al., 2004; Metcalf et al., 1978; Slotkin et al., 1982; Sparapani et al., 1996). In the present study, 3% DFMO in drinking water for 54 days resulted in an 80–90% reduction in the putrescine levels in the three sub-regions of the hippocampus and the

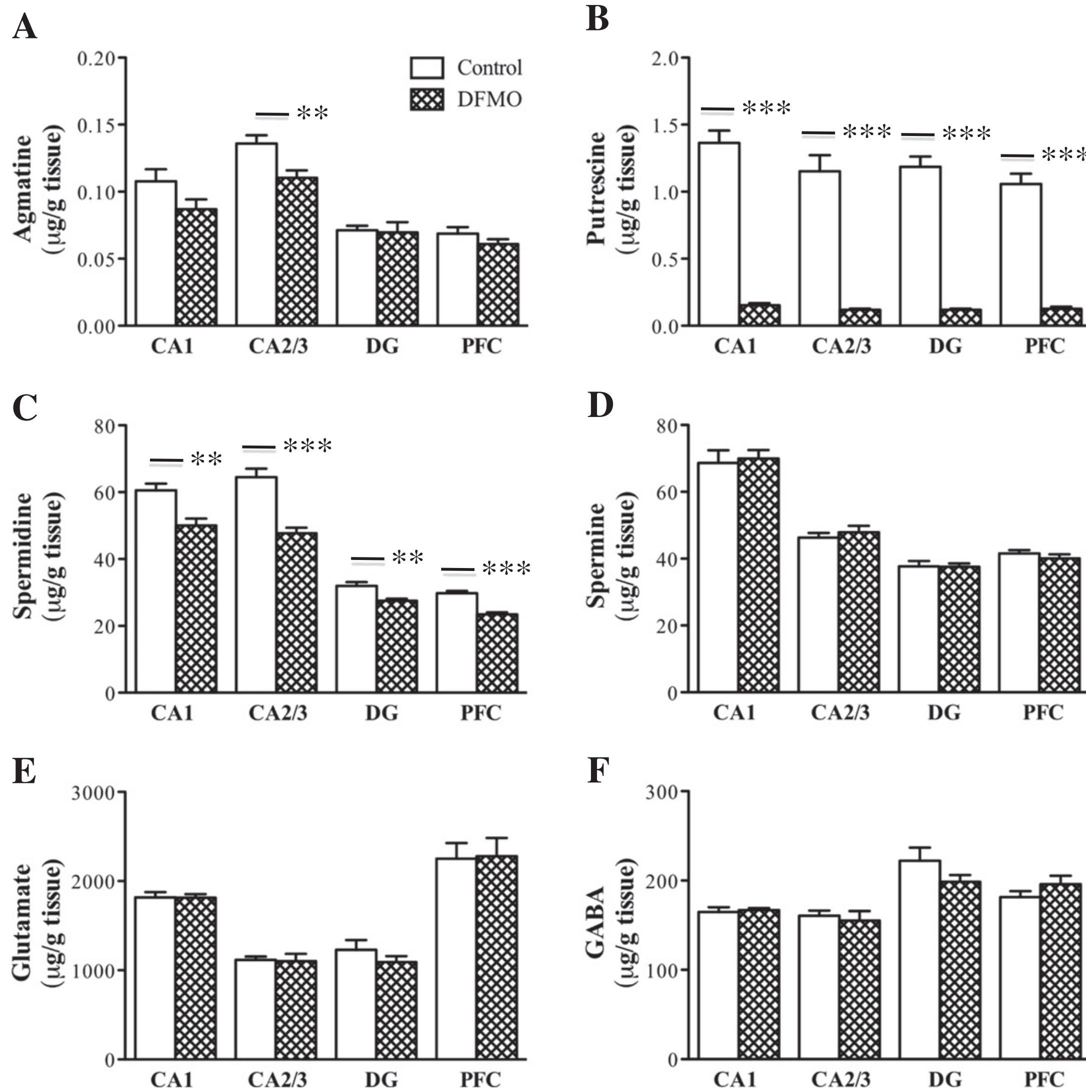

Fig. 6. Mean (±SEM) agmatine (A), putrescine (B), spermidine (C) and spermine (D), glutamate (E) and GABA (F) levels in the CA1, CA2/3 and DG sub-regions of the hippocampus and the prefrontal (PFC) in the control and DFMO groups (n = 10 in each group). There were approximately 80–90% and 20% reductions in the putrescine and spermidine levels, respectively, in the DFMO group relative to the control one in all of the brain regions examined. The level of agmatine was decreased in the CA2/3, but not other brain regions, in the DFMO group. There were no significantly differences between groups in the spermine, glutamate and GABA levels in any brain region examined. *Significant difference between groups: ** $p < 0.01$, *** $p < 0.0001$.

prefrontal cortex. The similar findings were reported by Malaterre et al. (2004), when the putrescine levels in the whole hippocampus were measured after 1-week and 4-week treatments of DFMO (3% in drinking water). Collectively, these findings suggest 3% DFMO in drinking water appears to deplete putrescine at a consistent rate at the time points of 7 to 54 days. Although the spermidine and spermine levels in the whole hippocampus did not change significantly at both the 1-week and 4-week time points in the study of Malaterre et al. (2004), we found approximately 20% reduction in the spermidine levels (with no changes in spermine) in the hippocampus and prefrontal cortex in the DFMO group when compared to the control one. It should be pointed out that the brain tissue was harvested when animals had 54 days of DFMO treatment in the present study, which is almost 4-week longer than the longest time point (4 weeks) in the study of Malaterre et al. (2004). Therefore, the difference in the duration of DFMO treatment prior to the tissue collection may account for the discrepancy between the two studies.

The present study assessed animals' performance in a number of commonly used behavioral tasks during the period of days 36–54 of the DFMO treatment. The DFMO group performed similarly to the control in the elevated plus maze, open field and object recognition tests, except for being slightly less anxious when tested on day 36 (but not day 54). These findings suggest that chronic treatment of DFMO (3% in drinking water) did not significantly alter animals' exploratory and locomotor activities, as well as their preference for the novel and displaced objects, but only affected animals' anxiety level transiently. It is of interest to note that the level of general activity in the open field on day 54 was markedly reduced when compared to that on day 36 for both groups. Previous research has demonstrated that animals perform differently in the elevated plus maze and open field with and without pretest handling (Schmitt and Hiemke, 1998).

In the reference memory version of the water maze task, animals were trained to escape from water by swimming to a fixed hidden platform using distal extramaze cues. The DFMO group generated longer path length relative to the control one across the 6 days of training. Because the swimming speed and the performance during the cued navigation in the DFMO rats were similar to that of the controls, the poor performance in the place navigation suggests DFMO-induced spatial learning deficit. It has been shown, however, that animals can use different strategies, such as circular search paths, to solve the water maze task. Thigmotaxis is the swimming along the wall of the pool, which is often seen during the first trials of water maze learning. Since the platform was located in the centre of the SE quadrant, animals should quickly learn to search for the central part of the apparatus rather than swimming along the wall continuously. It is of interest to point out that the DFMO group showed a higher level of thigmotaxis during the first 4 days of training in the place navigation relative to the control one, which may contribute to the performance deficit as indicated by the path length measurement. After completion of the place navigation, animal's memory to the platform location was assessed by conducting two probe tests. The DFMO group generated significantly shorter path length in the target quadrant and made markedly fewer number of platform crossings relative to the control one during Probe 2 (conducted 24 h after training), but not Probe 1 (conducted 120 s after training). Since there was no significant difference between the two groups in terms of thigmotaxic swimming during the last two days of training of the place navigation and during the cued navigation, the probe data suggest impaired spatial memory in the DFMO group at the longer (Probe 2), but not shorter (Probe 1), retention time. We also tested how the DFMO group performed relative to the control one in the working memory version of the water maze task. Although the DFMO rats tended to generate greater path length at both the 30-s and 180-s delays during the test phase relative to the controls, the differences were not statistically significant. Collectively, these findings suggest that chronic treatment of DFMO mainly affects spatial learning and memory in the reference memory version of the water maze task. The underlying mechanisms for such differential effects of DFMO will be investigated in the future.

It has been well documented that polyamines are essential for cell proliferation and differentiation, synthesis of DNA, RNA and proteins, protein phosphorylation, signal transduction, as well as the regulation of neurotransmitter receptors (for reviews see Oredsson, 2003; Wallace, 2000; Williams, 1997). Malaterre et al. (2004), for the first time, investigated the role of endogenous polyamines in adult neurogenesis in hippocampal DG and the subventricular zone (SVZ) of the lateral ventricle in rats. DFMO (3%) in drinking water for 1 week and 4 weeks induced a consistent decrease in neural progenitor cell proliferation in both DG and SVZ, and a significant decrease in newbown cell survival in the DG only. The DG is characterized by orthogonalization of sensory inputs to create a metric spatial representation and hence is important for spatial pattern separation (Kesner et al., 2004). It has been reported that colchicine lesions of the DG results in spatial learning and memory deficits (Sutherland et al., 1983; Xavier et al., 1999). Dupret et al. (2008) tested how specific ablation of newborn neurons in hippocampal DG affected spatial learning and memory in the water maze task. Interestingly, ablation of newborn neurons in the DG resulted in performance impairment in the water maze task when animals were trained to find a hidden platform in a fixed location from variable (similar to the place navigation procedure in the present study), but not constant, start positions. Given the role of the newborn cells in the DG in maintaining hippocampal function (Aimone et al., 2011; Clelland et al., 2009; Dupret et al., 2008; Van Praag et al., 2002), it is likely that DFMO-induced impairment in hippocampal neurogenesis as detailed by Malaterre et al. (2004) may account for the spatial learning and memory deficits observed in the present study.

Among the three polyamines, it has been shown that putrescine has a negative influence on the N-methyl-D-aspartate (NMDA) receptor function, whereas spermidine and spermine are the positive modulators of NMDA receptors (Rock and Macdonald, 1995; Williams, 1997; Williams et al., 1994). Since both putrescine and spermidine levels were decreased and the spermine levels were not changed in the DFMO group, we speculated that the NMDA receptor function might not be affected dramatically under the present experimental condition. When animals were tested in the water maze task, the DFMO group was significantly impaired during the place, but not cued, navigation, which contrasts against the deficits in the cued navigation induced by the NMDA receptor antagonists through the sensorimotor mechanisms (for a review see Cain, 1998). It is also of interest to mention that the present study measured the glutamate and GABA levels in the three sub-region of the hippocampus and prefrontal cortex, and found no significant differences between the control and DFMO groups in any region examined. These findings suggest that chronic DFMO treatment did not alter the glutamate/GABA ratio, and hence might not affect the neuronal excitability in the hippocampus and prefrontal cortex dramatically.

We found significantly decreased agmatine level in the CA2/3 subregion of the hippocampus and a trend of reduction in the agmatine level in hippocampal CA1 ($p = 0.08$) in the DFMO group relative to the control one. It is unclear at present why agmatine level changes in a region-specific manner, and what are the exact underlying mechanisms. Agmatine, in addition to being a precursor of putrescine, plays an important role in regulating the intracellular content of polyamines through the induction of antizyme, a small regulatory protein that inhibits ODC and down-regulates polyamine uptake (Satriano, 2003). Agmatine regulates the nitric oxide (NO) production by influencing the activity of three isoforms of NO synthase (NOS) (for a review see Halaris and Piletz, 2007), and may directly participate in the processes of learning and memory as a novel neurotransmitter (Leitch et al., 2011; Liu et al., 2008a, 2009a; Seo et al., 2011). It has been shown

that DFMO is an inhibitor of nitrite (the end product of NO) production by macrophages (Morgan, 1994), and that DFMO pre-treatment up-regulates endothelial NOS (Lin et al., 2010). Hence, future research is required to further investigate the interactions among DFMO (and/or polyamines), NO and agmatine.

It should be pointed out that both the behavioral and neurochemical results in the present study markedly differ from that of Gupta et al. (2009), in which acute putrescine depletion was induced by the i.c.v. injections of DFMO at the doses of 25 and 50 μg. In the latter study, the DMFO rats displayed increased anxiety level and impaired memory for the object displacement, with no spatial learning and memory deficits in a simplified version of the water maze task. DFMO treatment at both doses resulted in 80–90% reduction of putrescine level in the hippocampus and prefrontal cortex with minimal effects on the spermidine and spermine levels. There were increased glutamate and GABA levels in the CA2/3 and DG sub-regions of the hippocampus in the DFMO rats, and these changes appeared to be associated with anxiety-like behavior. By comparing the results of the Gupta et al. (2009) and present studies, it seems that acute and chronic DFMO treatments have differential behavioral and neurochemical effects. The exact mechanisms underlying these changes need to be explored in the future.

It should also be pointed out that in the present study DFMO treatment significantly affected animal's body weight gain. As the water requirement has positive correlation with body weight in rats (Cizek and Nocenti, 1965), a decrease in body weight over time would also decrease the water requirement for the DFMO rats. In the present study, however, animals' daily water intake normalized by body weight was slightly reduced in the DFMO group relative to the control one, which might be attributed to the unpleasant taste of DFMO (McWilliams et al., 2000). Since DFMO treatment affected animals' body weight gain and daily water intake, one may argue that chronic DFMO treatment might have caused undernutrition that could alter animals' behavioral performance. DFMO is the Food and Drug Administration (FDA) approved drug and has been used clinically as a therapeutic and chemopreventive agent for cancers (Babbar and Gerner, 2011; Meyskens et al., 2008; Simoneau et al., 2008). Brown et al. (1999) evaluated the toxicity of DFMO in female rats following 13 weeks of daily administration by gavage at a dose of 1000 mg/kg per day, and found decreased body weight gain and food consumption in DFMO rats, however with no histological evidence of liver lesions. Ferguson and Cada (2004) reported that developmental DFMO treatment slightly reduced adult cerebellar weight with few functional alternations. In the present study, the normalized brain, kidney and liver weights by body weight were not significantly different between the control and DFMO groups. Since 3% of DFMO in drinking water mainly affected behavioral performance in the reference memory version of the water maze, such task-specific effect cannot be interpreted as the consequence of general health deterioration and/or toxicity induced by chronic DFMO treatment. Rotta et al. (2003) reported that pre- and postnatal (up to 75 days) undernutrition significantly affected the weights of rats since birth up to 75 days, but did not affect animals' performance in the open field, elevated plus maze and inhibitory avoidance task. Andrade et al. (2002) investigated whether prolonged food restriction would cause deleterious alterations in the hippocampus and impair spatial learning and memory. Food restriction (40%) for 36 weeks did not alter the total number of dentate granule cells and hippocampal CA3 and CA1 pyramidal neurons, the morphology of the dendritic trees of hippocampal pyramids, and animals' performance in the place navigation and probe test of the water maze task.

In summary, the present study investigated the behavioral and neurochemical effects of DFMO administered orally. DFMO (3%) in drinking water for 54 consecutive days resulted in approximately 80–90% and 20% reductions in the putrescine and spermidine levels respectively in the hippocampus and prefrontal cortex. Behaviorally, DFMO treatment impaired the reference memory version of the water maze task, but did not affect animals' general behavior, object recognition, and performance in the cued navigation of the water maze task. These findings suggest that physiological levels of polyamines may be essential for spatial learning and memory, which merits further investigation to fully understand the functional role of endogenous polyamines in learning and memory.

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