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In utero methanesulfonyl fluoride differentially affects learning and maze performance in the absence of long-lasting cholinergic changes in the adult rat

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Abstract

There is increasing evidence that acetylcholinesterase (AChE) may have various specific developmental roles in brain development. Nevertheless, specific effects of AChE inhibition during early brain development have not been adequately described. Therefore, methanesulfonyl fluoride (MSF), an irreversible AChE inhibitor that shows high selectivity for the CNS was used to produce AChE inhibition in utero to study subsequent adult behaviors, sleep, and cholinergic markers. Rats exposed to MSF in utero showed a deficit in spatial learning tasks using appetitive motivation but, surprisingly, they performed equally well or better than controls when aversive motivation was used. One hypothesis was that MSF treatment in utero affected the response to stress. Tests of anxiety however showed no differences in basal levels of anxiety. Studies of sleep behavior, however, indicated a higher level of REM sleep which is only seen during the light phase of male rats exposed to MSF in utero as compared to controls. No differences in cholinergic markers in the brains of adults were found except that females exposed to MSF in utero had a higher level of ChAT activity in the synaptosomal fraction of the hippocampus. Even so, whether cholinergic alterations accompany the in utero MSF exposure remains to be determined. The failure to find widespread changes in cholinergic markers in the adult brains suggests changes in behaviors should be further investigated by testing the participation of postsynaptic mechanisms, measuring of cholinergic markers during earlier development periods and the possible participation of other neurotransmitter systems to clearly reveal the role of the cholinergic system following in utero MSF exposure.

Keywords: Acetylcholine; Acetylcholinesterase; Methanesulfonyl fluoride (msf); Development; Choline acetyltransferase; Choline uptake; Sleep

1. Introduction

Acetylcholine (ACh) is a well known neurotransmitter that has been implicated in virtually every aspect of behavioral and physiological regulation (Ninomiya et al., 2005; Gu, 2002; Taylor, 1998; Karczmar, 1998; Wolf, 1991). However, outside of its classical functions as a neurotransmitter, it has also been implicated in the regulation of growth in the developing CNS (Slotkin, 2004; Lauder and Schambra, 1999; Hohmann and Berger-Sweeney, 1998; Weiss et al., 1998), regulation of post-natal cortical development (Levitt et al., 1997), and modulation of axogenesis and synaptogenesis (Slotkin, 2004; Audesirk and Cabell, 1999).

Similarly, acetylcholinesterase (AChE) may have specific roles in the development of the nervous system such as regulation of neurite outgrowth, proliferation and differentiation of nerve cells (Brimijoin, 2005; Sternfeld et al., 1998) and it may also influence neural development (Brimijoin, 2005; Bigbee et al., 1999; Brimijoin and Koenigsberger, 1999; Koenigsberger et al., 1997; Brimijoin and Hammond, 1996; Karpel et al., 1996).

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In view of the potential importance of ACh and AChE in the development of the nervous system, it seems that long-term structural, behavioral and neurochemical deficits in brain development might result from inhibiting AChE (Brimijoin, 2005). Indeed, this strategy has been used to investigate the role of AChE on brain development (Aluigi et al., 2005; Slotkin et al., 2001). In addition, rats treated in utero with MSF (dams treated during pregnancy) show subsequent impairments as adults in working memory in the radial-arm maze, morphologic alterations in brain development (increase of frontal cortical thickness in females and delayed thickening in males) (Favela et al., 2003) and down-regulation of choline acetyltransferase (ChAT) (Byers et al., 2005). These results support the idea that interference with AChE during brain development can produce long-lasting structural, neurochemical, and behavioral deficits (Brimijoin, 2005).

The purpose of the present experiments was to better characterize the effects of in utero AChE inhibition on a wide range of adult behaviors, including the effects on learning and retention of spatial information, anxiety, and sleep.

MSF was selected for these studies because it is an irreversible inhibitor of AChE that shows high selectivity for the CNS (Moss et al., 1988, 1985) and is also highly selective (more than 20 times) for AChE compared to butyrylcholinesterase (Pacheco et al., 1995).

Lastly, three cholinergic markers (AChE activity, choline uptake, and choline acetyltransferase activity) in five brain regions (cortex, diencephalon, hippocampus, pons, and striatum) were examined in the adults to determine if in utero treatment with MSF produced long-lasting effects that could be detected in the adult and correlated with any observed behavioral changes.

2. Material and methods

2.1. Animals

Thirty two female and 16 male Sprague–Dawley rats (reared in the animal colony at the University of Texas at El Paso) were housed in wood-chip bedding with ad libitum access to food and water and bred to produce gravid females for in utero treatment. Four females and 2 males were caged together for one week to ensure insemination. Thereafter, the females were separated and placed in individual cages for the remainder of the pregnancy. The resulting pups were kept with their mothers until weaning at 21 days at which time male and female offspring were separated and housed in groups of 2–4 until they entered the study. A total of 154 offspring were produced from such parings, 80 males and 74 females. The number of individuals in each group was as follow: 36 control males, 44 MSF males, 37 control females, and 37 MSF females. As described below regarding individual experiments, the rats entered the studies between 2 and 6 months of age at which time they weighed between 200 and 500 g. Once in the study, the animals were housed individually in cages with access to food and water ad libitum at 22 °C and maintained on a 12 h light and 12 h dark circadian cycle, lights on at 0600. All testing was conducted between 09:00 and 16:00 h. The treatment of all animals was approved by the Institutional Animal Care and Use Committee of the University of Texas at El Paso in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. MSF treatment

Half of the pregnant females were injected subcutaneously 3 times a week with 0.5 mg/kg MSF (0.5 mg/ml in peanut oil) and the other half were injected with 1 ml/kg of peanut oil. These injections were given during the last 2 weeks of gestation and no further injections were given post-partum.

MSF (Lancaster Synthesis, Inc., Windham, NH) was dissolved in peanut oil (PO) (0.5 mg/ml) and stored at 4 °C. All other chemicals were from Sigma Chemical Company.

2.3. Behavioral tests

2.3.1. Radial eight arm food maze (8AFM)

The radial 8AFM was constructed of transparent Plexiglas™ with an octagonal center 35 cm in diameter and eight identical arms measuring 60 cm length × 15 cm wide × 30 cm high. The end of each arm had a food container 5 cm in diameter and 0.6 cm deep. The animals were placed on a reduced food diet one week prior to the test, (not losing more than 10% of their ad libitum weights) and were given a 4-hour acclimation to the maze the day prior to the experiment. The rats were given one trial per day, at the same time of day, for eight consecutive days. During each trial, a sweet food reward (Fruit Loops™) was placed in each of the eight food receptacles; the rat was placed in the center of the maze and allowed to move freely through the maze until all rewards had been consumed. A correct response was the first entry to an arm with consumption of the reward. Errors were scored when the rat reentered arms from which it had retrieved the reward on an earlier trial. The total time to complete the maze and the total number of errors required to retrieve all the rewards were recorded.

2.3.2. Radial eight arm water maze (8AWM)

The 8 arm radial food maze described above (8AFM) was filled with water (room temperature) to a depth of 20 cm and the food cups were replaced with a collapsible platform just below the water level. During each trial (same duration as the 8AFM) the rats were placed in the middle of the maze and allowed to swim freely through the maze. Once the rat entered an arm and reached the platform, it was allowed to stay on the platform for 10 s before the platform sank into the water. Performance in the maze was scored, as with food, by the total time and errors when the animal reentered an arm where the platform was down. When the animals had found all the platforms, they were towel dried and returned to their home cages.

2.3.3. Swim escape test

The swim escape (SE) task (Barraco and Klauenberg Irwin, 1978) consisted of a circular plastic tank 54 cm in diameter in the upper part, 41 cm in diameter at the base and 46 cm tall. The tank was filled with 30 cm of water (25 °C). A nylon rope

about 2.5 cm in diameter was placed in the middle with the upper part suspended from a support 60 cm above the water level while the lower end of the rope was anchored to the bottom of the tank. The rat was given three trials in which it was placed at the edge of the water-filled tank facing away from the rope and towards the investigator, released quickly, and allowed to swim until it found the rope and climbed out of the water. The time for escape was recorded starting at the moment that the rat was placed in the tank until it raised its entire body out of the water. A maximum time limit of 5 min was allowed after which, if the animal had failed to climb out of the water, it was manually placed on the rope and allowed to remain there for 10 s. The second and third trials were performed 72 and 96 h after the first trial, respectively. After each trial the animals were returned to their cages where they were towel dried and warmed.

2.3.4. Elevated plus maze (EPM)

The apparatus consisted of an elevated, plus-shaped runway built up of black Plexiglas™ elevated 66 cm above the floor. Two open arms and two closed arms, measuring 23 cm each, emerged from a central open platform of 15 cm per side. The height of the walls of the closed arms was 20 cm. Testing was conducted in a room lighted only with dim a red light. The test duration was 5 min and was conducted by placing the animal in the center of the EPM, where the four arms cross each other, facing a closed arm. The time spent in closed or open arms, in the central platform, and the number of crosses made between compartments were recorded. An entry was scored when both front paws were placed in an arm.

2.3.5. Open field test

Open field behavior was tested in a square clear Plexiglas™ box, 64 cm each side and 18 cm height. For scoring, the clear plastic floor was placed over a grid divided into 25 squares (13 cm) per side for 5 min. The animal was placed in a corner of the box and the time to leave the original position (latency), horizontal locomotion (number of crossings of the lines marked on the floor), amount of time spent in the periphery (the 16 marginal squares) or in the center (the 9 inner squares) were recorded.

2.4. Sleep studies

Nine MSF-treated and six control male rats were used in the sleep studies. After weaning they were housed, 2–3 rats per cage, with ad libitum food and water under a 12L:12D cycle with lights on at 06:30 h until they were prepared with the necessary electrode array for measuring their brain and muscle activity.

2.4.1. Animal surgery

At 2 months of age, a standard set of EEG and EMG electrodes were permanently implanted surgically under an anesthetic cocktail containing, 6.3 mg/ml xylazine, 0.25 mg/ml acepromazine maleate, and 25 mg/ml ketamine HCl at 2 ml/kg (IM). Three EEG screw electrodes (1 frontal and 2 parietal), one reference screw electrode (occipital), and two EMG electrodes (placed into the occipital muscles) were implanted. The electrode ends were inserted into a six-electrode pedestal and the whole assembly secured to the cranium and allowed 2 weeks of recovery.

2.4.2. Sleep recordings

During the two weeks allowed for recovery from surgery, the rats were acclimated to the recording chamber and the presence of the recording cable. Following recovery, baseline measures of sleep were recorded for each animal using a Grass Model 8 EEG recorder and a six-felectrode commutator. The EEG and EMG data were recorded on paper for later analysis. In these studies, the sleep of animals was recorded for 4 h during the light phase starting at 10:00 a.m. ± 30 min; and 4 h during the dark phase starting at 10:00 p.m. ± 30 min. Records were visually scored and quantified for wakefulness, drowsy, slow wave sleep, and rapid eye movement (REM) sleep in 6-second epochs. Total time, percent of time, number of bouts, duration of each bout, average waiting time between bouts, average bout duration, and the latency to the first bout of drowsy, slow wave sleep, and REM sleep were scored for each stage of sleep.

2.5. Biochemical procedures

Upon sacrifice, brains were quickly removed and placed in 50 ml of ice-cold sucrose (0.32 M sucrose containing 50 μM HEPES, and 1 mM EGTA) and visually dissected into frontal cortex, hippocampus, the striatum, pons and diencephalon, and immediately placed in ice-cold sucrose until they were patted dry and the wet tissue weight was recorded. Thereafter, they were immediately placed in a 2 ml of ice-cold fresh sucrose solution and homogenized using a Polytron (PRO Pro250) grinder set on the setting of 4. Aliquots of each brain part were removed for protein determination, total AChE activity, and total ChAT activity determinations. All samples were immediately frozen in a −80 °C freezer until assayed.

For the assays, the different brain homogenates were centrifuged at 1000 ×*g* for 10 min at 4 °C to pellet nuclei and large cellular fragments. Approximately 1.6 ml of the resulting supernatant was transferred to clean centrifuge tubes and spun at 10,000 ×*g* for 15 min at 4 °C to pellet the synaptosomes (nerve terminal fraction). The supernatant was discarded and the pellets kept on ice until assayed. The pellets were resuspended in 1 ml of the ice-cold sucrose solution and samples were removed for protein determination, synaptosomal AChE activity, and synaptosomal ChAT activity. These samples were also kept at −80 °C until assayed. The remaining synaptosomes were split in half and spun for 5 min at 14,000 ×*g*, decanted and placed on ice until the choline uptake measurement was made.

2.5.1. Protein determinations

Protein content was determined in duplicate according to the spectrophotometric method of Bradford (1976) at 595 nm no sooner than 10 min after adding the Bradford reagent. A standard curve was prepared using concentrations of 0, 1, 5, 7.5, and 10 μg/ml bovine serum albumin (BSA Fraction V).

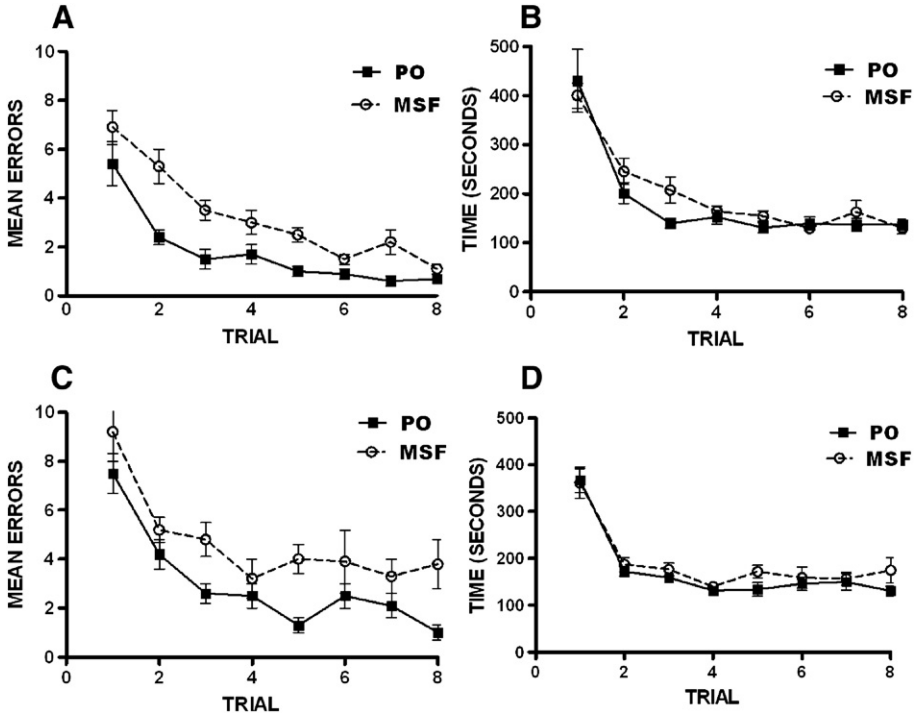

Fig. 1. Effect of in utero MSF exposure on performance in the radial 8 arm food maze. Panel A represents the errors committed by male rats on each trial (one trial per day). Panel B represents the time to complete the maze for male rats on each trial (one trial per day). Panel C represents the errors committed by female rats on each trial (one trial per day). Panel D represents the time to complete the maze for female rats on each trial (one trial per day). Each point represents the mean ± SEM of: males, 21 peanut oil rats and 38 MSF rats and for the females, 37 peanut oil rats and 37 MSF rats.

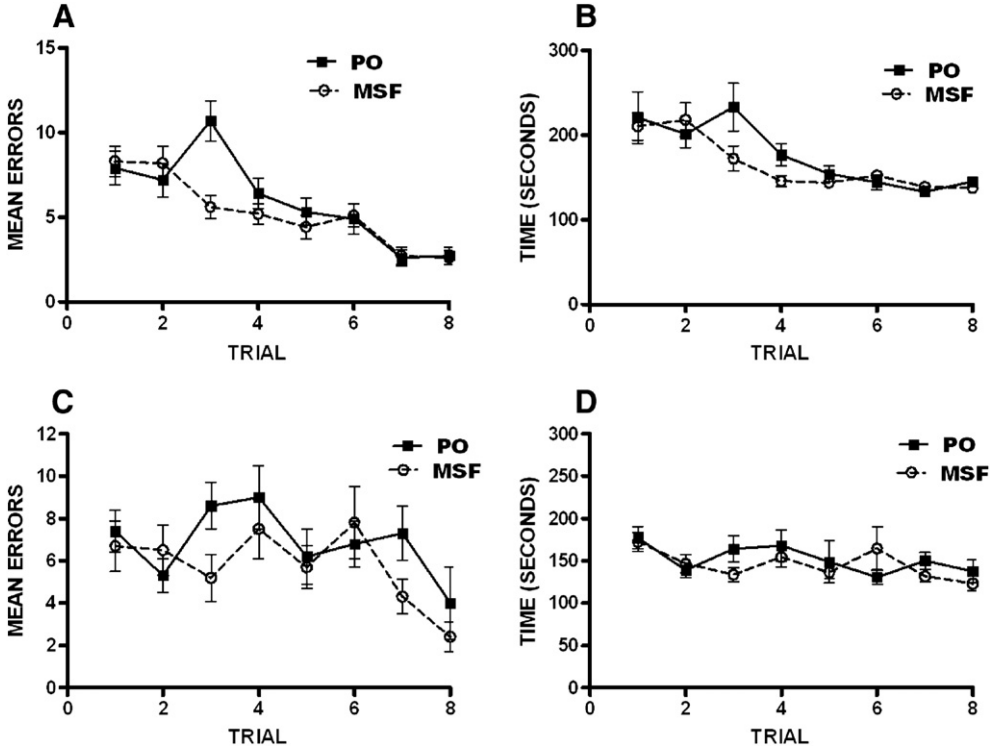

Fig. 2. Effect of in utero MSF exposure on performance in the radial 8 arm water maze. Panel A represents the errors committed by male rats on each trial (one trial per day). Panel B represents the time to complete the maze for male rats on each trial (one trial per day). Panel C represents the errors committed by female rats on each trial (one trial per day). Panel D represents the time to complete the maze for female rats on each trial (one trial per day). Each point represents the mean ± SEM of: males, 21 peanut oil rats and 34 MSF rats and for the females, 20 peanut oil rats and 20 MSF rats.

2.5.2. AChE

Triplicate AChE assays of cortex, hippocampus, pons, striatum and diencephalon samples were conducted according to the spectrophotometric method of Ellman et al. (1961). Samples were resuspended using 500 µl of 0.1 M (Na) PO_4 buffer pH 7.4. One hundred µl of 0.075 M acetylthiocholine iodide (in deionized water) and 50 µl of 0.01 M dithiobisnitrobenzoic acid (DTBN) (in phosphate buffer) were added to 750 µl of buffer to make a total of 900 µl before reaction was started by the addition of 100 µl of the resuspended sample. The absorbance at 412 nm was recorded at 5, 7,9,11 and 13 min at room temperature. Estimates of Vmax for the acetylthiocholine substrate were calculated for each brain region by least squares linear regression. The activity was expressed as nmol/min/mg protein.

2.5.3. Choline uptake

One pellet from each brain region was resuspended in 500 µl of a Krebs solution (120 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 9.9 mM dextrose, 25 mM $NaHCO_3$) saturated with 95% O_2 and 5% CO_2 to give a pH of 7.4 and prewarmed to a temperature of 38 °C. A second pellet was suspended in 500 µl of the same Krebs solution but it also contained 10 µM hemicholinium-3. Twenty µl of a solution containing 250 µCi of choline chloride [methyl-3H] dissolved in 198 µl of Krebs solution was added to both samples (with and without hemicholinium) to give a final concentration of 2 µM choline and incubated at 38 °C for 5 min and vacuum filtered through Durapore membrane filters (0.45 µm HV). The filters were then immediately washed three times with a total volume of 5 ml of a saline solution (4 °C), placed in scintillation vials with 3.5 ml of scintillation liquid (Biosafe II), and counted by liquid scintillation for 5 days. Sample quench was estimated using 10 ml of a 3H–H_2O standard. The transport of 3H–Ch was determined based on the specific activity of the choline and corrected for the amount of synaptosomal protein.

2.5.4. Choline acetyl transferase

ChAT activity was determined using the radio-enzymatic method of Fonnum (1969). Buffer A contained 300 mM NaCl, 0.2 mM Physostigmine, 0.5 mg/ml BSA, 12.5 mM Choline, and 0.2% Triton X-100. Buffer B contained 0.25 mM AcCoA and 0.1 µCi 3H–AcCoA, dissolved in buffer A. Buffer C contained 10 mM $NaPO_4$ and 0.2 mM ACh. One hundred eighty µl of Buffer A was used to resuspend the pellet from the total sample or the P2 pellet previously obtained as described above, then 25 µl of Buffer B was added to start the reaction and the mixture was incubated at 38 °C for 5, 10 and 15 min. At these time points 50 µl of the reaction mixture was removed and added to 250 ml of ice-cold buffer C to stop the reaction. To the stopped reaction, 400 µl of heptanone containing 10 mg/ml of tetraphenyl borate was added and the tube vigorously vortexed at least 4 times. The resulting mixture was then centrifuged for 3 min at 14,000 ×*g* to separate the aqueous and organic layers. Following this, 300 µl of the organic layer was added to 3.5 ml of scintillation cocktail and then quantified by liquid scintillation spectrophotometry 72 h later (time was chosen based on preliminary experiments). A tube containing no sample was also processed at 15 min to determine the blank number of counts that transfer to the organic layer when no radio-labeled ACh was formed. The samples were quenched by using 10 µl of a 3H–H_2O standard. Estimates of ChAT activity were calculated for each brain region by least squares linear regression at the three time points in the organic layer. The rate of product formation was then calculated based on the specific activity of the added Acetyl CoA. All measures were corrected for mg of protein in the appropriate sample.

2.6. Statistics

2.6.1. Behavioral experiments

For the radial 8 arm food and water maze and the swim escape tests, rats were compared (using SPSS 13.0 package for Windows, SPSS Inc., USA) based on treatment, gender and trial using a 3-way repeated measures ANOVA with treatment (MSF, PO) and gender (males, females) as between-subject factors and trial as the within-subject variable. For relevant main effects or interactions one-way ANOVA and post-hoc test were run. Linear and quadratic trend analyses were used to assess progressive effects across repeated measures. Partial eta squared (η^2) are listed for effect sizes of interactions' *F*-tests.

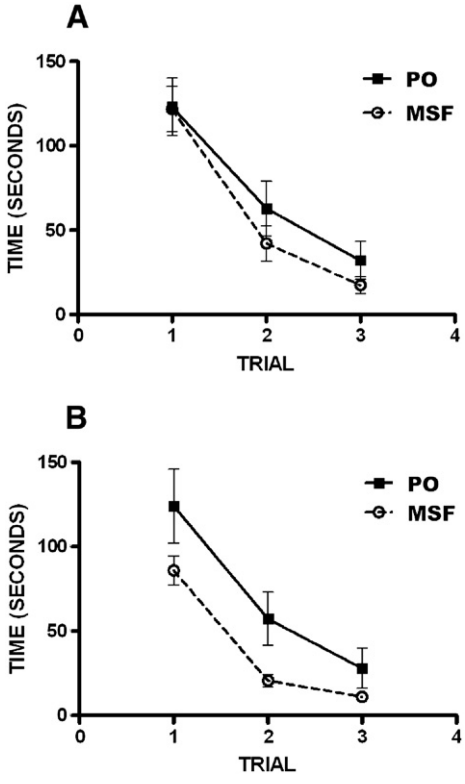

Fig. 3. Effects of in utero MSF exposure on performance in the swim escape test. Panel A illustrates the time required for males to climb out of the water per trial (trial 1 day 0, trial 2 day 3, and trial 3 day 4). Panel B illustrates the time required for females to climb out of the water per trial (trial 1 day 0, trial 2 day 3, and trial 3 day 4). Each point represents the mean ± SEM of: males, 23 peanut oil rats and 26 MSF rats and females, 18 peanut oil rats and 19 MSF rats.

For the elevated plus test and the open field tests, rats of a given gender were compared based on treatment using a Student *t*-Test to determine statistical significance with regards to time spent in the open to closed arms and the number of total crosses made by the animals. Data are given as means and standard errors. Significance was assumed at the level of $p<0.05$ for main effects.

2.6.2. Sleep experiments

The means of each sleep parameter were compared between in utero MSF exposure and control rats using the Student *t*-Test ($p<0.05$).

2.6.3. Biochemical experiments

The means for each measure, of each brain region, were compared between MSF-exposed and control rats using the Student *t*-Test ($p<0.05$).

3. Results

3.1. Behavioral experiments

3.1.1. 8AFM

Both males and females exposed to MSF in utero show significant impairment of performance in the 8AFM, committing more errors than the controls (MSF main effect, $F(1,131)=13.12$, $p<0.001$), when data was reexamined it was confirmed that main treatment effect was present in both (males, $F(1,57)=15.32$, $p<0.0001$ and females $F(1,72)=21.77$, $p<0.0001$). A significant gender effect is also noticed ($F(1,131)=14.28$, $p<0.001$); however interaction result between gender and MSF was not significant ($p=0.86$). A significant trial effect ($F(7,125)=31.8$, $p<0.01$) is also shown with a significant linear ($F(7,125)=114.4$, $p<0.001$) and quadratic ($F(7,125)=39.47$, $p<0.01$) trial change present indicating that while there was an overall significant decrease in committing errors, the trend is not totally linear and it has leveled off (Fig. 1, panels A and C). No gender–trial, MSF–trial or gender–MSF–trial interactions were observed.

Along with committing more errors, MSF-exposed rats also tended to take a little longer completing the task than the control rats. Results show a significant overall difference in the time employed to finish the task (MSF main effect ($F(1,131)=3.34$, $p<0.05$), (Fig. 1, panels B and D). The main effect of gender was also significant ($F(1,131)=3.36$, $p<0.05$), and when lower-order ANOVAs were run they revealed that the difference was significant for males only ($F(1,57)=3.969$, $p=0.0469$), although almost significant for the females ($F(1,72)=3.650$, $p=0.0566$). No interaction gender–MSF was observed. Furthermore, a significant trial effect ($F(7,125)=7.14$, $p<0.05$) showing linear ($F(7,125)=129.7$, $p<0.001$) and quadratic ($F(7,125)=134.3$, $p<0.001$) trial changes, together with a trial–gender interaction ($F(7,125)=2.85$, $p=0.006$) with a significant linear component ($F(7,125)=6.185$, $p=0.14$) were also present; however, the magnitude of the effect size of this interaction ($\eta^2=0.022$) is small (Cohen, 1988). There was no trial–MSF significant interaction present.

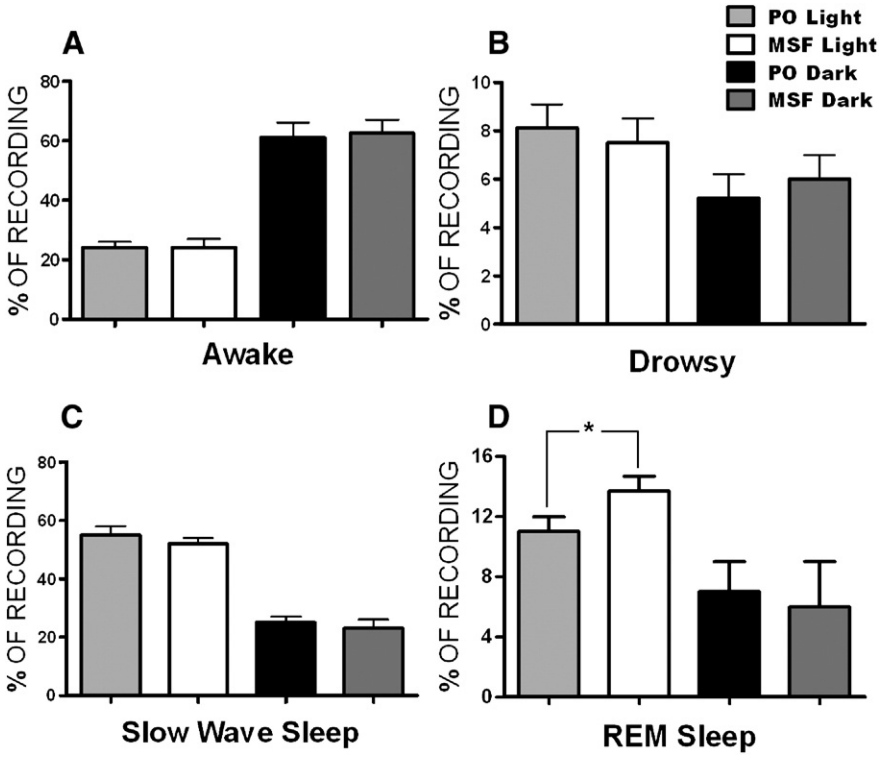

Fig. 4. The effect of in utero MSF on percent of time spent in given states of consciousness. Panel A illustrates the percent of recording time spent awake. Panel B illustrates the percent of time spent drowsy. Panel C illustrates the percent of time spent in SWS. Panel D illustrates the amount of time spent in REM sleep. Each bar represents the mean ± SEM of 6 peanut oil control rats and 9 MSF-exposed rats. PO Light = peanut oil light phase, MSF Light = MSF light phase, PO Dark = peanut oil dark phase, and MSF Dark = MSF dark phase (*$p<0.05$).

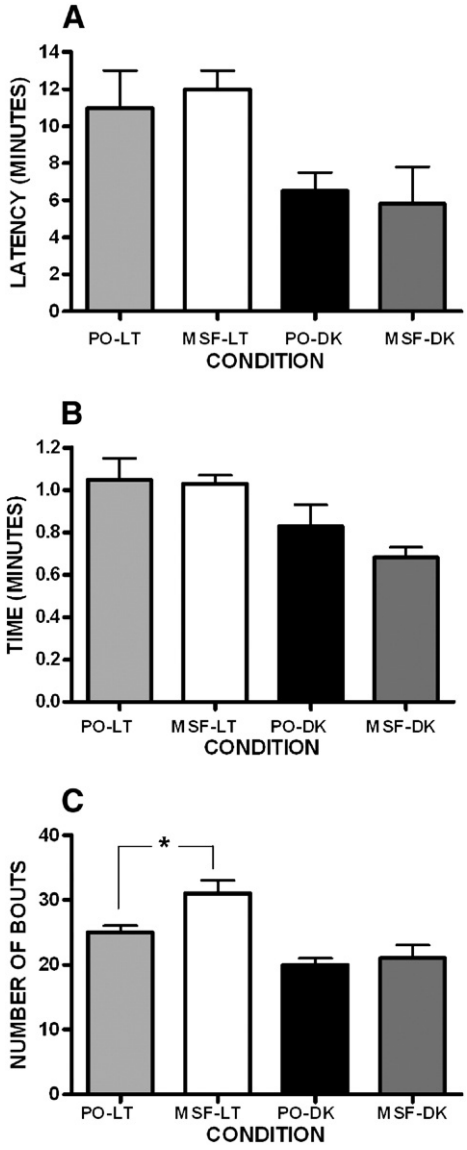

Fig. 5. The effect of in utero MSF exposure on REM sleep parameters. Panel A shows the REM sleep latencies. Panel B shows the average REM sleep durations. Panel C shows the number of REM sleep bouts. Each bar represents the mean ± SEM of 6 peanut oil rats and 9 MSF-exposed rats. PO-LT = peanut oil light phase, MSF-LT = MSF light phase, PO-DK = peanut oil dark phase, and MSF-DK = MSF dark phase (*$p<0.05$).

3.1.2. 8AWM

In utero MSF exposure did not affect performance accuracy in the radial-arm maze during the acquisition training period, (MSF main effect $F(1,97)=2.32$, $p=0.15$). (Fig. 2 panels A and C) Similarly, a significant effect for gender was not found ($F(1,97)=1.28$, $p=0.24$). Gender–MSF interaction was also absent ($p=0.69$). When looking for trial effects, a significant effect was found ($F(7,91)=8.87$, $p<0.01$) and a trial–gender interaction observed ($F(7,91)=4.73$, $p<0.01$) with a significant linear ($p<0.01$) but not quadratic ($p=0.52$) trend with the number of errors steady decreasing over time as can be observed with the MSF-treated animals (Fig. 2, panel A). In this case the magnitude of the effect size of the interaction fall in between small and medium ($\eta^2=0.050$). There was no significant interaction between trial and MSF.

The amount of time to complete the maze (Fig. 2 panels B and D) shows similar results to those shown for errors, with no significant MSF main effect displayed [$F(1,97)=1.98$, $p=0.162$], nor with any significantly interaction. No gender main effect was observed ($F(1,97)=3.86$, $p=0.54$). Analysis of trials showed a significant trial effect ($F(7,91)=9.91$ $p<0.01$) with a significant linear ($p=0.047$) but not quadratic ($p=0.639$) component. Also a trial–gender interaction was found ($F(7,97)=2.67$, $p=0.02$). Separating the results by sex revealed that the effects were restricted to males and, in fact, within that sex, the second trial is the significant one ($p=0.024$), eta squared is 0.039 indicating a small to medium effect size of the interaction.

3.1.3. Swim escape

A main MSF effect ($F(1,110)=3.83$, $p<0.05$) was found in the animals performing this task assessed by escape's time. When data was examined by gender showed that the significant MSF effect was limited to females ($F(1,43)=9.973$, $p=0.0021$), (males $p=0.259$) (Fig. 3 panel B). No significant gender effects or gender–MSF interaction was reached. In addition, a significant trial effect was found ($F(2,109)=125.52$, $p<0.001$) with both linear ($F(1,110)=166.29$, $p<0.001$) and quadratic ($F(1,110)=30.27$, $p<0.001$) significant effects. No trial–gender or trial–MSF interactions were found.

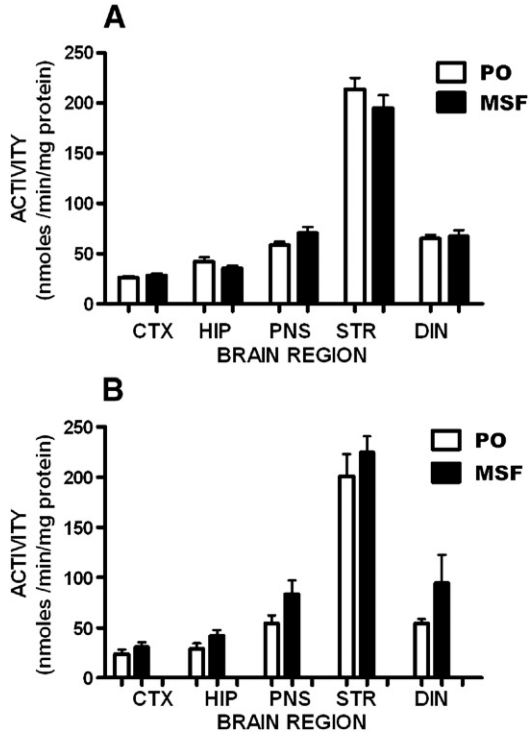

Fig. 6. Effect of in utero MSF exposure on AChE activity in males (A) and females (B). Panels A and B show the AChE activity found in the synaptosomal fraction in five brain regions: cortex (CTX), hippocampus (HIP), pons (PNS), striatum (STR) and diencephalon (DIN). Each bar represents the mean ± SEM of acetylcholinesterase activity.

3.1.4. Elevated plus maze and the open field test

When compared to their respective controls both males and females exposed to MSF spent equal amounts of time in the open and closed arms of the elevated plus maze as revealed by the ratio of time (±SEM) spent in open to closed arms for each group (MSF males 0.94±0.10 vs. 0.70±0.15 of PO males and MSF females 1.07±0.20 vs.0.93±0.10 of PO females), no differences between genders were noticed.

Similarly, no differences related to gender or in utero MSF exposure were found in the total number of crossings between the edge and middle quadrants of the open field test when compared to controls (MSF males 18.63±1.41 vs. 15.50±2.01 of PO males and MSF females 18.62±1.66 vs.15.89±1.38 of PO females).

3.2. Sleep experiments

As shown in Figs. 4 and 5, in utero treatment with MSF made no major changes in wakefulness, drowsy state, or slow wave phases of consciousness (Fig. 4, panels A, B, and C, respectively). However, a significant increase in REM sleep (T=2.991, df=13, p=0.0104) (Fig. 4, panels D) occurred primarily at the expense of drowsy and slow wave sleep (SWS) but not wakefulness. The increase in REM sleep was due to the occurrence of more REM sleep bouts (T=2.334, df=13, p=0.0363), and not due to an increase in the mean length of the REM sleep bouts nor as a shortening of the REM sleep latency (Fig. 5).

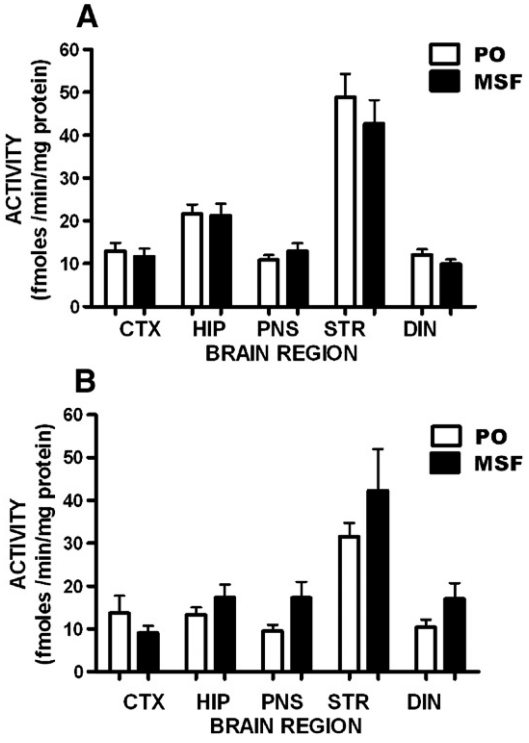

Fig. 7. Effect of in utero MSF exposure on ^{3}H–Ch uptake. Panel A shows the ^{3}H–Ch uptake found in males in five brain regions: cortex (CTX), hippocampus (HIP), pons (PNS), striatum (STR) and diencephalon (DIN). Panel B shows the ^{3}H–Ch uptake found in females in the same five regions. Each bar represents the mean±SEM of 11 control male rats, 12 MSF-exposed male rats, 10 control female rats, and 10 MSF-exposed female rats.

3.3. Biochemical experiments

3.3.1. Proteins

No differences between MSF-exposed and control rats of either gender were found with regard to the amount of protein or weight in the brain regions studied (cortex, hippocampus, pons, striatum and diencephalon).

3.3.2. AChE

Results from the AChE measurements indicate that were no significant changes in the level of this enzyme in any of the brain regions from in utero exposure to MSF by either males or females (Fig. 6).

3.3.3. Choline uptake

No differences were found in the transport rates of either gender of drug exposed rats as compared to controls (Fig. 7).

3.3.4. ChAT activity

There were no differences in the level of ChAT activity in the total tissue or the synaptosomal fractions of the various brain regions investigated in males (Fig. 8). Although not statistically significant, the enzyme levels in the pons, hippocampus, and striatum of MSF-exposed male rats were higher and almost reached significance. MSF-exposed females, however, showed significantly increased levels of ChAT only in the synaptosomal fraction of the hippocampus (Fig. 8).

4. Discussion

Previous studies and from our own results suggest that learning and memory are affected in the pups of MSF-treated mothers, suggesting changes at the level of the hippocampus, the main structure involved in these spatial functions. However, it was interesting to note that there was a deficit in learning and memory in MSF-exposed rats of both sexes when the motivation was for food reward, as measured in the 8AFM a spatial short (working) memory task in order to detect hippocampal damage (Barnes et al., 2000; Gerlai, 2001; Vann et al., 2000). In contrast, a better or equal performance of the MSF-exposed rats was observed when the stimulus was aversive (8AWM). One of the differences between the 8AFM and the 8AWM is that these two tasks require different levels of motor activity, motivation, and attention processing (Berger-Sweeney, 2003). The tasks also differ with respect to the degree of anxiety that is produced, while the 8AFM hardly generates anxiety in the animals, the 8AWM naturally produces a moderate degree of anxiety, as is corroborated by the attempts of the experimental animals to escape the maze. Furthermore, the water maze requires that the animal makes a spatial map with the external cues, while rats in the food maze rely not only on external cues but also odors and other intra-maze clues (Berger-Sweeney, 2003).

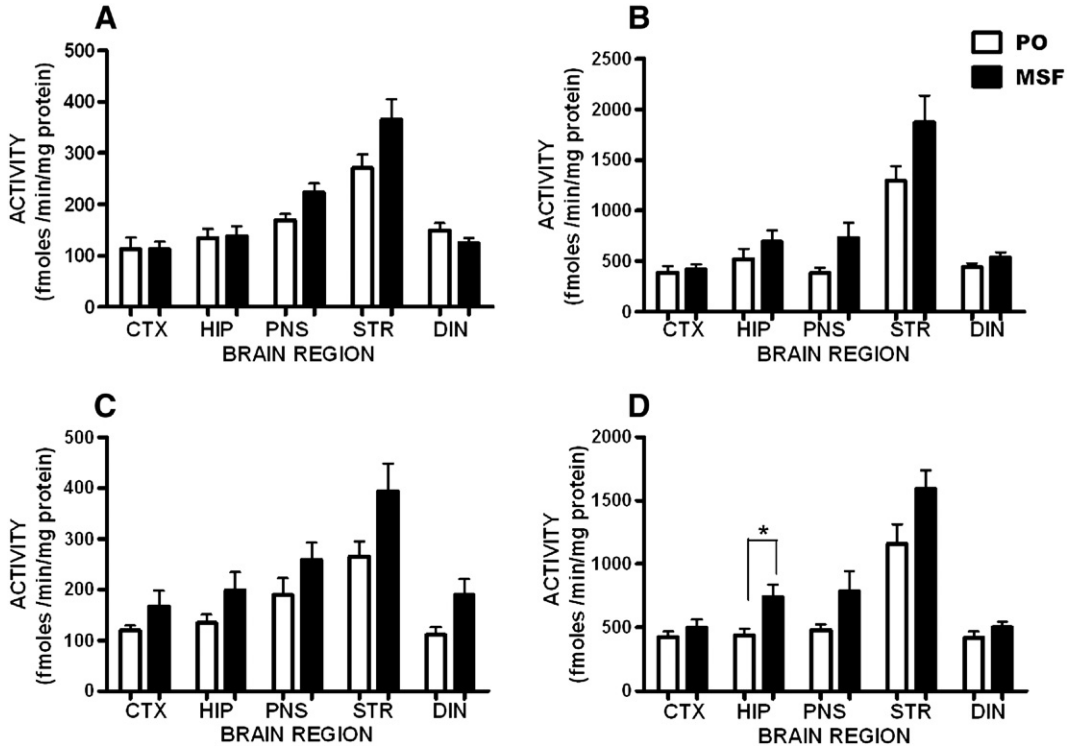

Fig. 8. Effect of in utero MSF exposure on ChAT activity in males (A and B) and females (C and D). Panels A and C show the ChAT activity found in total tissue homogenates in five brain regions: cortex (CTX), hippocampus (HIP), pons (PNS), striatum (STR) and diencephalon (DIN). Panels B and D show the ChAT activity found in the synaptosomal fraction in the same five regions. Each bar represents the mean ± SEM of 7 control female rats and 7 MSF-exposed female rats (*$p < 0.05$).

Prior studies linking cholinergic function to anxiety levels (Srikumar et al., 2006; Degroot and Dallas, 2002; Millan, 2003; Sienkiewicz-Jarosz et al., 2000) initially led us to hypothesize that the different effects of in utero MSF exposure as shown in the 8AFM vs. the 8AWM may have been due to an altered stress response, in this case a blunted response to anxiety when exposed to noxious stimuli. Clearly we found no such change in the open field maze or the elevated plus maze, though the animals were tested only at basal levels of anxiety and not in responses to induced stress (e.g., 8AWM). It may be necessary to conduct future studies using several anxiety paradigms to create an anxious state in the animal (conditioned anxiety assessment) and compare performance in the 8AWM.

The swim escape test developed like an alternative to shock avoidance/escape procedures (Barraco and Klauenberg Irwin, 1978) is different from the 8AWM since it involves only a one trial learning strategy and only internal cues. However, the results show basically the same response as the 8AWM, although in this case female MSF-exposed rats showed significantly better performance. The SE task is simpler than the 8AWM and it could allow, like inhibitory avoidance tasks does (Izquierdo et al., 2002), for a clearer way to observe the difference on learning ability. Therefore, even when a single exposure to a swim tank can induce stress in rodents it may decrease potential interference from different elements (such as changing levels of catecholamines and corticosterone) caused by the stress of repetitive exposure to the maze. If an anxiolytic tendency were produced by in utero MSF exposure, this might be a better test paradigm than the 8AWM.

Although rats exposed to MSF in utero perform better under aversive conditions in trials of learning and memory, the results from the EPM, a reliable paradigm used to assess anxiety (Ohl, 2003), indicate that MSF-exposed rats are not different from controls. Also the failure to find differences in the EPM suggests no differences in psychomotor ability or in the basal level of anxiety. The open field test confirmed these results as no differences were found in the total number of crossings between the edge and middle quadrants. These results indicate that MSF-exposed rats of both genders have an equal level of anxiety as the controls, at least under these baseline conditions.

Sleep behavior was studied because it is well known that REM sleep mechanisms have a precise cholinergic regulation in the pons and the participation of different neurotransmitters is well defined, and secondly, is subject to less variations, under basal conditions. Sleep behavior, therefore, allowed study of potential changes in cholinergic function in an alternative brain area not directly associated with learning and memory.

As might be expected, there were no major changes in wakefulness, drowsy state, nor slow wave phases of consciousness since the regulation of these stages is controlled by a dynamic interaction of several other neurotransmitters besides ACh, like serotonin, dopamine, and catecholamines (Lechin et al., 2004; Zoltoski et al., 1998). However, the data for REM sleep was different. The results clearly show changes in REM sleep. There was an increase in the amount of REM sleep (during the light phase) and an increase in the number of REM bouts (while the mean length of REM sleep bouts remained unchanged). These results might be explained by a

hypercholinergic effect in the pons and are consistent with the notion that in utero MSF exposure increases the cholinergic tone of certain pontine areas that regulate REM sleep.

Since MSF is primarily an irreversible inhibitor of AChE, measurement of the effects of in utero MSF exposure on adult levels of this enzyme were of interest. Our measurements of AChE activity were restricted to brain areas known to have a good concordance between cholinergic innervation and AChE, measures of AChE activity may be indicative of the general cholinergic status of a tissue. According to our behavioral results, we were essentially expecting changes in cholinergic markers at the level of the pons and the hippocampus but not in the diencephalon, since no change in the parameters measuring hypothalamic function were found (food consumption, body weight gain, basal metabolism, and hypothermic responses to cholinergic agonists) during previous studies in our lab (Sierra, 2004). We also did not expect any changes in the striatum, although having substantial cholinergic activity, given that there were no obvious changes in locomotor activity. However, some changes in the cortex could be anticipated due to gender differences observed in cortical development (Favela et al., 2003). Nevertheless, there were no significant differences in the levels of AChE activity in any brain regions studied.

An increased cholinergic tone may result from presynaptic changes, possibly through increased ACh release or an increase in cholinergic terminals. Therefore, choline uptake was measured to assess presynaptic function. Because a good correlation exists between the level of choline transport and the level of ChAT activity, this enzyme was also assessed. The results of choline reuptake studies were similar to the results for AChE activity where no differences were found. It was interesting, however, the choline reuptake measures did not correlate well with ChAT activity where higher levels were found in drug exposed hippocampi from females (but not in males or other brain areas). The meaning of these findings is not clear. It is known that ChAT levels are notably decreased in a number of pathologies, such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, schizophrenia and sudden infant death syndrome (Oda, 1999), but an increase of ChAT activity is not a consistent change connected to a particular physiologic state or pathology, although some reports mention that enhanced acetylcholine synthesis accompanies learning (Oh et al., 1996; Park et al., 1992). However it has been reported that MSF treatment – after experimentally induced stroke in rats – showed more intense staining of septal ChAT-immunoreactive neurons correlating with a better response in learning and memory tasks (Borlongan, Sumaya and Moss, 2005). These findings suggest that MSF, probably by cholinergic system's enhancement, lessened stroke-induced deficit in learning and memory. Whether this conservation of cholinergic functions could be related to ChAT's increase observed in our study remains to be clarified; on the other hand this study is important since it supports the idea that measurement of cholinergic markers proximal to MSF treatment in contrast with our long-term measurements, could be a different approach to more clearly elucidate the connection between MSF and brain cholinergic system.

Summarizing, the present experiments, aimed to observe on adult rats the effects of in utero MSF treatment on behavior and cholinergic markers, showed changes at several levels (physiologic, biochemical and morphologic) that have been attributed to a higher cholinergic tone; however current results suggest that in utero MSF exposure does not translate to long-lasting cholinergic changes in the adult brain and thus an explanation of such changes deserves further examination that considers the participation of postsynaptic mechanisms, measuring of cholinergic markers during earlier development periods and even the possible contribution of other neurotransmitter systems.

5. Conclusions

The characterization of rats with in utero MSF exposure presents a complex task, mainly when the integration of the physiological, behavioral and neurochemical findings is attempted. However, a couple of conclusions can be drawn: 1) neurochemical changes in these animals deserve even more detailed and extended studies. The presence of few statistically significant changes, associated with a strong tendency to high levels of ACh in different brain areas suggest that even more extensive studies should be done, including evaluations of postsynaptic measures as muscarinic and nicotinic receptors; and, 2) although rat performance in the swim escape task and in the radial-arm water maze could suggest a blunted response to anxiety, the results of performance in the open field and the elevated plus maze did not support this hypothesis, indicating that MSF-exposed rats have an equal level of anxiety as the controls, at least under baseline conditions. Further research using conditioned anxiety assessment might help elucidate this problem.

Summarizing, in utero MSF-treated animals show a series of changes at several levels (physiologic, biochemical and morphologic) that have been attributed to a higher cholinergic tone, however such changes, still have not been conclusively demonstrated and thus, the possible participation of other neurotransmitter systems must be tested.

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