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# Sex Related Differences in Rat's Behavior and Brain Morphology After the Toxic Effect of Manganese

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**Abstract**

Exposure to heavy metals is a common phenomenon, which is due to contaminated drinking water and industrial activities. The toxic effect of different forms of metals is not only related to the environment, but also related to the metabolism and detoxification mechanism of the organism. In order to explore the difference of manganese toxicity on male and female rats, we studied the impact of manganese compounds on rat behavior and brain morphology. Four-week-old Wistar rats with body weight between 80-120g (n=42, including 21 males and 21 females) were studied. Wistar rats were assigned to four groups: rats in control groups (male, female) were given regular water, while rats in other groups drank water with final manganese concentration of 15 mg/ml (male, female) for three months. To study exploratory and anxiety behavior rats were tested in open field, home cage and elevated plus maze. To estimate learning and memory status multi-branched maze was used. The behavioral disturbance of male rats was more noticeable than that of female rats in the same group. It is found that excessive manganese ions also had more toxic effects on male animals than females. It was revealed that manganese poisoning increased Mn contents in the brains of both genders, caused slight damage of neurons, and produced notable gliosis. However, in hippocampus there were bioaccumulation differences between gender. Excess amount of manganese in the brain had a strong impact on learning processes. It was concluded that, under this experimental design, Mn exposure causes metal deposition in CNS. The effect of this dose of manganese was gender-dependent and males had more pronounced behavioral damage compared to females, but females had an indication of motor damage. Gender differences in neuron morphology were not due to differential accumulation of Mn in brain regions. But the effect of manganese exposure was not similar in all observed brain regions (Motor Cortex, Prefrontal Cortex, Hippocampus-CA1, CA3 area and Dentate Gyrus).

***Keywords:*** manganese; rat; behavior; neurotoxicity; gender.

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## 1. Introduction

Manganese (Mn) is an essential metal found in abundance in the environment and is necessary for numerous biochemical processes in the human body. Its function arises secondary to its inclusion in protein structures as a cofactor. Without its presence, the immune function of the human body, biochemical regulation of energy consumption, growth potential, coagulation and hemostatic function, as well as the mechanisms for removing the by-products of aberrant oxidative stress would be significantly weakened [1].

Optimal nutritional sources of Mn come primarily from plants, but supplementation with vitamins or healthy food is another important contribution. Drinking water often contains trace amounts of Mn with defined thresholds measured to prevent toxic effects. Another beneficial source of manganese for newborns is breast milk and formula, which prevents manganese deficiency during the critical period of infant development [2,3].

Manganese is an essential element, but excessive exposure to manganese is toxic. The main toxic effects associated with this metal are extrapyramidal side effects. Those disturbances closely resemble the symptoms of Parkinson's syndrome. These side effects are due to the changes in its deposition in certain components of the basal ganglia and changes in the activity of dopaminergic neuronal enzymes. Other notable effects include cardiotoxicity, hepatotoxicity, and increased infant mortality [4,5].

Sex differences in the prevalence, severity and progression of diseases of the nervous system are becoming increasingly important in biomedical research, motivated by gender-dependent behavior and physiology. Substantial evidence from recent neuroimaging studies supports gender differences in the structure and function of the human brain, starting in utero and continuing throughout the life. Long-term studies demonstrate persistent sexual dimorphism in brain trajectories, especially in the early adolescence. Endogenous developmental programming, developmental experience and/or environmental influence may become one of the reasons of arising sex differences in brain development. Sex and gender-related behavioral differences in infancy and childhood have been reported, which are associated with differences in early exposure to chemicals. A better understanding of gender-specific differential vulnerability to neurotoxicants may provide insight into sexual brain dimorphism, behavior, mental health, and mental disorders [6].

Studies of the population not exposed to high Mn levels have shown conflicting results. According to some studies men have decreased absorption of Mn from the gastrointestinal tract, lower ferritin levels, and slower Mn clearance than women. Several studies show that, in a healthy, unaffected population, serum manganese levels are higher in males than in females [7].

However, there are some more resent reports showing no gender difference in baseline serum manganese levels. One recent study examined the intellectual function of children living in the Mexican mining region of Mn. Children exposed to Mn had higher levels of Mn in their hair and blood (20 ×) than children not exposed to high levels of Mn. Girls exposed to manganese performed worse on the revised Wechsler Intelligence Scale for Children than boys exposed to manganese. Although there were no gender differences in Mn levels in exposed children, this suggests that cognitive ability may be affected differently in boys and girls. Gender differences are

not identified and unique to Mn. Human studies have also found gender differences in the storage and sensitivity of other metals. Similar features are indicated for cadmium (Cd), lead (Pb), Mn, mercury (Hg), and nickel (Ni) [8].

Animal studies also show gender differences in toxicokinetic and metal sensitivity. For example, male and female rats accumulate Mn differently in body tissues after exposure to Mn by inhalation; however, there were no differences in Mn accumulation in the striatum or other regions of the basal ganglia. Gender differences in toxicokinetic after a single oral dose of methylcyclopentadienyl manganese tricarbonyl (MMT) gasoline supplement showed that female rats accumulate higher MMT levels than male rats, due to slower MMT clearance. Erickson and his colleagues (2004a) exposed young rats to manganese by air for 13 weeks and found gender-specific changes in glutamine synthetase protein and mRNA expression, metallothionein mRNA, and glutathione levels in many areas of the brain. Gender differences in the toxicokinetic of Mn are present regardless of the route of administration and the type of Mn. Like humans, rodents show gender differences when exposed to other metals such as Pb and Hg [7].

We decided to evaluate potential gender-related differences in response to Manganese exposure.

## 2. Materials and methods

### *Animals and experimental design*

42 Wistar rats - 21 females and 21 males (P-28-30 at the initial day of experiments) were used in our experiment. Rats in control groups drank regular water, and rats in experimental groups got water containing Mn in dose 15 mg/ml (Sodium arsenite (NaAsO2) purchased from Sigma-Aldrich Cat. N S7400) for 3 months.

Rats were kept on regular light/dark cycles throughout the procedures with ad libitum access to food. Care of animals during/after procedure(s) animals were transferred to the testing room and allowed to adapt to this room prior to testing. Animals were monitored while in the arena and returned to the home cage immediately after testing.

All experimental procedures were approved by Animal Studies Committee of Georgian I.Beritashvili Center of Experimental Biomedicine and are in accordance with guidelines of the EC Ethical Directives.

### *Behavioral tests*

Animals were subjected to following behavioral tests:

### *Open field test*

This test is used to evaluate the exploratory and anxiety behavior of rat.

Equipment: The Open Field is a circular arena with a diameter of 100 cm with walls 30 cm high. The floor is divided up into quarters with a central circle of 33cm diameter in the middle of the arena. Normal laboratory up

lighting is required. Each animal is placed in the central circle and is observed for 5 min. The following events are recorded: ambulation - the number of grid lines crossed with all four paws; number of entries into the center of the open field; rearing – the number of times the animal stood on its hind limbs; hole reflex – looking into holes; grooming and defecation.

***Elevated Plus Maze (EPM)***

The EPM is a widely used rodent behavioral test that is utilized to assess anxiety-related behavior. The EPM apparatus consists of four arms: two open, and two closed arms, the apparatus is elevated 50–70 cm from the floor. Each arm is 50 cm long and 5 cm wide, and the closed arms were shielded by 25 cm high side end walls. The four arms were linked at a central square (the junction). Briefly, rats are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer simultaneously for 5 min. Other ethological parameters (i.e., rears, head dips and stretched-attend postures) can also be observed. An increase in open arm activity (duration and/or entries) reflects anti-anxiety behavior. In our laboratory, rats are exposed to the plus maze on one occasion; thus, results can be obtained in 5 min per rodent.

*The Resident-Intruder - RI test* (20) was used to determine the level of animal intraspecific territorial aggression. The test consists of the following: 1 weeks before testing the rat (so called "resident") is placed in the experimental chamber (40x24x35 cm) and after 30 min the other rat (so called "intruder"), weighing 20-30 grams less than the "resident" is placed into the chamber. Duration of the test is 10 minutes. The aim of the test is to record the following indices: First attack latency time, Number of attacks, Duration of attacks, Lateral threat, Offensive upright and keep down, Clinch, Chase/flight, Investigating opponent, Ano-genital sniffing, Grooming, Exploration.

***Learning processing multi-branched maze***

The process of learning is studied by means of multi-branched maze consisting from footbridges, mounted on props of 30 cm height. To move on an optimum trajectory animal is learning by a trial-and-error method. With the purpose of adaptation, all groups of animals for a few days are placed in a nest-box prior to the beginning of experiments. This nest-box is located at the exit platform of maze. At the beginning of the experiment an animal is placed on the start-platform and it has to find out the correct way to the nest-box. Process of learning proceeds without any food reinforcement and it can be described as follow: each passage of the crotch (when the animal has an opportunity of a choice of a direction) serves as a stimulus for further movement. Getting in a deadlock branch of the maze (error) and necessity of returning should be considered as a punishment for error and, probably, forces rat to avoid errors and to continue search for a right direction. It is possible to consider deliverance from nonethologic conditions (being on a maze platform) at the moment of hit in a nest-box as a reward and it serves as a motivation for maze learning. Each group of animals during 10 days was trained to pass a maze. The maze task was presented to each animal 5 times a day with at least 30 min intervals. The learning process is estimated by maze test performance (to reach the nest-box) within 5 min – by number of errors made during ambulation through the maze (enter into the blind-alley section) and by the time of maze

passage. For estimation of learning process, Mn was given to experimental animals for 3 months before the maze test. For evaluation of memory tests, animals were subjected to maze session 2 months later after the termination of the learning test.

***Nissl staining***

Animals were deeply anesthetized with ketamine (100mg/kg) and then perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Excised brains were post-fixed at 4°C in the same fixative for another 24 h and then cryoprotected in a 30% sucrose-solution. For Nissl staining 15-µm coronal sections were cut on a cryostat (Microm HM 500 M). Every 6th section was collected and mounted on a poly-L –lyssine coated glass slides. The slides were remained to dry, rehydrated with 100% alcohol, 95% alcohol, and distilled water. Subsequently, the sections were stained in 0.1% Cresyl violet (Sigma-Aldrich, Cat. N C504 solution). The sections were then differentiated in 95% ethyl alcohol, dehydrated in 100% alcohol, and rinsed in xylene. Finally, the sections were mounted and observed under a light microscope (Leica DM LB). Cell counting in hippocampal CA1, CA3 areas, Dentate Gyrus, prefrontal and motor cortex was conducted blindly. For this purpose, the systematic random sampling was employed. The 2-dimensional counting grid (250 µm x 250 µm) at the magnification 400x was used. Totally 10-12 sections from each level within experimental and control animals were selected (35 randomly chosen range of visions at the same site of all sections from each animal).

***Statistical analysis***

Statistical analysis was performed using GraphPad Prism version 7.00 La Jolla California USA. Behavioral data from the different trial days were analyzed using repeated measures one-way ANOVA followed by post hoc Tukey's multiple comparison tests. Unpaired t-test with Welch's correction was used to determine a statistically significant difference between the two groups. $P<0.05$ were considered statistically significant. All data are presented as Mean ± SEM.

**3. Results**

Obtained data support the hypothesis that excess manganese in the body is one of the immediate causes of enhancement of interspecific predatory aggressive and violent behavior in rats.

Aggression increased in 37.5 percent of female animals and 42 percent of male animals (Fig.1). It should also be noted that, aggressive attacks in male rats were more pronounced and strongly expressed.

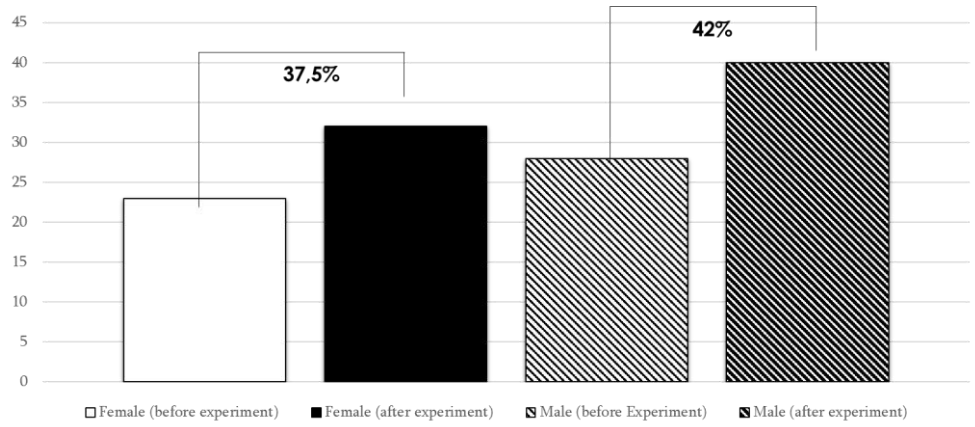



**Figure 1:** Increased aggression rate in females and males.

The open field test is a straight-forward test to investigate activity, anxiety-related behavior, and exploratory behavior in rodents. According to the crossed lines, which is an indicator of locomotor activity, the decrease in activity was more noticeable in females than in male rats (Fig.2).

The number of entries in the center shows the level of emotional tension. Female rats showed much lower levels of emotional tension than male rats, which exhibited higher levels of anxiety (Fig.3).

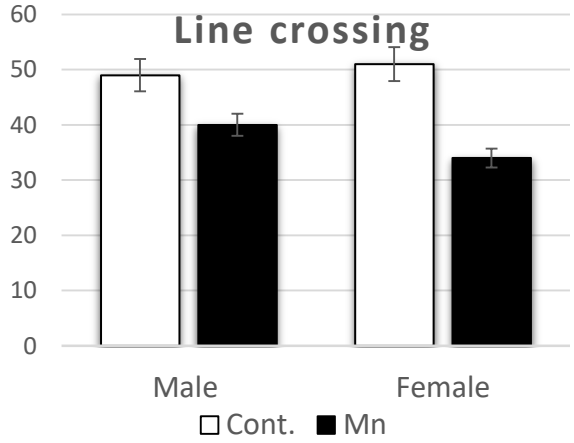



**Figure 2:** Number of line crossing in open field.

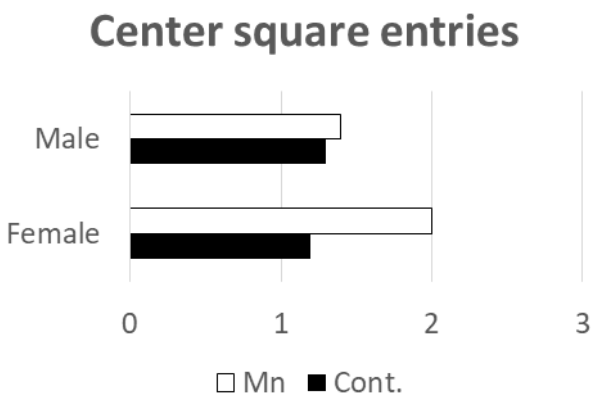



**Figure 3:** Number of entries in Center of open field.

In addition, grooming duration and frequency were increased by 20-30% in animals of both sexes. However, defecation rate was not significantly different from that of control animals.

The Elevated Plus Maze (EPM) test is used to assess anxiety-related behavior in rodent models of CNS disorders. The preference for being in open arms over closed arms (expressed as either as a percentage of entries

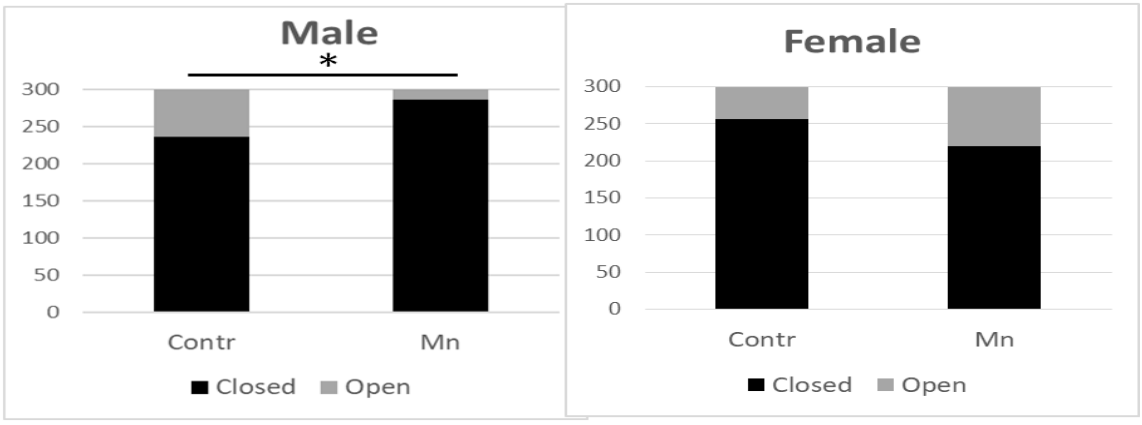
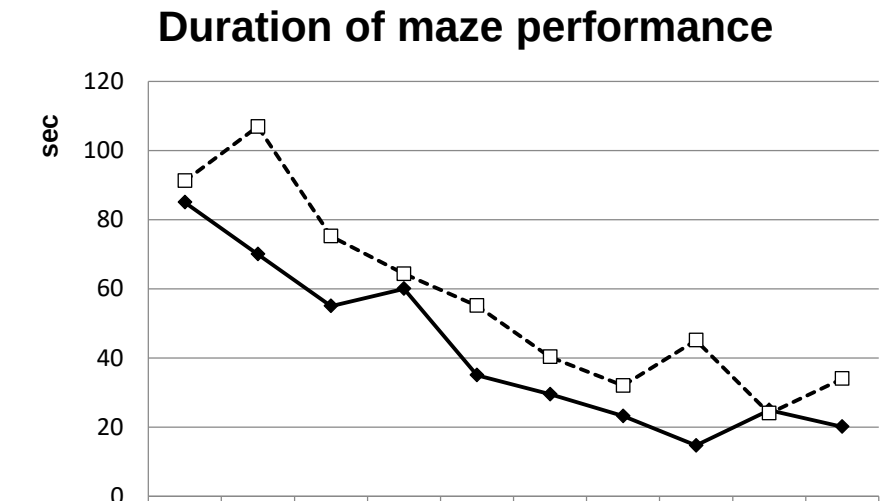



**Figure 4:** Results of the Elevated Plus Maze.

and/or a percentage of time spent in the open arms) is calculated to measure anxiety-like behavior.

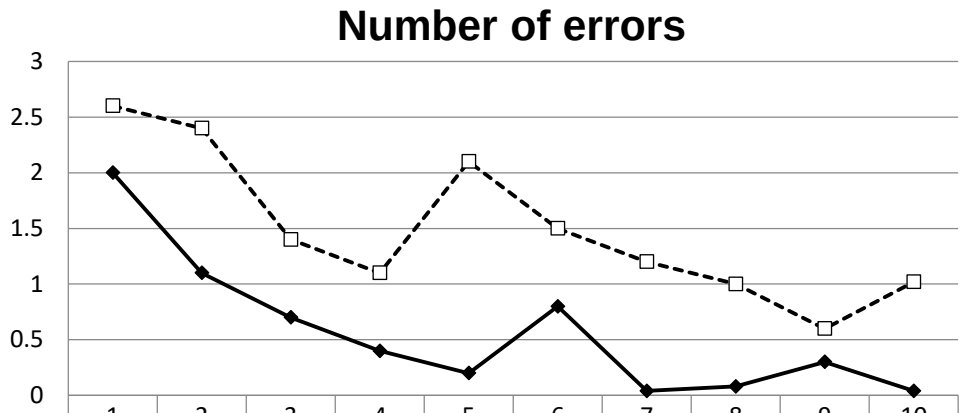
Male rats showed a higher anxiety-like response to manganese as evidenced by: lower number of entries into the open arm of the EPM and less time spent into open arms. It should be noted that manganese exposed male rats spent statistically significant more time ($P<0.05$) in the closed arms than control rats. On the contrary, female rats spent more time in open arms compared to control rats, although this difference was not statistically significant (Fig.4).

As for the evaluation of the learning process in the multi-branched maze, manganese exposed rats lagged behind control animals in maze performance, but this impairments were different in female and male rats. Male rats made more mistakes in the learning process than control ones, and at the end of the session they could not learn to pass the maze without errors (Fig.5,6). The female rats in the last days of the labyrinthine session almost reached the same rate as the control animals, but there was a difference in the time required to cross the maze (Fig.7,8). They needed about twice as much time to complete the task as control ones. If control rats needed less than 20 seconds at the end of the session, manganese exposed rats needed more than 40 seconds. These values were maintained for 2 months since the memory test was performed.



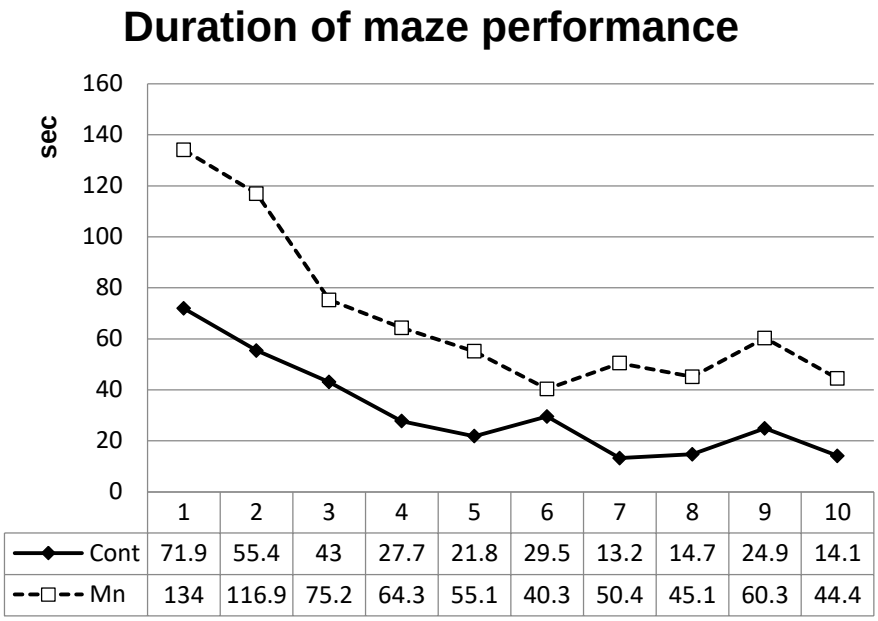

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Cont | 85 | 70 | 55 | 60 | 35 | 29.5 | 23.2 | 14.7 | 24.9 | 20.1 |
| Mn | 91.2 | 106.9 | 75.2 | 64.3 | 55.1 | 40.3 | 32 | 45.1 | 24 | 34 |

**Figure 5:** Duration of multi-branched maze performance in male rats.



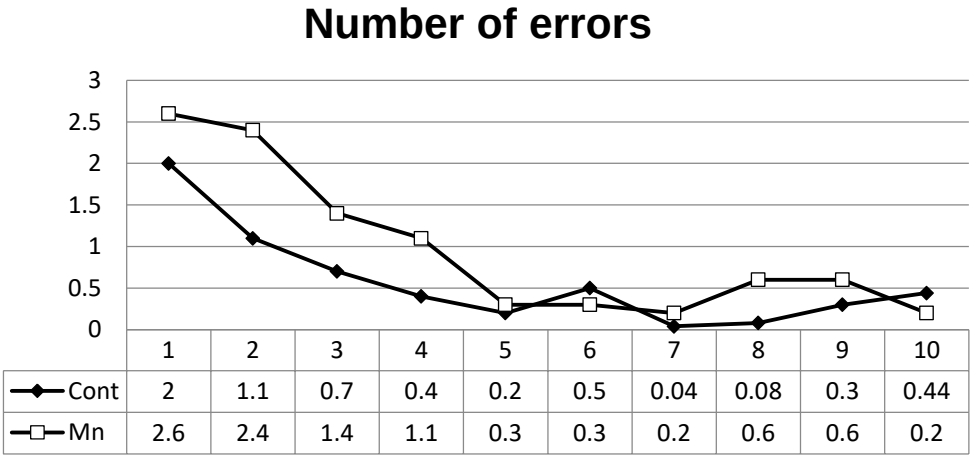

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Cont | 2 | 1.1 | 0.7 | 0.4 | 0.2 | 0.8 | 0.04 | 0.08 | 0.3 | 0.04 |
| Mn | 2.6 | 2.4 | 1.4 | 1.1 | 2.1 | 1.5 | 1.2 | 1 | 0.6 | 1.02 |

**Figure 6:** Number of errors in multi-branched maze made by male rats.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Cont | 71.9 | 55.4 | 43 | 27.7 | 21.8 | 29.5 | 13.2 | 14.7 | 24.9 | 14.1 |
| Mn | 134 | 116.9 | 75.2 | 64.3 | 55.1 | 40.3 | 50.4 | 45.1 | 60.3 | 44.4 |

**Figure 7:** Duration of multi-branched maze performance in female rats.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Cont | 2 | 1.1 | 0.7 | 0.4 | 0.2 | 0.5 | 0.04 | 0.08 | 0.3 | 0.44 |
| Mn | 2.6 | 2.4 | 1.4 | 1.1 | 0.3 | 0.3 | 0.2 | 0.6 | 0.6 | 0.2 |

**Figure 8:** Number of errors in multi-branched maze made by female rats.

Manganese ion levels were estimated by atomic absorption spectrometry. Specimens were carbonized by moist mineralization. After measuring the optical densities of a sample and standard solutions of different concentrations, manganese contents were read from the resulting curve. These data are given in the tables (Table1 and Table 2):

**Table1:** Manganese ion accumulation in different brain regions of male rats, *p<0.05.

| Brain region | Control | $MnCl_2 \cdot 4H_2O$ |
|---|---|---|
| **Neocortex** | **0.028±0.001** | **0.083±0.037*** |
| **Striatum and Diencephalon** | **0.051±0.025** | **0.063±0.005** |
| **Mesencephalon and Medulla Oblongata** | **0.029±0.005** | **0.036±0.002** |
| **Hippocampus** | **0.077±0.002** | **0.420±0.021*** |

**Table2:** Manganese ion accumulation in different brain regions of female rats, *p<0.05.

| Brain region | Control | $MnCl_2 \cdot 4H_2O$ |
|---|---|---|
| **Neocortex** | **0.030±0.001** | **0.081±0.025*** |
| **Striatum and Diencephalon** | **0.049±0.025** | **0.061±0.005** |
| **Mesencephalon and Medulla Oblongata** | **0.030±0.005** | **0.033±0.001** |
| **Hippocampus** | **0.073±0.002** | **0.411±0.015*** |

Based on the obtained data, it is obvious that highest accumulation of manganese ions was in the hippocampus and the neocortex. This applies to both female and male rats.

To evaluate morphological alterations - we counted the number of neurons in these areas. Cell counting in hippocampal CA1, CA3 areas, Dentate Gyrus, prefrontal and motor cortex was conducted. Statistically significant decrease of neurons was noticeable in the CA3 field, but this only applied to male rats (Fig.9, 10). A statistically significant decrease was also observed in Dentate Gyrus in both female and male rats (Fig.11, 12). As for the neocortex, there was a decrease in both - prefrontal and locomotor cortex, but this decrease was not statistically significant.

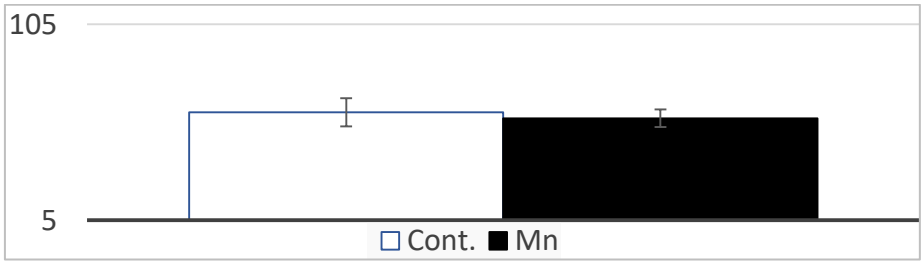



**Figure 9:** Results of the CA3 subregion (control and manganese exposed female rat's brain).

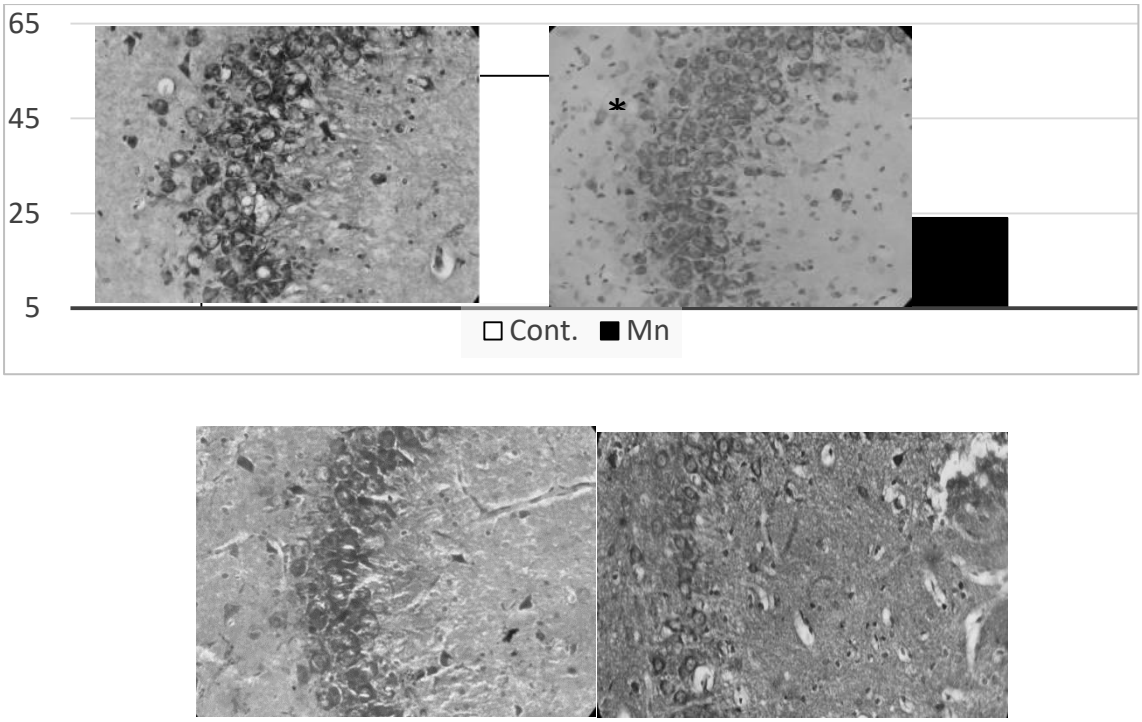



**Figure 10:** Results of the CA3 subregion (control and manganese exposed male rat's brain).

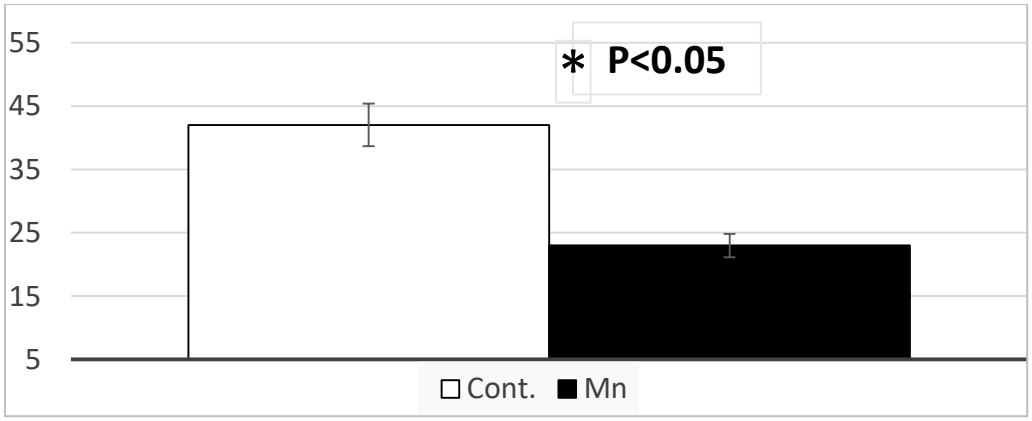



**Figure 11:** Results of the CA3 subregion (control and manganese exposed male rat's brain).

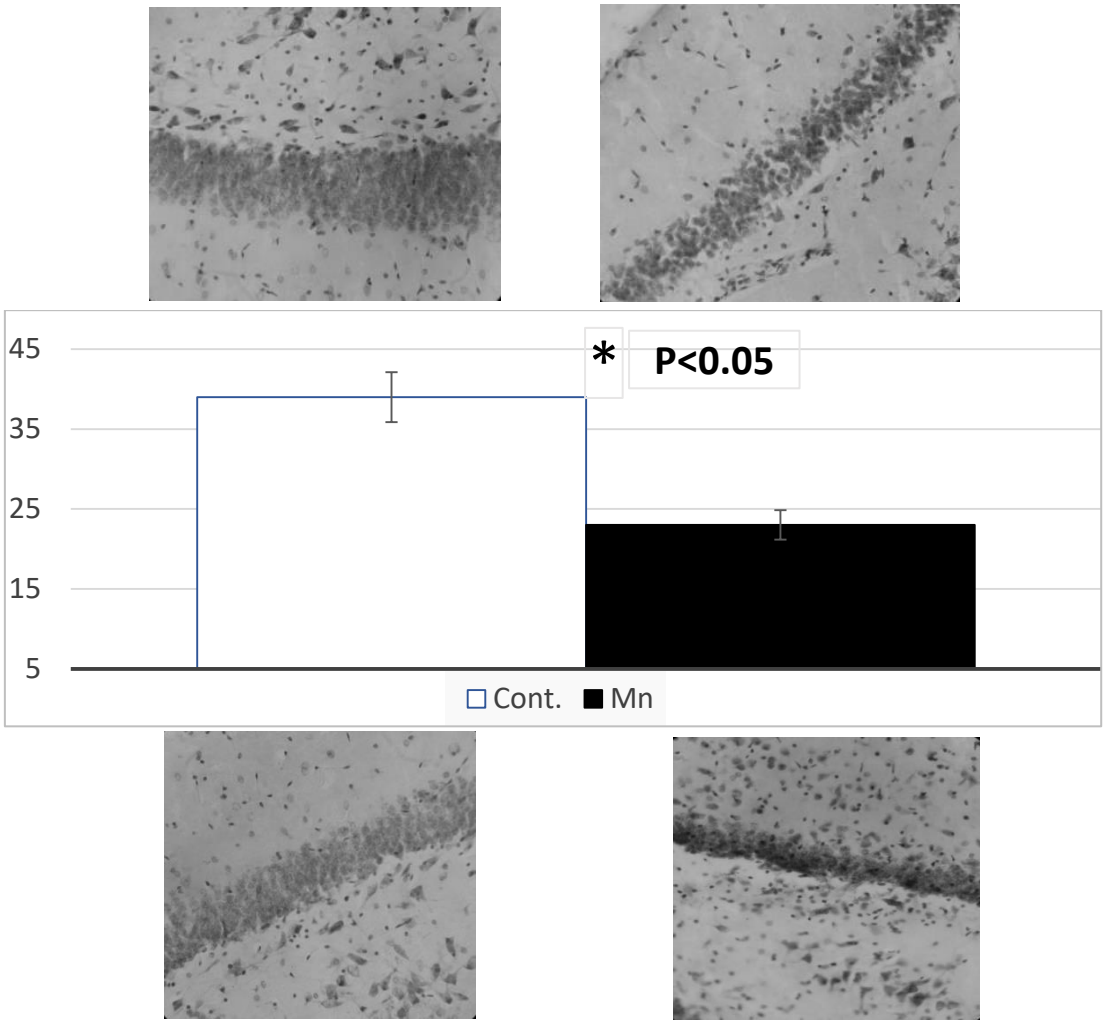



**Figure 12:** Results of the the dentate gyrus subregion (control and manganese exposed female rat's brain).

## 4. Discussion

The results of the present study indicate that manganese compounds causes different anxiety-like responses in male vs. female rats. Thus, male rats appear to be more responsive and show a greater degree of anxiety-like behavior following the exposure to manganese ions. This anxiety like behavior was reflected in lower number of entries into the open-arm maze of the EPM, lower number of entries into central zone of OF. Avoidance of entry into the open arm of EPM or central zone of OF are common indication of an anxiety response. Our findings confirm the existence of sex-dependent differences following the exposure to manganese.

Numerous studies have confirmed sex or sex-related differences in the expression of emotional behavior.

For Parkinson's disease, gender differences are described in terms of disease spread, age of onset, and progression. It is known that the symptoms of the disease are less pronounced in women. But in the study of manganese neurotoxicity little attention has been paid to this issue. This has not been the main focus in the field of manganese toxicology.

It is well known that studies on animal models are often conducted to study Parkinson's disease, and these studies have shown that gender differences play an important role in the development of the disease. In particular, estrogen has been found to play a protective role in neuropathology and behavior. Survival of dopamine neurons after exposure to dopamine neurotoxic agents used in animal models of Parkinson's disease depends on the gender. For example, a study examining the gender impact of 6-hydroxydopamine (6-OHDA) lesions on behavior and neuropathology found that male rats were more sensitive to this toxic substance than female rats. Our studies also showed that male rats were more sensitive to manganese ions than female rats. Interestingly, male rats were more prone to depression, but in some situations they were more aggressive than female rats.

In addition, it is known that cases of depressive disorder, anxiety, inability to suppress fear are more common in women than in men. Two-thirds of patients with depressive or stress-related disorders are women. However, preclinical studies related to anxiety rates indicate a higher sensitivity to stress stimuli in males than in females. Experiments on rats have revealed a snumber of cases that adult female rats spend more time in the open arms of the cross-maze than adult male rats, which also occurred during our experiment. Thus, the difficulty is due to the fact that in many cases the results are contradictory and there is no clear conclusion about the direction of development of mental disorder.

It should also be noted that according to some data, sex influences the differences in the distribution of Mn in the body [9]. Women have a high prevalence of Mn levels in different countries, which may be related to the better food absorption. In our experiments we didn't find any noticeable differences between the levels of manganese ion accumulation in brain regions.

Based on the data obtained by our experiments, the prominent decrease in the amount of neurons was detected in the brain regions of male rats. While in females, neuronal loss was mostly observed among dopaminergic neurons, as females expressed significant impairments in locomotor activity. Scientific data indicates that Manganese ions target dopaminergic neurons, especially in the striatum and cause alterations in their morphology. The same scenario (damaged dopaminergic neurons) is characteristic to Parkinson's disease.

## 5. Conclusions

Intoxication with manganese compounds has a significant impact on the aggressive behavior and emotional state of individuals. Aggressive behavior and alterations in the emotional state is expressed in both male and female rats. Decreased locomotor activity is observed in female rats. Rats intoxicated with manganese ions lag behind control animals in learning ability. Disorders in the learning process are more pronounced in male individuals. Accumulation of manganese ions in areas of the brain is particularly pronounced in the hippocampus and cerebral cortex. Changes in the number of neurons in the hippocampus were observed in the CA3 field and the dentate gyrus.

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reality: The sustainability of scientific research during the Covid-19 pandemic"

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