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Impact of Degeneration of the Prelimbic and Infralimbic Cortices on Psychomotor Activity and Cognitive Function in Rats Stereotaxically Injected with Ibotenate

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Abstract

Background: **Little is understood about how mild cognitive impairment affects motor function (MCI). Memory and psychomotor functions are assumed to be regulated by the medial prefrontal cortex (mPFC). The mPFC consists of a total of three distinct subareas. Thus, the purpose of this work was to specifically damage the prelimbic (PrL) and infralimbic (IFL) subareas utilizing a rodent stereotaxis injection of a relatively low dose of ibotenate.** ***Aim:*** **to investigate the impact of the PrL and IFL subareas lesions on psychomotor activities and memory among stereotaxically injected rats with ibotenate.** ***Materials and Methods:*** **For this study, a total of 36 white albino rats were employed. Three groups were designed: the normal control group (GI), the bilateral sham group (GII) which included injected rats with phosphate-buffered saline (PBS) in the PrL and IL subareas, and the bilateral lesioned group (GIII) which included injected rats with ibotenate in the PrL and IL cortices. Then rats were undergone through the open field test (OFT) and hole board test (HB). Transcardial perfusion and brain extraction for tissue preparation and histological evaluation were done at the end of the experiment.** ***Results:*** **the group with bilateral PrL and IFL lesions had intact psychomotor functions and spatial reference memory, but they started to lose short-term memory.** ***Conclusions:*** **PrL and IFL subareas of the mPFC degeneration don't impair psychomotor functions or spatial reference memory but can impact short-term memory in rats.**

Keywords: mild cognitive impairment, medial prefrontal cortex, rodent stereotaxis, ibotenate

Introduction

Multiple memory systems that are independent of one another makeup memory. Non- declarative memory, also known as implicit memory, is a component of these systems and refers to knowledge of how to carry out an action. Additionally, declarative or explicit memory refers to factual knowledge of individuals, places, and things as well as the significance of this information. Semantic memory, which is a memory of facts, and episodic memory, which is a memory of events and firsthand knowledge, are further categories for declarative memory(1). Amnestic mild cognitive impairment (MCI) is a clinical diagnosis that describes elderly people who have short-term or long-term mem-

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ory impairment but no major daily functional impairments[2]. Within five years, 50% of MCI patients will develop Alzheimer's disease (AD)[3]. MCI has been associated with decreased functional connectivity between the posterior cingulate cortex (PCC) and medial prefrontal cortex (mPFC) at the cellular level[4]. Rodent mPFC shares a strong anatomical resemblance with primates' dorsolateral PFC (dlPFC), which is composed of the anterior cingulate, prelimbic (PrL), and infralimbic (IFL) cortices[5]. It seems that the mPFC exerts top-down executive control over a variety of cognitive processes and inputs and has substantial connections to the thalamus, amygdala, and hippocampus in subcortical areas[4]. However, there is limited agreement on how pathological situations directly alter the functional c onnectivity of the medial prefrontal cortex[4]. Recent studies indicate that patients with MCI have reduced psychomotor function, although it is unclear how this affects their risk of developing AD in the future[6]. Using digital stereotaxis tools, this study focuses on the PrL and IFL subareas of the mPFC. In multiple studies, stereotaxic surgery was used to create site-specific lesions, inject anatomical tracers, or implant electrodes or micro-dialysis probes. It has proven to be a great tool in neuroscience research[7]. Additionally, compared to traditional surgery, digital stereotaxic surgery is superior since it performs more quickly and with less chance of human error[8,9]. Ibotenate, a potent neurotoxin derived from the amanita muscaria plant and employed as a potent brain-lesioning agent, was used in the current work to cause PrL and IFL degeneration[10]. It severely damages cholinergic neurons when administered directly into the brains of mice, rats, or monkeys[11].

Therefore, the purpose of the current study was to: 1) Examine how the rats' psychomotor activities are impacted by the lesions to the PrL and IFL.2) investigate the impact of infralimbic and prelimbic cortical damage caused by ibotenate on rat non-associative explicit memory, including the exploration and habituation of new contexts and spatial reference memory.

Materials and Methods

Animals

The Animal House of The Faculty of Medicine, Suez Canal University (FOMSCU), Egypt, provided 36 male white albino rats that were 3 to 4 months old and weighed 260–300 grams. Prior to surgery, the rats were kept in groups of four per cage; thereafter, they were kept individually. The rats were kept in an environment with a controlled temperature (24°C) and a 12-hour light-dark cycle (lights on at 6:30 am), and testing was done during the light phase. The rats had free access to normal pellet animal food and tap water. All methods were authorized by the research ethics committee, FOM/SCU code 3992. Animals were handled in accordance with The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research[12]. The number of rats used in each experiment was kept to a minimum to minimize any pain, suffering, or discomfort experienced by the animals. Animals were grouped into 3 groups (12 rats each): Group I: Normal control without any intervention. Group II: Bilateral sham-operated group, phosphate-buffered saline (PBS). (5µl/side) was injected bilaterally in the PrL and IFL cortices. Group III: Bilateral lesioned, ibotenate (5µl/side) was injected bilaterally in both sides of PrL and IFL cortices.

Stereotaxis Surgery

IM injection of a combination of xylazine 10 mg/kg and ketamine 80 mg/kg (SANDOZ, code 4550) was used to anesthetize the animals (ADWIA, code 190356). In order to clearly define the bregma and lambda, which enable stereotactic identification of the mPFC coordinates, animals were positioned into a stereotaxic apparatus (David Kopf Instruments, USA). Small craniotomies were created in the side of interest above the area of interest using a dental drill, and ibotenic acid (a powerful neurotoxin) was manually injected at a rate of 1 l/minute using a 26-S Hamilton syringe and 10UL needle. Rats' Paxinos Atlas[13] depicts the location of the lesion (Fig.1).

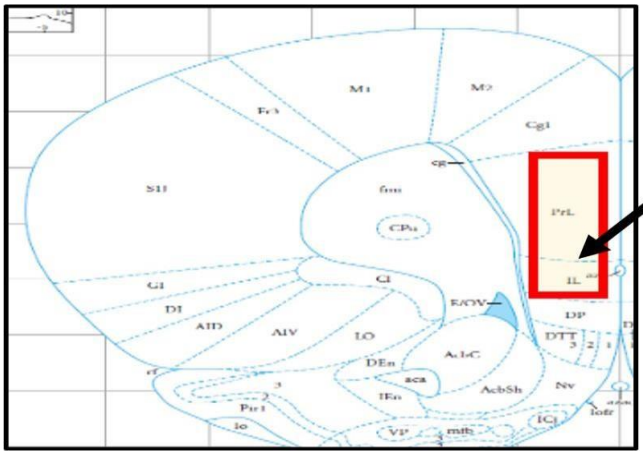


Figure 1: targeting PrL and IFL cortices for mild cognitive impairment induction.
PrL= prelimbic cortex, IFL= infralimbic cortex

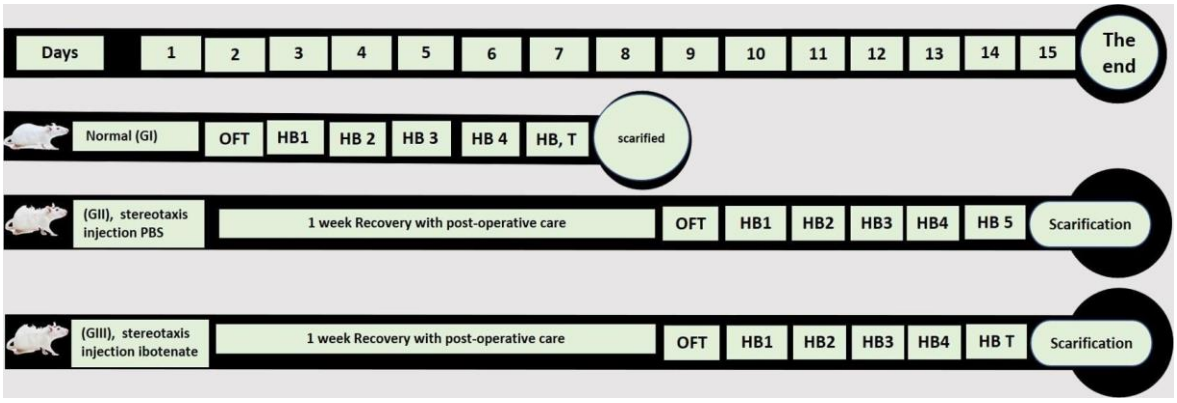


Figure 2: Timeline of the study

The needle was gradually lowered to the following places while taking all necessary precautions to prevent the neurotoxin's backflow. PrL: AP + 3.2 mm ML ± 0.6 mm DV – 2.6 mm from the skull. IL: AP + 3.2 mm ML ±0.6 mm DV – 4.3 mm from the skull. Following surgery, all animals received oral Voltaren 75 mg/3 ml once daily, topical bivatracin spray twice daily, and systemic ceftriaxone (250 mg/1 ml) once daily. The animals were allowed a minimum of seven days to recover before starting behavioral training as shown in (figure 2).

Behavioral tests

All rats underwent an open field test (OFT) and a hole board test (HB).

Open field test (OFT)

It was done as per the protocol of Quillfeldt, 2016[14]. The rectangular box was made from plastic and measured size 50 X 60 X 60 cm as in (figure 3a).

The floor was subdivided into regular sectors by visible lines. Animals were put into the arena for a training session that lasted 10 minutes.

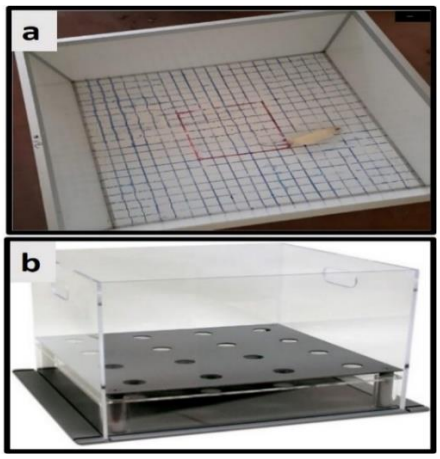


Figure 3: behavioral tests apparatuses
Open field test (OFT) ***(a)****.*
*Hole board (HB) test (****b****).*

Two hours later, each animal has transported again to the same arena for a test session that lasted 10 minutes. During both sessions, the measured parameters were the number of rearings exhibited by the animal, the total number of crossings between floor sectors, the number of self-grooming, time to leave the first quadrant (TLQ), and the number of fecal boli and urination.

Hole board test (HB) for spatial reference memory

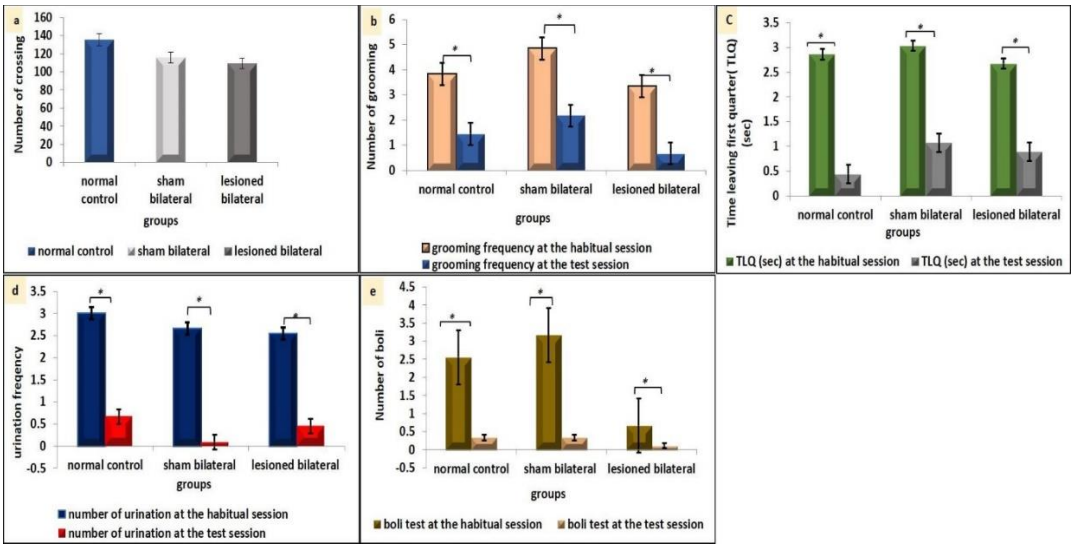
HB test was done as the protocol of Malikovi ć et al., 2019[15]. A black wood maze with a translucent plexiglass wall surrounds it. A board at the center of a box is shown in (figure 3b). The board typically has 16 holes that are evenly spaced. The animals were acclimated to the hole board for two days by allowing them to freely explore the maze for 15 minutes each day while having access to food pellets in all holes. After that, four holes were marked with a colored ring (contrasting with the floor) and were baited with a removable reward (a piece of cheese). Training/ consisted of 3 days (four trials on day one, four trials on day 2, and two retention trials on day3) with an intertrial interval of 30 min for individual rats. In each Trial, the animal was placed gently into the middle of the arena. The trial duration was until all 4 pellets were eaten. The apparatus was Cleaned with tap water and a paper towel after every trial to avoid a bias based on olfactory cues. The pattern of baited holes remained the same during the entire test. Mean reference. memory index (RMI) from trials 9 and 10 = total visits of baited holes / total visits of all holes.

Trans-cardiac perfusion and fixation

By injecting xylazine (30 mg/kg) intraperitoneally, rats were rendered unconscious. After administering 30 ml of Phosphate Buffer Saline (PBS) intracardially to the rats, 4% paraformaldehyde was then administered. The brains were held in and extracted[16].

Tissue preparation & histology

The coronal section of the brain was divided into two equal halves. For histological analysis, paraformaldehyde 4% was used to fix the damaged area in the anterior half of each group. 50 wide coronal sections of the brain were removed. All of the wax was removed using the hydrocarbon solvent xylene, and the pieces were then rinsed in water (hydrated). so that aqueous reagents can penetrate the tissue and cells with ease. Slices then adhered to coated coverslips following the preparation of the sections for staining. Hematoxylin and eosin (H&E) staining were used to identify the spot and extent of the lesion. Then Cresyl violet stain was additionally used to show the Nissl substances in the soma and dendrites of mPFC neurons and to accurately assess the cells' number and size[17].



Number of crossings parameter during training session of OFT among all rats in different study groups **(a).**number of grooming in first session *vs.* second session/ group **(b).** TLQ in first session *vs.* second session/group **(c).** number of urinations in the first session *vs.* second session/group **(d).** number of boli in first session *vs.* second session/group **(e).** *Data are presented as Mean ± SEM.*Statistically significant by Kruskal Wallis test followed by LSD in (a).*Statistically significant by Wilcoxon Signed-Rank test in (b-e). Abbreviations: (SEM)= standard error of mean. (OFT)= open field test. (LSD)= least significant difference. (TLQ) time leaving first quarter.*

Statistical analysis

The SPSS application (Statistical Package for Social Science), version 26, was used to computerize and statistically evaluate the data that had been gathered. Using the Shapiro-Walk test, the distribution of the data was checked for normality. The mean standard error of the mean was the format used to express quantitative data (SEM). The Wilcoxon Signed-Rank test was employed for OFT to compare the variations in quantitative data between each group's first and second sessions. P <0.05 indicate significant differences.

Results

Behavioral results:

Open field test: Parameters of the open field test are the number of rearings, number of crossings, time leaving first quarter (TLQ), number of grooming, number of boli, and number of urinations. The number of crossings parameter in the training session was analyzed using the Kruskal Wallis test followed by the least significant difference test (LSD), revealing insignificant differences (P>0.05), among all rats in different study groups. This finding excludes any motor affection of lesioned group (G III) as in (figure 4a). The number of grooming, TLQ, number of boli, and number of urination parameters was compared in each study group during both the first session (training) and second session (test) using Wilcoxon signed-rank test. Data revealed a significant decrease of previously mentioned parameters (p<0.05) in the test session compared to the training session (figures 4, b-e). The difference in the number of rearings and the number of crossings during both training and test sessions as seen in (figure 5 f, g) were compared in each group to assess memory affection. Using *Wilcoxon signed-rank test*, the Pre-surgery and sham groups (GI, GII) showed a significant reduction in the number of rearing and crossings in the first session (p<0.05)

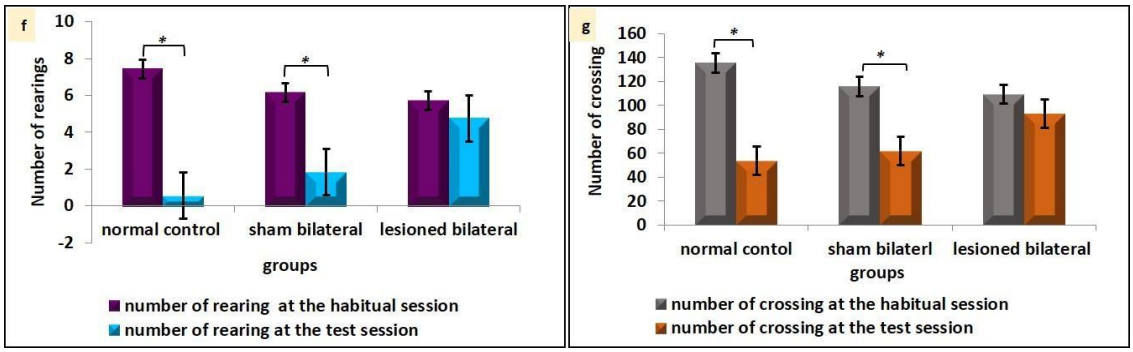


Figure.5: Number of rearings in the first session vs. in the second session/ group **(f)**. number of crossings in the first session vs. in the second session /group **(g)**. *All data presented as Mean± SEM.*Statistically significant as p<0.05 by Wilcoxon Signed-Rank test. Abbreviations: OFT= open field test. TLQ=time leaving the first quarter. SEM=standard error of the mean*

compared to 2nd session, indicating intact memory. The bilateral lesioned group (GIII)showed an insignificant reduction in both the number of rearing and crossings in the first session compared to the second session (p>0.05). This finding indicates complete amnesia with the bilateral lesion of mPFC.

Hole board test

Data from the hole board test among all rats in different study groups were assessed using the Kruskal-Wallis test. Data revealed that there is no statistically significant difference between all study groups regarding spatial reference memory (p =0.081) (table 1).

Histological assessment

The histological analysis of normal and sham control groups showed normal cellular architecture with normal cortical layers integrity by H&E staining, normal neuronal structure with normal soma and axons, and normal pyramidal cells distributed in the prefrontal cortical area as shown in (figure 6. h-k) hile, the lesioned group using ibotenate (5µL/side) showed remarkable pathological alterations with disorganized cellular architecture in form of degeneration, vacuolations, oligodendroglia, cellular necrosis, and pyknosis as shown in (figure 6. l, m). Cresyl violet stains the nissil (chromophil) granules of the neurons and renders purple color to the neurons. The soma of the neurons showed coarse nissil bodies in their cytoplasm and a large nucleus with a striking nucleolus as shown in (figure 7. n-q). Lesioned groups of cresyl violet staining showed cell loss, vacuolations, and small and more packed neuronal cell bodies (figure 7. r, s).

Discussion

Despite evidence that the medial prefrontal cortex contributes to working memory, it is uncertain whether the amnestic mild cognitive impairment disease, which is a stage between nor mal aging and Alzheimer's disease (AD), is caused by mPFC damage. AD attacks these patients at a rate of 10–15% per year[18].

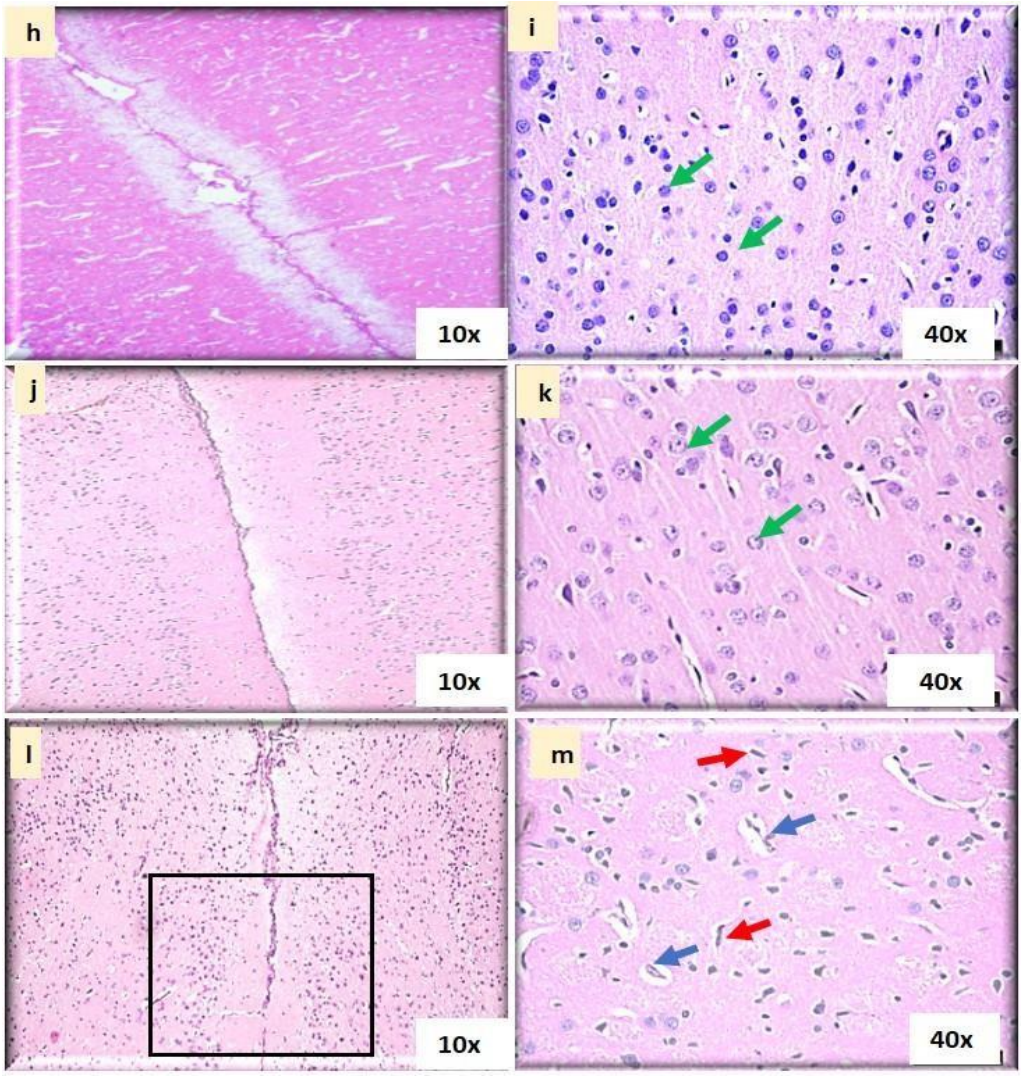


Figure 6: Photomicrographs of H&E, normal control rats (GI) *(h-i) and sham operated bilateral PrL and IFL rats (GII)* (j-k) showed normal pyramidal cells (green arrows) distributed in the prefrontal cortical area (Magnifications 10x and 40x). Bilateral lesioned rats (GIII) showed ibotenate induces numerous cellular ballooning, degenerated neurons surrounded by vacuoles (blue arrows). Red arrows, refer to pyknotic cells and cellular necrosis (Magnifications 10x and 40x)

According to Wu et al. (2016)[19], learning new skills and motor control may also be impacted in MCI. The current study was applied to thirty-six white albino male rats. Aimed to assess the impact of prelimbic (PrL) and infralimbic (IFL) cortices lesions on psychomotor activities and memory among rats stereotaxically injected with ibotenate. The histopathological confirmation of the prelimbic (PrL) and infralimbic (IL) subareas of mPFC lesions by ibotenate was confirmed by H & E staining that showed remarkable degeneration, vacuolations, cellular necrosis, and pyknosis at the areas of injection. Also, Cresyl violet (CV) staining revealed low Nissil bodies, cellular atrophy, and shrunken cells distributed in these areas. This characteristic ibotenate-induced lesion was in agreement with Hennebert et al. (2004)[20]. who reported cavitations and tissue disruption after the injection of ibotenic acid. Also Martínez-Torres et al. (2021)[21]. documented fewer pyramidal neurons

and cellular loss after ibotenic acid injection in the medial prefrontal cortex. In the first session OFT, the number of crossings showed no statistically significant difference between any of the rats in the various research groups. This result rules out any motor impairment in groups that have had lesions. In the second session compared to the first session, there was a significantly lower number of grooming, time leaving the first quarter (TLQ), boli, and urination metrics for all rats in the various study groups. This result rules out worry and any emotional instability within the injured group. In other research, such as Felix-Ortiz et al. (2016)[21]. it was shown that mPFC inhibition reduced anxiety-like behavior, and this result is consistent with their findings. Goes et al. (2018)[22]. concluded that mPFC lesions reduced anxiety in all animals, regardless of trait anxiety level, even highly nervous rats. This result is in contrast to Pati et al.'s (2018) study, which found that elevated pulse maze testing can demonstrate a decrease in anxiety following acute mPFC activation[23].

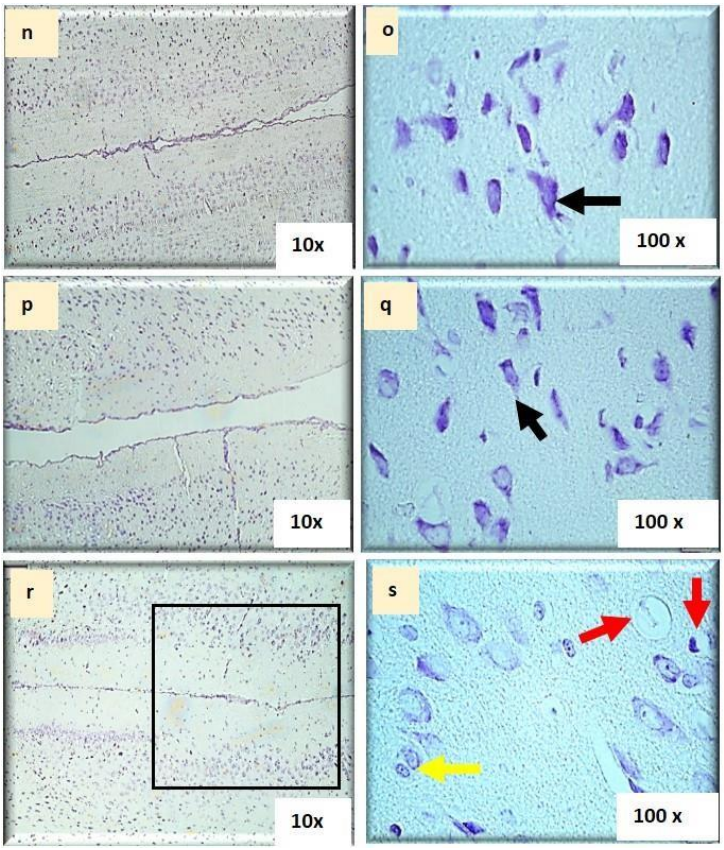


Figure 7: cresyl violet staining, normal control (GI) (n-o), and sham-operated bilateral PrL and IFL rats (GII) (p-q) pyramidal cells are indicated by the (black arrows) in a normal neuron's structure of mPFC subareas (10x and 100x). Rats (GIII) with bilateral lesions displayed Ibotenate toxicity by the loss of neurons (red arrow) revealing the presence of fusiform-shaped apoptotic neurons in the lesioned subareas. (Yellow arrows) indicated that the neuronal cell bodies were much smaller and more densely packed (10x and 100x)

Table 1: Descriptive data of hole board among all study groups

Test	variable	control	Sham bilateral	Lesioned bilateral	P value
Hole board	Reference memory in	0.7186±0.01399	0.5983±0.02798	0.5133±0.02108	0.081

Data are presented as mean ± SEM; *Statistically significant as P < 0.05; ANOVA, Kruskal Wallis test was used

The reason why Pati and his colleagues' study differs from the current one is that they looked at the effects of acutely injecting clozapine-N-oxide (CNO) 30 minutes before a high pulse maze and OFT behavioral testing. There is no indication of whether the activation of the PrL is transient or permanent. However, in the present investigation, PrL and IL subareas in mPFC were inactivated one week prior to OFT. In other words, this does not contradict our findings because it is likely that the anxiolytic effect was caused by the abrupt PrL stimulation. To investigate the memory of exploration and habituation to a new context, the difference in the number of rearings and crossings during both the first and second sessions of OFT were compared in each group. The present data suggested that the bilateral lesioned group had complete amnesia. This puts the focus on the close relationship between mPFC malfunction and amnestic cognitive impairment. This is in agreement with the study of Jobson et al. (2021)[4], who reported that previous cross-sectional studies suggested that mPFC functional connectivity abnormalities are consistently found in the default mode network across both aging and neurocognitive disorders such as Alzheimer's disease. Regarding spatial reference memory, the hole board test was used to calculate the mean reference memory index (MRMI). The current study found that there is no significant difference in the MRMI between all rats in the study groups, concluding that the spatial reference memory does not rely on the mPFC by itself. This is consistent with the findings of Bannerman et al. (2008)[22], who claimed that the mPFC is not directly involved in spatial reference memory. In contrast to the study of Peyton et al. (2019)[23]. who concluded that mPFC has a role in spatial reference memory, this discrepancy is mostly due to different induction designs; the current study's excitotoxic lesion targets were PrL and IFL cortices by stereotaxis procedure, while in Peyton's study, the induction was systemically by intraperitoneal injection of lipopolysaccharide (LPS). So, the present study suggested that encoding of spatial reference memory may occur in structures other than prelimbic and infralimbic cortices.

Conflict of interest: None

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