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Effects of Pretreatment with Clozapine on Spatial Memory of Rats with Lesioned Dorsal Hippocampi

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Abstract

The atypical antipsychotic clozapine improves spatial memory in rats with partial hippocampal lesions. However, the effectiveness of treatment with clozapine before brain injury is unknown. To assess the possibility that chronic pretreatment with clozapine might preserve spatial memory after a hippocampal lesion, we injected rats with either saline or clozapine for one month prior to partial hippocampal lesioning and then assessed spatial learning. Our initial results suggest that pretreatment with clozapine might minimize spatial memory deficits following partial hippocampal lesions. This finding has implications for the prevention and treatment of spatial memory deficits.

Effects of Pretreatment with Clozapine on Spatial Memory of Rats with Lesioned Dorsal Hippocampi

Humans experience a number of neurological disorders that affect the brain. Current treatment for these diseases often relies on reactive drug administration. However, in a perfect world, pretreatment would prevent individuals from ever developing different types of brain disease. While the present study seeks to analyze the effects of pretreatment with clozapine on spatial memory in a rat model of hippocampal damage, the ultimate goal is that such treatment might one day be applied to humans with analogous dysfunction. Specifically, might the drug improve spatial dysfunction in people with Alzheimer's disease? Also, although schizophrenia is not characterized by spatial dysfunction, there are some models of the disease in rats that exhibit such deficits. Will clozapine alleviate this type of spatial dysfunction? These are the types of questions that this study ultimately seeks to move toward answering. Although the present study is specific in nature, relating to spatial memory in rats with hippocampal damage, it represents a preliminary step in addressing larger questions such as the viability of pretreatment as a protective tool in the human brain.

Navigation, a spatial memory function, is heavily controlled by the hippocampus (O'Keefe and Nadel, 1978). Without a properly functioning hippocampus, an individual would lose the ability to navigate through a familiar city; the ability to learn the layout of a new city would also be lost. In terms of specific brain regions controlling spatial function, the posterior hippocampus is activated upon using previously learned spatial information; the anterior hippocampus is more involved in the encoding of new spatial information (Maguire et al., 2000). Although there are other areas involved in spatial functioning (such as the parietal lobe), the hippocampus plays a particularly crucial role. The loss of the hippocampus (regardless of the cause) results in the loss of spatial memory and spatial functioning overall.

London taxi drivers have larger posterior hippocampi than people who do not drive taxis (Maguire et al., 2000). However, average people that do not drive taxis have larger anterior

hippocampi (Maguire et al., 2000). This is related to the fact that licensed taxi drivers in London know the streets and are recalling this spatial information (controlled by the posterior hippocampus), whereas controls are not as familiar with them and have to encode this information (a function of the anterior hippocampus) (Maguire et al., 2000). More support for the idea that the hippocampus is responsible for spatial functioning comes from small mammals and birds. Animals that engage in behavior that requires spatial memory (like locating a food cache) demonstrate increased hippocampal volume when compared against animals that do not exhibit such behavior (Lee et al., 1998). Rats require spatial memory in order to learn and recall the location of food in a maze.

In the rodent model, hippocampal damage results in the loss of spatial functioning (Bardgett et al., 2006). The rat model of hippocampal damage is achieved through ablation of the medial temporal lobe—specifically the hippocampus (Bardgett et al., 2006). The appropriate lesion to model focal hippocampal damage is the neurotoxic lesion, because it spares extrahippocampal circuits (Ramos, 2008; Bardgett et al., 2006). Electrolytic lesions damage extrahippocampal structures and their projections and cytotoxic lesions do not provide as much regional specificity as the neurotoxic lesion (Ramos, 2008). The dorsal hippocampus is the area of interest for modeling spatial impairments in rats (Pouzet, 2002; Ramos, 2008). The CA1 area of the dorsal hippocampus in particular exhibits a high correlation between damage and loss of spatial memory function (Dillon, Qu, Marcus, & Dodart, 2008; Bardgett et al., 2006).

A number of methods for treating spatial memory deficits in the rat model of hippocampal damage are currently in use. One category of drugs being tested is the atypical antipsychotic such as clozapine and its relative, risperidone. Risperidone and clozapine alleviate spatial memory impairment due to structural hippocampal damage (Bardgett et al., 2006). These findings are relevant for the treatment of hippocampal lesions in rats. Clozapine facilitates prefrontal cortex neurotransmission and aids in the functioning of remaining hippocampal cells (Bardgett et al., 2006). Specifically, clozapine

increases the efflux of acetylcholine in the rat hippocampus (Bardgett et al., 2006). This neurotransmitter release may allow the remaining hippocampal neurons to better encode spatial information (Bardgett et al., 2006).

Pharmacological treatment of brain damage and dysfunction of any type largely centers on reactive drug administration, while the method of pretreatment as a protective tool remains surprisingly ignored. For instance, clozapine restores spatial ability when administered after hippocampal damage takes place (Bardgett et al., 2006). Studies like this prove clozapine's effectiveness at restoring function after dysfunction has manifested. However, the question as to whether clozapine can protect the brain when administered preventatively has not been explored yet. The proven success of post-traumatic drug administration raises the possibility that pretreatment might also demonstrate such success.

Clozapine has different effects on memory based on the state of the brain before administration. Clozapine impairs spatial memory in normal rats (Bardgett et al., 2006). This impairment manifests itself in slightly longer reaction times. However, when administered to a rat with existing hippocampal damage, clozapine improves performance on tests of spatial memory. In fact, chronic clozapine treatment completely reverses the memory deficit due to hippocampal damage, such that the spatial performance is *indistinguishable* from intact rats (Bardgett et al., 2006). However, lesioned rats receiving acute (test-day) clozapine treatment perform worse than lesioned rats given saline (Bardgett et al., 2006). For clozapine to aid consistently in spatial memory it needs to be administered chronically, before behavioral assessment (such as in the Y maze).

The purpose of this study is to determine whether chronic administration of clozapine before a dorsal hippocampal lesion will preserve spatial memory functioning, as measured by behavioral performance in a Y maze. It is hypothesized that rats receiving chronic pretreatment with clozapine before dorsal hippocampal damage will outperform rats receiving only saline before dorsal

hippocampal damage in the Y maze, indicating improved spatial functioning. Additionally, because clozapine may also enhance PFC neurotransmission, prefrontal cortex cell density will be analyzed to determine whether clozapine pretreatment leads to changes in neuron number. This is a secondary, exploratory investigation. Although a better measure of increased activity in the PFC might include analysis of receptor number and/or post-synaptic density size, such procedures require the use of an electron microscope. Instead, counting cellular density is an achievable approximation for this investigation.

Method

Subjects

Ten adult male Sprague-Dawley rats (250 – 300g) were acquired from Jackson Laboratories for use in this study. The rats were housed in pairs in the Regis University Animal Colony and received food and water *ad libitum* except where noted. Lighting was maintained on a 12 hour light/dark schedule with the lights on at 6:00 AM. One rat died during surgery, so all data shown reflect N = 9. All procedures were performed under a protocol approved by the Regis University Institutional Animal Care and Use Committee (IACUC).

Materials

Y Maze

A Y maze consisting of three arms 120° apart from each other measuring 4.75” wide at the top of the arm and 1.5” wide at the bottom was used to train and test the rats. The walls were 6.75” high and each arm was 29” long. The arms were covered with opaque lids. The percentage of correct choices made in the Y maze measures spatial functioning.

Procedure

Pretreatment

The rats were randomly assigned to receive daily intraperitoneal injections of either clozapine (2mg/kg) or saline (0.9%) for one month. Administration of clozapine and saline ceased one day before the lesion process.

NMDA Lesion

After pretreatment, each animal was anesthetized with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (12 mg/kg). Then the rats' heads were shaved and treated with Septosine solution and the researcher applied Vaseline to the rats' eyes. Afterward, the rats were placed into a stereotaxic device and an incision was made from between the eyes to behind the ears. The incision site was irrigated with Lidocaine, and the skull was wiped clean and the location of bregma was determined. Each rat received six injections of 2 μ l of *N*-methyl-D-aspartate (NMDA) (12.5 mg/ml) at -3.0 AP, \pm 2.0 ML, -3.6 DV; -3.8 AP, \pm 2.8 ML, -3.6 DV; and -4.6 AP, \pm 3.5 ML, -3.9 DV (all measurements relative to bregma). The researcher then stapled or sutured the incision, removed the rats from the stereotaxic device, and placed the rats under heating lamps to encourage recovery. The rats' recovery was largely unremarkable and lasted under an hour in most cases.

Training

Four days after surgery, all ten rats received three trials each on the Y maze for four days. The rats were initially placed into an empty arm (the starting point). Food cups were placed in the remaining two arms with one Honey-Nut Cheerio® in each cup. After being placed in the start arm, the trial ended when the rats had eaten both cheerios or 90 seconds had elapsed. The intertrial interval was 20 minutes. After the four days of training, all rats were able to eat both food rewards within 90 seconds.

Testing

Eight days after training, the rats were tested for six trials on the Y maze each day for five days. Using the same materials and procedure as mentioned in the training section, the rats were placed into

the start arm and were allowed to choose whether to go right or left. Regardless of the arm they chose, the animals ate the Cheerio on that side. They were then removed for 15 seconds, and replaced into the start arm. The rats' subsequent choice was correct if they entered the arm that they had previously not entered—indicating that the rats remembered which arm still had the Cheerio in it. If the rats re-entered the arm that they chose on the initial run, they were allowed to investigate the empty food cup and were removed from the maze.

Perfusion

Following the behavioral testing, the rats were perfused as follows. Using 108 mg/kg nembutal, the experimenter euthanized the rats then exposed the heart with a transverse incision across the abdomen and lateral incisions under the arms. The ribcage was pulled up, revealing the thoracic cavity. The researcher then perfused the rat by inserting a needle into the base of the heart and cutting the aorta. The rats were first perfused with saline and then 4% paraformaldehyde solution. After five minutes of perfusion with paraformaldehyde, the researcher decapitated the rat and then stored the brain in 4% paraformaldehyde for four days. The brains were then transferred into 10% sucrose in 4% paraformaldehyde. The concentration of sucrose in the storage solution was increased from 10% to 30% over the course of four days. Thirty micron frozen coronal sections were cut and mounted onto gelatin coated slides and stained with thionin.

Microscopy

The researcher viewed all slides of the rat brains to verify that the dorsal hippocampi had been lesioned properly and to determine cell density in the prefrontal cortex. In order to determine the prefrontal cortex cell density, the researcher counted the ratio of neurons to glia on three fields of 40x magnification per rat. The fields were arbitrarily selected from slides showing the prefrontal cortex. The experimenter then determined the mean of each count in order to compare the ratio of neurons to glia between saline rats and clozapine rats.

Results

Chronic administration of clozapine had no significant effect on the behavioral assessment of spatial memory in the Y maze. The saline group received a mean performance of 65% correct choices ($SD = 0.150$). The clozapine group received a mean performance of 80% correct choices ($SD = 0.137$). Although the difference in performance between the groups was not statistically significant $t(7) = 1.60$, $p = 0.16$; Cohen's $d = 1.21$, it occurred in the hypothesized direction (that clozapine-treated rats would outperform saline-treated rats in the Y maze). Additionally, the effect size was large. See *Figure 1* for these results. After removing the rats that received ineffective, undetectable dorsal hippocampal lesions, another statistical analysis including only the rats with either unilateral or bilateral lesions revealed different, yet still statistically insignificant results. The rats in the saline group scored 64% on average ($SD = 0.171$). The rats in the clozapine group scored 77% on average ($SD = 0.189$). See *Figure 2* for these results. These results were insignificant, yet the effect size remained large $t(4) = 0.82$, $p = 0.46$, Cohen's $d = 0.87$.

Microscopy revealed that damage to the dorsal hippocampus was partial. Neither group showed consistent dorsal hippocampal damage. Three rats (two from the clozapine group, one from the saline group) showed no signs of lesion whatsoever, five rats (two from the clozapine group, three from the saline group) had unilateral dorsal hippocampal damage, and only one rat (from the saline group) had bilateral NMDA lesions in the dorsal hippocampus. See *Figure 3* for an example of a dorsal hippocampal lesion.

Histology also proved to be problematic. All slides showed tearing of brain tissue, and identification of damage to dorsal hippocampal tissue was speculative in some cases due to the lightness of the stain on the slides. Despite these shortcomings, lesions were identified in six of the nine rats.

There was no significant difference in the prefrontal cellular density between the clozapine

group and the saline group $t(4) = 1.54$, $p = 0.20$, Cohen's $d = 1.1$. On average, the clozapine group had 137 neurons per mm^2 . The saline group had 82 neurons per mm^2 on average. See *Figure 4* for an example of an arbitrarily selected prefrontal cortex field at 40X magnification. *Figure 5* shows the prefrontal cortex cell density comparison.

Discussion

This investigation did not yield evidence to support the hypothesis that chronic clozapine pretreatment protects spatial memory functioning before hippocampal damage. Behaviorally, there was a minor difference in the expected direction in the Y maze performance between the two groups. An analysis of effect size revealed that a larger sample size might make the difference statistically significant between the two groups. This finding is consistent with previous research in that the clozapine did minimally (though not significantly) improve spatial memory performance when administered chronically before injury (Bardgett et al., 2006).

Previous researchers have demonstrated the improvement on delayed spatial alternation tasks following chronic administration of clozapine, at the cost of increased latency of response. The sedative properties of clozapine slowed the rats' response times in the Y maze in previous studies (Bardgett et al., 2006). In this study, response times were not recorded. Despite experiencing an increased latency of response in previous research, the clozapine group outperformed the saline group in terms of spatial choices in the Y maze (Bardgett et al., 2006). While administration of clozapine leads to a reduction in response speed, the benefits of improved spatial functioning outweigh this minor setback (Bardgett et al., 2006). In terms of applying the drug to humans, users would need to be cautious when considering activities in which these sedative effects might be harmful—such as when driving.

Several limitations affect the meaningfulness of the results. Each rat received six penetrations in this study, whereas other studies of this nature included eighteen NMDA penetrations (Bardgett et al., 2006). As such, the partial dorsal hippocampal damage under investigation in this study was not as

widespread as it was in other studies. To compensate for the reduced number of penetrations, the dosage of NMDA was increased in order to enlarge the size of each lesion. Despite this compensatory measure, three rats showed no lesion and were eliminated from the behavioral analysis. In addition to reduced lesion effectiveness, the lightness of the stain on the microscope slides interfered with determining the presence of dorsal hippocampal lesions in all animals. A darker stain would ensure more definitive verification of lesions. Lastly, prefrontal cortex cell density was measured by analyzing the ratio of neurons to glia in three arbitrarily selected fields of PFC. This was done in an attempt to investigate how clozapine increases neurotransmission in the prefrontal cortex. The trend did occur in the predicted direction. Although the clozapine group did appear to have a greater number of neurons in the prefrontal cortex than the saline group, better measures of neurotransmission exist. For future studies, a better investigation of clozapine's effect on neurotransmission would take into account the size of the post-synaptic density and/or receptor number.

Pending successful replications, the ultimate goal of this study is that pretreatment with clozapine, which might preserve spatial functioning, be applied to humans that are either predisposed to or show signs of deficits related to reduced hippocampal functioning (such as in Alzheimer's disease) and NMDA/DA abnormalities (such as in schizophrenia).

Alzheimer's disease (AD) involves damage to the hippocampus and results in memory deficits (Lee, Jerman, & Kesner, 2005). People with AD exhibit notable difficulty in performing spatial recollection; in fact, people with AD scored 25% lower on spatial recall and 28% lower on delayed recall on a modified spatial version of the Buschke controlled learning task than did unaffected individuals (de Toledo-Morrell et al., 2000). This performance decrease is important. Perhaps pretreatment with clozapine in individuals that are genetically predisposed to AD might preserve spatial functioning.

An endophenotype of schizophrenia that includes spatial memory deficits using the mouse

model is the metabotropic glutamate receptor 5 (mGluR5) knock-out mouse (Gray et al., 2009). These mice exhibit abnormal locomotor patterns such as psychomotor agitation and deficits in performance of short term spatial memory on tasks such as the Y maze (Gray et al., 2009). The mGluR5 mice are unable to recognize novel arms in short term measures of spatial memory such as the Y maze. Additionally, they exhibit longer term spatial learning and recall deficits in the Morris Water Maze (Gray et al., 2009; Lu et al., 1997). After being trained to learn the location of the platform in the Morris Water Maze, controls spent more time in the target quadrant than other quadrants and crossed the platform site often (Lu et al., 1997). The mGluR5 knock-out mice did not stay longer in the target quadrant and did not persistently search for the platform (Lu et al., 1997). Although this knock-out mouse is not analogous to humans with schizophrenia, mGluR5 closely interacts with and regulates NMDAR function; aberrant regulation and/or expression of this receptor leads to schizophreniform deficits (Gray et al., 2009). The link between rodent and human work is further strengthened in that in a limited group of people with schizophrenia, there is a small increase in mRNA for mGluR5 in the frontal cortex (Gray et al., 2009). Accordingly, there is some merit in using these knock-out mice in an attempt to research schizophrenia and especially spatial dysfunction.

Rat models exist for both hippocampal damage (AD) and NMDA hypofunction and dopamine hyperfunction (schizophrenia) (Bardgett et al., 2006; Ozawa et al., 2006). The latter is achieved through either the aforementioned mGluR5 knock-out or administration of NMDA antagonists and dopamine agonists (Bardgett et al., 2006; Moghaddam, 2004), or through prenatal maternal injection with polyribonucleosinic-polyribocytidilic acid (Ozawa et al., 2006). Although Alzheimer's disease is not fully approximated through a simple hippocampal lesion because the nature and extent of the damage differs between humans with the disease and animals with hippocampal ablation, the common link of structural hippocampal damage leads to similar deficits such as spatial dysfunction. For the purposes of this investigation, simple hippocampal damage induces spatial learning and memory dysfunction

which is ameliorated by clozapine.

Clozapine works on a number of levels in the brain, facilitating increased functioning in models of both AD and schizophrenia. In terms of treating deficits resulting from hippocampal damage, clozapine enhances neurotransmission in the prefrontal cortex (PFC) and aids in the functioning of remaining hippocampal cells (Bardgett et al., 2006). Relevant for the treatment of schizophrenia is the fact that clozapine is a dopaminergic antagonist that acts especially at the D₄ receptors (Mansbach et al., 1998). In terms of up-regulating the hypofunctional NMDA receptor system (NMDAR) typical of schizophrenia, clozapine indirectly facilitates this process by inhibiting a glycine transporter which increases glycine's binding to its positive modulation site on the NMDA receptors (Javitt, 2004). In the presence of its co-agonist glutamate, glycine affects channel opening time and the rate of desensitization, thereby up-regulating NMDAR function (Javitt, 2004). Chronic treatment with clozapine results in the up-regulation of hippocampal and cortical NMDAR binding (Gray et al., 2009). Additionally, clozapine acts as an inverse agonist at some serotonin receptors (Purohit et al., 2005). Indeed, clozapine improves both positive and negative symptoms of schizophrenia (Semiz et al., 2007; Capuano, Crosby, & Lloyd, 2002).

In addition to pharmacological pretreatment as a preventative measure against loss of hippocampal function, there are also some lifestyle factors that are associated with reducing the risk of cognitive deficits—especially due to AD. Individuals who have attained higher levels of education experience a lower risk for dementia than do individuals without this level of education (Le Carret et al., 2005). The odds of a clinical diagnosis of dementia in individuals with neuropathological AD diminish by roughly 0.82 to 0.87 with each additional year of formal education (Roe et al., 2007). As education increases, so does the cognitive reserve that an individual has at his/her disposal. This cognitive reserve is made up of a greater synapse count, a more flexible neural network, and even larger brain size overall (Roe et al., 2007). The greater the cognitive reserve, the greater the delay in

clinical expression of the pathology (Roe et al., 2007). In addition to education, cardiovascular fitness and exercise lead to the preservation of brain volume (Burns et al., 2008). Physical fitness promotes increased vascularization in the brain and an increase in growth factors in areas important for memory (Burns et al., 2008). In mice, physical activity spurs neurogenesis in the hippocampus. While this effect has yet to be replicated in humans, evidence seems to indicate that fitness serves as a protective factor against AD (Burns et al., 2008).

If it were the case that a larger sample size demonstrated a statistically significant difference between the spatial memory performance of the two groups in the present study, the paradigm of pretreatment with clozapine as a means of preserving spatial functioning and spatial memory would have implications for people predisposed to suffering from reduced hippocampal volume and also for those at risk for experiencing NMDAR hypofunction and/or DA hyperfunction. By taking this drug on a daily basis before the symptoms of a degenerative, chronic disease like AD appear, spatial functioning deficits might be lessened. This would be a significant step forward in terms of protecting those that are aware of an impending disorder.

Again, while this study analyzed spatial dysfunction in the rat model, the eventual goal is that pretreatment with clozapine be applied to humans that are either predisposed to or show signs of deficits related to reduced hippocampal functioning and, separately, NMDA/DA abnormalities. Previous studies have shown the effectiveness of clozapine treatment in humans, so pretreatment with clozapine might prepare a human for an impending disorder characterized by NMDA receptor malfunction, dopaminergic hyperfunction, and hippocampal damage on the whole. To further improve prognosis, perhaps the combination of pharmacological pretreatment and lifestyle change might be successful at increasing the human health span against diseases of the hippocampus (Burns et al., 2008; Roe et al., 2007).

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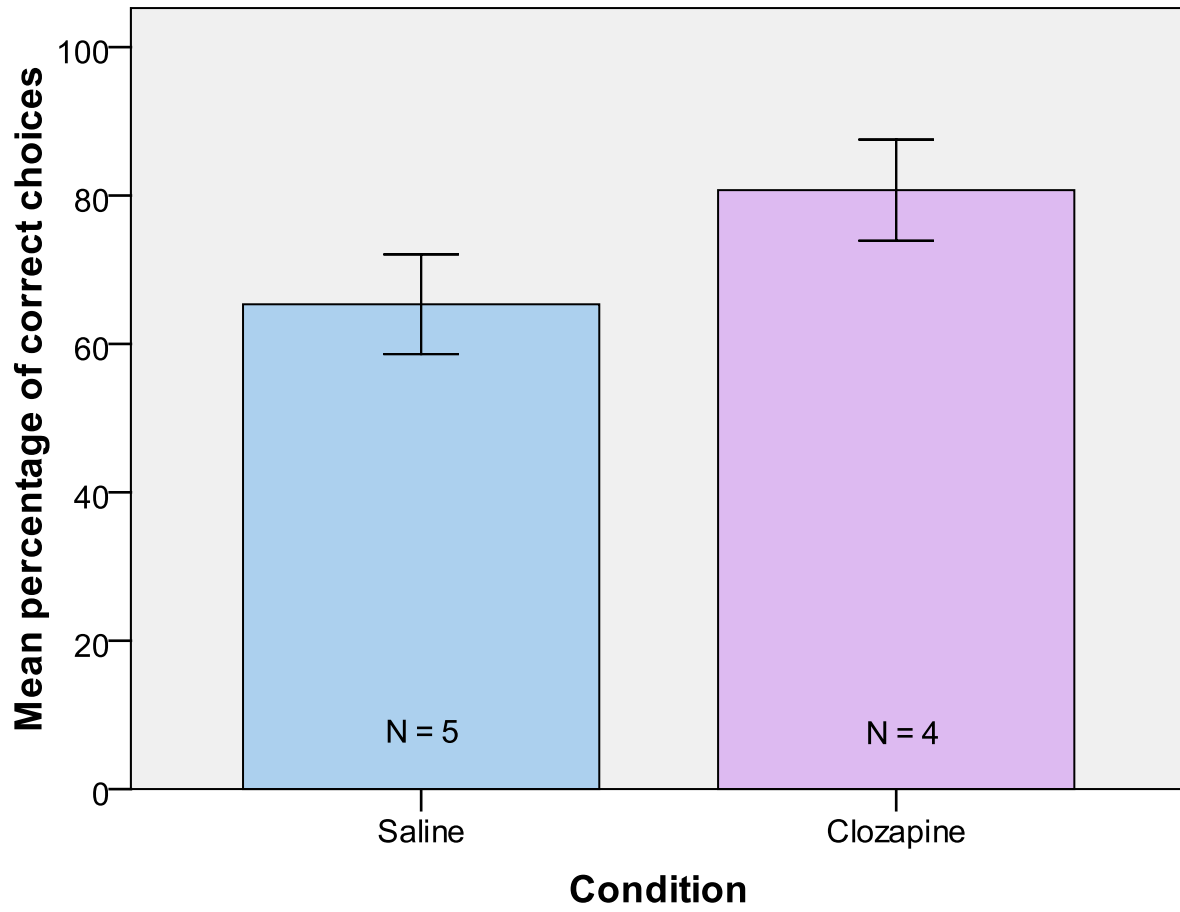


Figure 1. Mean Y maze performance between the two groups, uncorrected for lesion status.

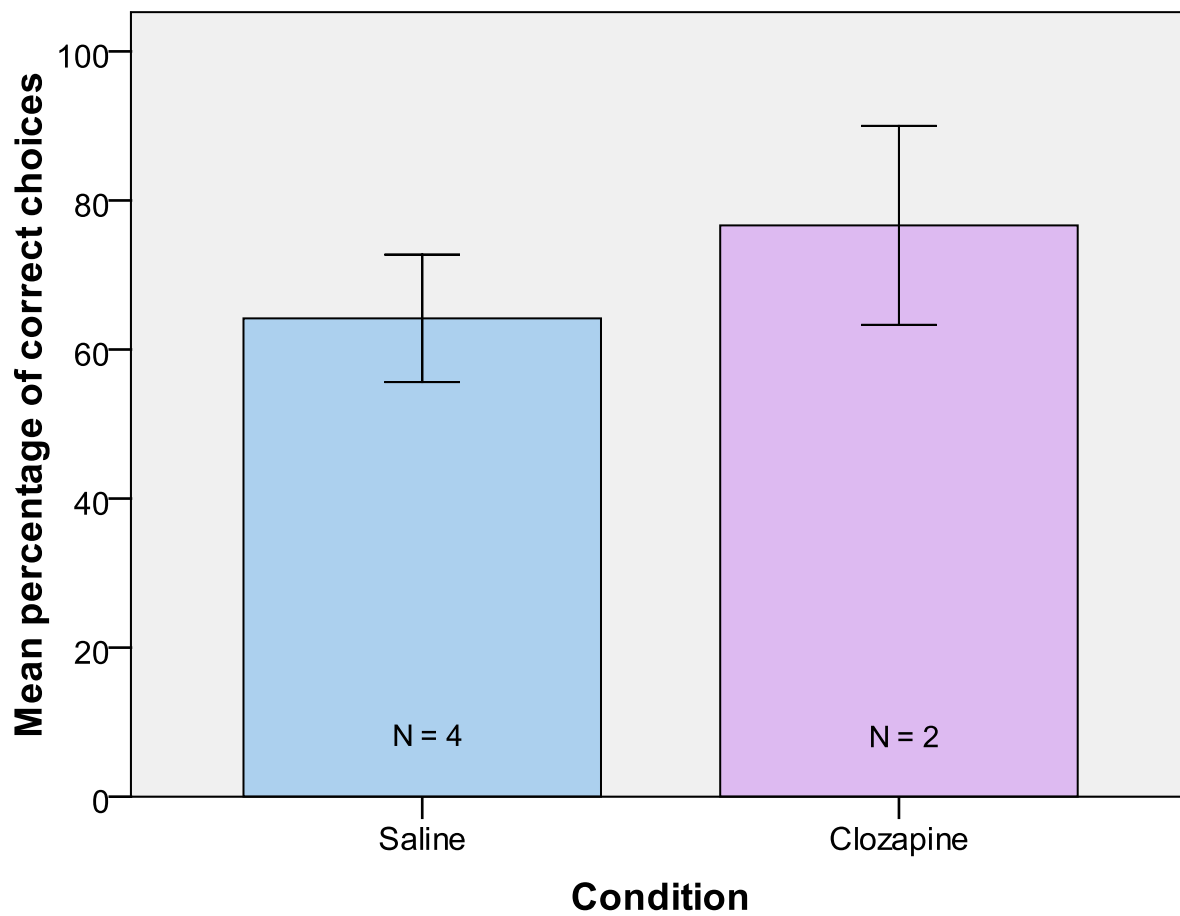


Figure 2. Mean Y maze performance between the two groups, showing only lesioned animals.

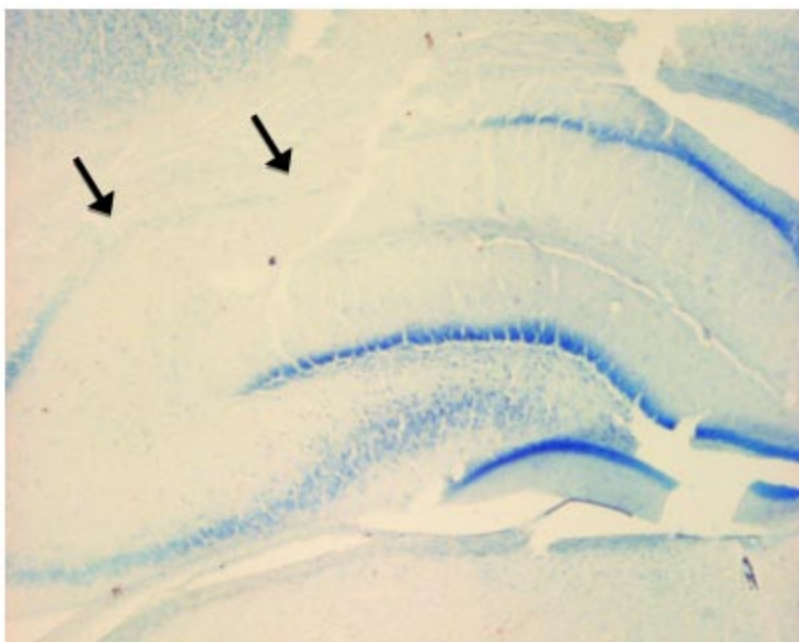


Figure 3. Dorsal hippocampal lesion between the arrows.

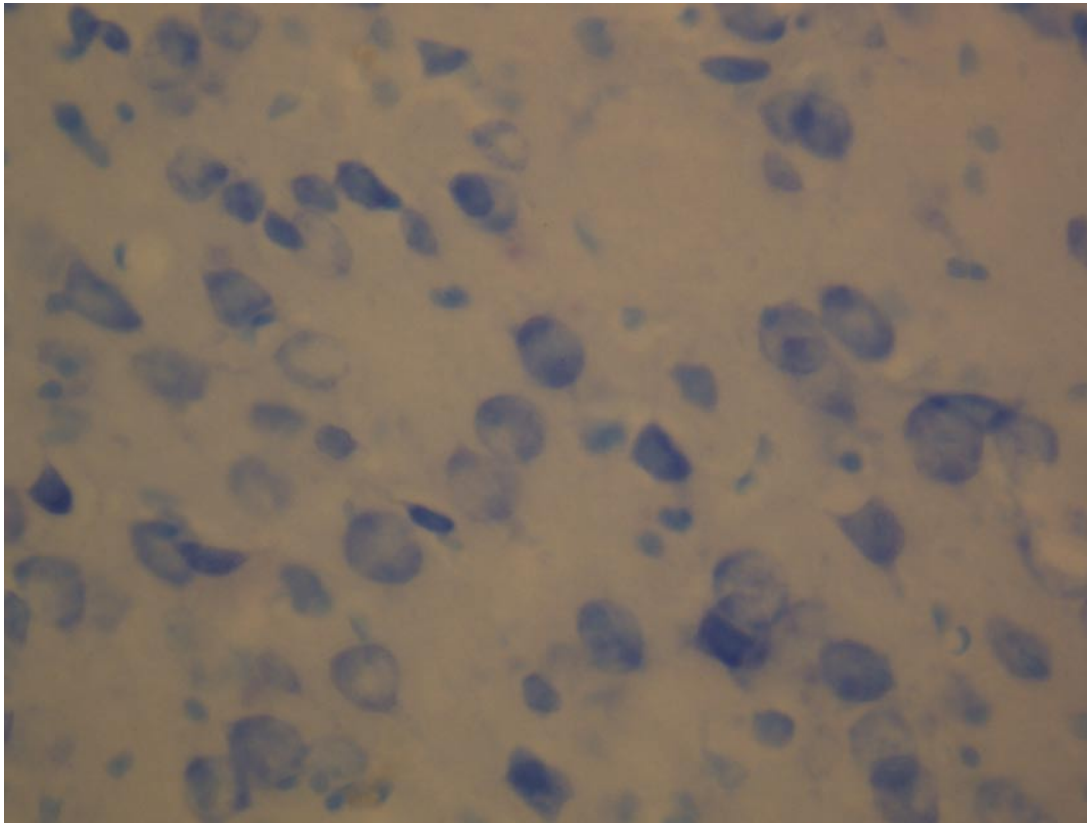


Figure 4. Prefrontal cortex cell density count field (40X).

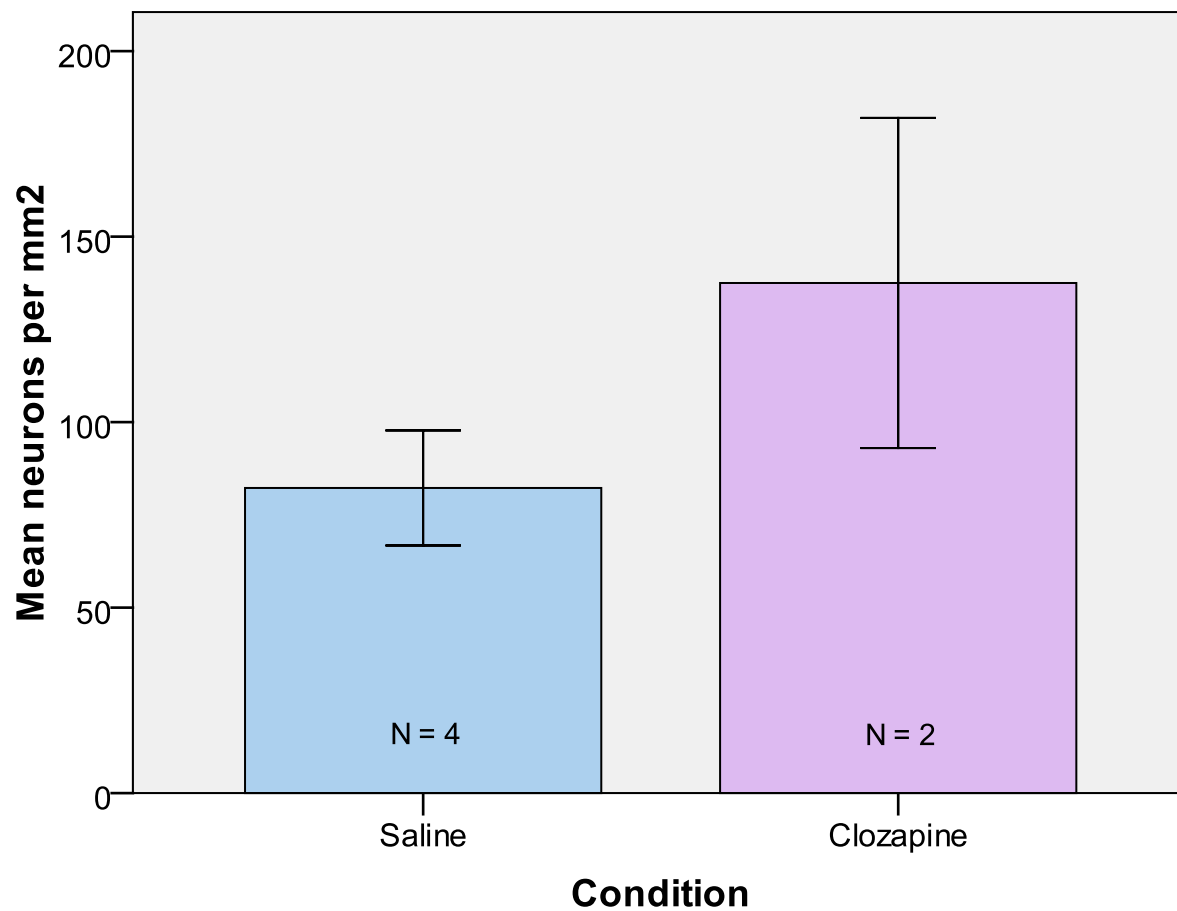


Figure 5. Mean prefrontal cortex neuronal density for both groups, showing lesioned animals only.