THE EFFECTS OF THE TIME OF THE DAY AND THE PINEAL GLAND ON THE ANXIETY LIKE BEHAVIOUR AND THE LEARNING PERFORMANCE OF THE MALE WISTAR ALBINO RATS

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by
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ABSTRACT

THE EFFECTS OF THE TIME OF THE DAY AND THE PINEAL GLAND ON THE ANXIETY LIKE BEHAVIOUR AND THE LEARNING PERFORMANCE OF THE MALE WISTAR ALBINO RATS

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The previous studies on the anxiety like behavior in rats by using elevated plus maze and open field have provided rather unequivocal results since they obtained data at different times of the day. These studies have never investigated the effects of the different times of day in a single research paradigm even though the results of such studies are generally attributed to the different measurement times. Thus, we aimed to examine the effects of the time of the day and the pinealectomy on anxiety like behavior in male Wistar albino rats by using elevated plus maze and open field in the first study. Experiments were performed with control and pinealectomy groups at the four different time points (06:00, 12:00, 18:00 and 24:00 hours; LD 12:12, lights on at 0600 and off at 1800). In open field, the main effect of the measurement time was significant on the total distance travelled, the mobility and the velocity. In elevated plus maze, the main effect of the measurement time was significant on the total distance travelled, the total entry to closed arms, and mobility. In the second study, we examined the effects of pinealectomy, constant release melatonin implants and timed melatonin injections on spatial

memory in male Wistar rats by using Morris water maze. Pinealectomy, melatonin injection and melatonin implantation groups were performed including controls for all groups. After a week of the pinealectomy and melatonin implantation surgeries, we applied the injections and made the training trials throughout four days. At the fifth day, experiments were performed and recorded. Spatial memory performance of the rats was impaired by the pinealectomy and melatonin injections since they elongated the latency and shortened the time passed in the correct quadrant. Melatonin implantation did not affect the spatial memory performance of the rats. The latency and the time passed in correct quadrant were not statistically different from their controls. Beside the contribution of these results obtained from these two studies to the behavioural science, these results were also important for representing a procedural mistake in the previous studies.

Key Words: Anxiety, Elevated plus maze, Memory ,Morris water maze, Open field

ÖZET

GÜNÜN ZAMANI VE PİNEAL BEZİN WİSTAR ALBİNO RATLARDA ANKSİYETE BENZERİ DAVRANIŞLAR VE ÖĞRENME PERFORMANSI ÜZERİNE OLAN ETKİLERİ

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Ratlarda, yükseltilmiş artı labirent ve açık alan düzenekleri kullanılarak yapılan önceki çalışmalarda, veriler günün farklı zamanlarında toplandığından açık ve net sonuçlar sağlanamamıştır. Bu çalışmaların sonuçları genellikle farklı ölçüm zamanlarına katkıda bulunmalarına rağmen, bu çalışmalarda, tek bir araştırma paradigmasında, günün farklı zamanlarının etkileri araştırılmamıştır. Birinci çalışmamızda, yükseltilmiş artı labirent ve açık alan düzeneklerini kullanarak pinealektomi ve günün farklı zamanlarının anksiyete benzeri davranışlar üzerine olan etkilerini açıklamayı amaçladık. Kontrol ve pinealektomi grupları oluşturuldu ve bu gruplardaki ratlardan günün dört farklı zamanında ((06:00, 12:00, 18:00 and 24:00; ışıklar 06:00'da açıldı, 18:00'da kapandı) ölçümler alındı. Açık alan testinde ölçüm zamanlarının belirgin etkisi toplam katedilen mesafe, hareketlilik ve hızda görülmekteydi. Yükseltilmiş artı labirent testinde ise, ölçüm zamanlarının belirgin etkisi, toplam katedilen mesafe, kapalı kol giriş frekansı ve hareketlilikte

görülmekteydi. İkinci çalışmamızda, Morris su labirenti kullanarak ratlarda pinealektominin, implantasyon yolu ile sürekli melatonin salınımının ve zamanlı melatonin enjeksiyonlarının uzaysal hafıza üzerine etkilerini araştırdık. Kontrolleri de içeren pinealektomi, melatonin implantasyonu ve melatonin enjeksiyonu grupları oluşturuldu. Pinealektomi operasyonları ve melatonin implantlarının yerleşkleştirilmesinden bir hafta sonra dört gün boyunca alıştırma eğitimi yapıldı. Melatonin enjeksiyonları her eğitimden önce uygulandı. Beşinci gün ölçümler alındı. Pinealektomi ve melatonin enjeksiyonu ratların uzaysal hafızasına platformun bulunduğu çeyrekte geçirilen süreyi kısalttığı ve latansı uzattığı için zarar verdi. Melatonin implantasyonu hafızayı etkilemedi. Platformun bulunduğu çeyrekte geçirilen zaman ve latans istatistiksel olarak kontrol gruplarından farklı değildi. Her iki çalışmadan elde edilen sonuçlar davranış bilimine olan katkılarının yanında, yöntemsel olarak yapılan bir yanlışı ortaya çıkarması bakımından da önem arz etmektedirler.

Anahtar Kelimeler: Anksiyete, Yükseltilmiş artı labirent, Hafıza, Morris su labirenti, Açık alan

TO MY PARENTS

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TABLE OF CONTENTS

ABSTRACT ..... **iii**
ÖZET ..... **v**
DEDICATION ..... **vii**
ACKNOWLEDGMENT ..... **viii**
TABLE OF CONTENTS ..... **ix**
LIST OF TABLES ..... **xi**
LIST OF FIGURES ..... **xii**
1. INTRODUCTION **1**
1.1. PHOTOPERIOD 1
1.2. PINEAL GLAND 1
1.3. MELATONIN 2
1.4. ANXIETY 5
1.5. SPATIAL MEMORY 6
1.6. THE OBJECTIVESé ...6

2. MATERIAL AND METHODS **7**
2.1. Animal Care 7
2.2. Experimental Protocol 7
2.3. Pinealectomy 8
2.4. Melatonin Administrations 9
2.5. Open Field 9
2.6. Elevated Plus Maze 10
2.7. Morris Water Maze 10
2.8. Statistical Analyses 12

3.RESULTS **13**
3.1. Open Field Measurements 13
3.2. Elevated Plus Maze Measurements 19
3.3. Morris Water Maze Measurements 25

4. DISCUSSION 34
5. CONCLUSION.....46
REFERENCES.....50

LIST OF TABLES

Table 1. Mean (SEM) performance values on open field maze text of male ratsé é 47

Table 2. Mean (SEM) performance values on elevated plus maze test of male ratsé é .48

Table 3. Mean (SEM) performance values of the rats in Morris water maze testé é é 49

LIST OF FIGURES

Figure 1. A. Total distance travelled in the open field 14

Figure 1. B. The amount of mobility in the open field 16

Figure 1. C. The velocity of the male rats exposed to different times of the day in the open field.....18

Figure 2. A. Total amount distance travelled in the elevated plus maze 20

Figure 2. B. The number of entries into closed arms in the elevated plus mazeé ...é é é é 22

Figure 2. C. The amount of mobility in the elevated plus maze.....24

Figure 3. A. Total distance travelled in the Morris water maze 26

Figure 3. B. Time spent to find the platform in the Morris water maze 27

Figure 3. C. Time spent in the quadrant of the platform in the Morris water maze 29

Figure 4. A. The entrance frequency to the quadrant of the platform in the Morris water maze.....32

Figure 4. B. Mobility time in the Morris water maze.....33

1. INTRODUCTION

1.1. PHOTOPERIOD

In mammals, some parameters of endogenous circadian rhythm (e.g., locomotor activity, body temperature, and release of some hormones) are generated and controlled by a circadian oscillator, located in the hypothalamic Suprachiasmatic nuclei (SCN). These circadian rhythms disappear by the ablation of the SCN (Refinetti et al., 1994) and some of them (e.g., locomotor activity) reappear upon transplantation of fetal SCN tissue (Le Sauter and Silver, 1994). Circadian rhythms are synchronized by some zeitgebers such as light/dark cycle, temperature, food availability and hormones (Goldman, 1999; Prosser and Bergeron, 2003; Rusak and Zucker, 1979). In light of the abundance of descriptive and mechanistic data on circadian changes in behavior, surprisingly little is known about the extent to which circadian changes affect more complex psychological processes in animals. Anxiety, for instance, influences a number of behaviors that could reasonably be of adaptive significance in a seasonal context (Pyter and Nelson, 2006). Thus, there has been a great interest of whether or not there is a circadian rhythm in the anxiety like behaviour of the mammals.

1.2. PINEAL GLAND

Pineal gland is a small pine-shaped structure located in the epithalamus of the brain and melatonin is the main hormone of this gland that informs the body about the environmental light and dark regimen (Klein, 1993). The production of melatonin shows a diurnal fashion; with highest concentrations during the dark phase of the day and this rhythm

is similar in all animals whether they are nocturnal or diurnal. The removal of the pineal gland (pinealectomy) abolishes the rhythmic endogenous melatonin release and decreases the plasma levels of melatonin significantly (Hoffman and Reiter, 1965). Melatonin modulates many physiological functions, including sleep, as well as circadian, visual, cerebrovascular, reproductive, neuroendocrine and neuroimmunological functions (Arendt, 2000; Borjigin et al., 1999; Brzezinski, 1997; Masana and Dubocovich, 2001; Vanecek, 1999; Wirz-Justice, 2001).

1.3. MELATONIN

Melatonin is produced and released from the pineal gland into the blood especially at night and is referred to as the chemical experience of darkness (Reiter,1981; Stetson and Watson-Whitmyre,1984). The melatonin activity of the pineal gland is determined primarily by the release of norepinephrine (NE) from postganglionic sympathetic nerve endings that terminate in the gland. The release of NE is associated with darkness, when the suprachiasmatic nucleus (SCN) is relieved of the inhibitory signal from the eyes due to the interaction of photons with the retinas. In as much as NE release onto the pinealocytes occurs at night, melatonin synthesis likewise occurs primarily during darkness. Therefore, the concentration of melatonin in the blood is greater at night than during the day.

Melatonin modulates many physiological functions, including sleep, as well as circadian, visual, cerebrovascular, reproductive, neuroendocrine and neuroimmunological functions (Arendt, 2000; Borjigin et al., 1999; Brzezinski, 1997; Masana and Dubocovich, 2001; Vanecek, 1999; Wirz-Justice, 2001). The amphilicity of the melatonin is allowing the molecule to enter any cell, compartment or body fluid (Poeggeler et al., 1994). In addition to physiological functions, melatonin seems to influence the behavioural processes (Krause and Dubocovich, 1990), learning, stress, anxiety like

behaviors, and depression (Krause and Dubocovich, 1990; Mantovani et al., 2003; Naranjo-Rodriguez et al., 2000; Loiseau et al., 2006) and to produce some psychotropic effects in rodents, such as sedative (Golombek et al., 1996), anticonvulsant (Golombek et al., 1996), antidepressant (Mantovani et al., 2003; Ergun et al., 2006), and anxiolytic (Naranjo-Rodriguez et al., 2000; Papp et al., 2006) effects.

With regard to behavioural processes, melatonin binding sites have been found in the regions implicated in cognition and memory in the brain (Cardinalli et al., 1979; Weaver et al., 1989). Previous studies have shown that passive and active avoidance learning are affected by melatonin (Martini, 1971; Kovács et al., 1974). Melatonin that decreases recognition time, leads to a facilitation of short-term memory (Argyriou et al., 1998). The melatonin administered group shortened the mean latency on the third and fourth days of training trials compared to the control group and spent more time in the correct quadrant compared to the control group (Gönenç et al., 2005). Raghavendra and Kulkarni (2001) have shown that melatonin improved chronic ethanol-induced amnesia. Taken together, these findings have suggested the beneficial effect of melatonin on cognition and memory. However, inconsistent evidence was also present in the literature. A more recent research has demonstrated that low dose of melatonin given from weaning did lead to learning and memory deficit in rats (Cao et al., 2009). This difference in above mentioned findings can be due to some related factors (e.g., test, measurement, model or paradigm, dose differences, and etc.). For instance, the former studies used different learning paradigm such as active and passive avoidance test (Martini, 1971; Kovács et al., 1974), whereas the latter one (Cao et al., 2009) employed the Morris water maze. This important difference can be attributed to the Morris water test that has been considered to be affected by both some exogenous factors (nutrition procedure, stress, infection, and etc) and endogenous factors (i.e., sex, age, strain differences) (Hooge and De Deyn, 2001). Another important

reason for preferring the Morris water test is that it is one of the most suitable tests for the investigation of spatial memory (Hooge and De Deyn, 2001). Since our focus was on spatial memory, we used the Morris water maze test.

It is well recognized that the removal of the pineal gland abolishes the rhythmic endogenous melatonin release and decreases the plasma levels of melatonin significantly (Hoffman and Reiter, 1965). Parallel to studies on the effects of regulatory function of melatonin on the learning performance, pinealectomy has been received a research attention in the literature. For instance, one research has shown that pinealectomy itself did not have a detrimental effect on cognitive performance in rats, whereas the interaction of it with the other lesion (i.,e, lesion on habenula) did impair cognitive performance (Lecourtier et al., 2005). A great number of studies have consistently demonstrated that pinealectomy did not have a significant role on the acquisition and extinction of the active avoidance behavior (Appenrodt and Schwarzberg, 2003), anxiety behavior (Appenrodt and Schwarzberg, 2000), passive avoidance learning (Appenrodt and Schwarzberg, 1999), open field exploratory activity (Kovács et al., 1974), and social recognition (Appenrodt et al., 2002). On the other hand, whether or not pinealectomy decreases the learning capacity on the Morris water maze is still not known and no published research has been reported about it so far. The present study, which has its unique modifications in its content, may contribute to understanding of the role of pinealectomy on cognitive performance.

A number of studies have investigated the effect of melatonin implants on animals. All of these studies have focused on the effect of it on reproductive system (Hoffman, 1974, Turek et al., 1975; Karakaк and Gündüz, 2003). No study has been reported about the effect of melatonin implants on spatial memory. Implantation leads to the constant release of melatonin. Therefore it would be an interesting research inquiry of whether or not the

constant melatonin release via implantation has an effect on the spatial memory performance of rats.

1.4. ANXIETY

Anxiety is a common experience in daily life which causes substantial suffering in the general population. The data from Western Countries report the prevalence estimates as high as 10 to 15% of the population (Regional Health Forum). This high percentage of the prevalence of the anxiety disorder in general population is elevating the importance of the results of the animal experiments especially for the drug treatments.

The animals used in the anxiety studies tend to show different activity patterns. However, these different activity patterns were taken into consideration at the different times of the day depending on the nature of the experiments. For instance, some of these experiments were performed when the animals were in their active period (Ashkenazy et al. 2009; Ashkenazy-Frolinger et al. 2009; Einat et al. 2006), but others were not (Benabid et al. 2008; Nava and Carta, 2001; Prendergast and Nelson, 2005; Pyter and Nelson, 2006). Most research was performed with nocturnal rodents (mostly rats) during the light period while some of the effects of daylight cycles or melatonin levels in nocturnal animals may differ greatly from those effects in diurnal species (including humans) (Ashkenazy et al. 2009). For instance, in nocturnal rodents a nighttime light pulse reduces activity levels, whereas light triggers activity in diurnal mammals (Redlin, 2001). Although previous studies have provided evidence for the seasonal variations in anxiety like behaviors, there is no reported study that examines the possible circadian variations. Given the consideration that the time of the day which experiments are performed is important on the results of the anxiety studies, in the present study, we examined the effects of the time of the day and the pinealectomy on anxiety-like behaviour in male Wistar rats by using elevated plus maze and open field.

1.5. SPATIAL MEMORY

In cognitive psychology and neuroscience, spatial memory is the part of memory responsible for recording information about one's environment and its spatial orientation. For example, a person's spatial memory is required in order to navigate around a familiar city, just as a rat's spatial memory is needed to learn the location of food at the end of a maze. It is often argued that in both humans and animals, spatial memories are summarized as a cognitive map. Research indicates that there are specific areas of the brain associated with spatial memory. Many methods are used for measuring spatial memory in children, adults, and animals.

1.6. THE OBJECTIVES

The aim of the study 1 is to examine the effects of the time of the day and the pinealectomy on anxiety-like behaviour in male Wistar rats by using elevated plus maze and open field.

The aim of the study 2 is to investigate the effects of melatonin injection, pinealectomy and melatonin implantation on the spatial memory performance of the rats in the Morris water maze.

2. MATERIALS AND METHODS

2.1 Animal Care

Adult male Wistar rats (200 ï 250 g) were obtained from our laboratory colony maintained at the Abant Izzet Baysal University (AIBU). They were exposed from birth to 12L (12 hour of light, 12 hour of darkness, lights off at 1800 hr). Animals were maintained in plastic cages (16x31x42 cm) with pine shavings used as bedding. Food pellets and tap water were accessible ad libitum. The procedures in this study were carried out in accordance with the Animal Scientific procedure and approved by the Institutional Animal Care and Use Committee. All lighting was provided by the cool-white fluorescent tubes controlled by automatic programmable timers. Ambient temperatures in the animal facilities were held constant at 22 ± 2 °C in air-ventilated rooms.

2.2 Experimental Protocol

In the first study, twenty male adult rats were used and were randomly divided into two groups as control and pinealectomy. Anxiety like behaviors of Wistar rats were measured at different time points of the day [at 06:00 (200 lux), 12:00 (200 lux), 18:00 (5 lux) and 24:00 (5 lux) hours] as repeated measures by a means of open field test and elevated plus maze test. 06:00 and 12:00 measurements were performed during the light period and 18:00 and 24:00 measurements were performed during the dark period. In pinealectomy group, we started the measurements after three weeks of the pinealectomies, when surgery wounds healed up completely and animals behaved as normal (i.e., normal locomotor activity, absence of any anxiety like behaviors) as controls which were not sham-pinealectomized. There were at least three week intervals among the measurements in order to eliminate the familiarity of the rats with the test. Our previous observations showed us that the three week interval is approximately enough time to eliminate the ratôs familiarity with the test. Also, studies on

human subjects in psychology have frequently suggested the three week interval for test-re test reliability of some tests or measurements. Rats were first exposed to open field test and then elevated plus maze test was applied. The measurements in the dark period were performed under red dim light.

In the second study, the forty five male adult rats were used and were randomly divided into three groups as melatonin injection, pinealectomy and melatonin implantation including controls (sham operated) for all groups. In sham pinealectomy, animals were exposed to the same surgical procedure with the experimental group except for the removal of the pineal gland. In sham melatonin implantation, they were implanted with the tubes including only beeswax. In sham injection group, they were injected with the same amount of saline solution. Spatial memory of Wistar rats were measured at 1800 hr by a means of Morris water maze test. In pinealectomy and implantation groups, we started the experiment after a week of the pinealectomies and implantations, when surgery wounds healed up completely.

2.3 Pinealectomy

Before surgery, the rats were anesthetized subcutaneously with Ketamine (20mg/kg, Sigma Chemical Company, St. Louis, Missouri, USA) and intraperiotoneally with pentobarbitol (32.5 mg/kg, Sigma Chemical Company, St. Louis, Missouri, USA). The depth of anesthesia was monitored by the frequent testing of leg reflexes and muscle tonus.

Pinealectomy of rats was performed according to the method of Hoffmann and Reiter (Hoffman and Reiter, 1965); aspiration was used to control the hemorraging. Anesthetized rats were placed in a stereotaxic apparatus to stabilize the head during surgery. After the head was shaved the surgical area was sterilized with 70% ethanol, an incision was made in the scalp. Muscle attachments were removed from the dorsal skull. After drying the skull, an incomplete circular cut was made with a dental drill burr at the λ (lambda) suture and a piece

of cranium covering the pineal gland was folded forward anteriorly. The fine-tipped forceps was used to extend into the confluence of the sinuses to grasp and remove the pineal gland. After removal of the pineal gland, the bone flap was replaced and a small square of absorbable gelatin sponge (Gelfoam, Up John, Kalamazoo, MI) was applied to the skull surface to help promote clotting. The scalp was closed with stainless steel surgical clips. After surgery, the incision was treated with Newskin adhesive to prevent any contamination. At the end of the experiment, pinealectomized animals were decapitated and checked for the security of the pinealectomy.

2.4 Melatonin Administrations

Melatonin (Sigma) was dissolved in ethanol and further diluted in saline; it was injected in a dose of 100 µg/kg (17:00 pm) intraperitoneally for 5 days.

Implants were prepared according to the methods of Horton et al., 1992. Crystaline melatonin (sigma) was dissolved in melted beeswax (1 mg melatonin/25 mg beeswax). Then the mixture was aspirated into 15 cm lengths of PE 320 tubing (2.69 mm i.d. x 3.5 mm o.d.; intramedic, clay Adams, Porsipony, NJ). When the beeswax had cooled to room temperature and hardened, the tubing was cut into 10 mm capsules. Control capsules were prepared in a similar way but filled with beeswax only. Implants were inserted s.c. in animals under ketamine and pentobarbital anesthesia through a small dorsal skin incision at a shaved area on the back. The wound was closed by a steel wound clip.

2.5 Open Field

Open-field test is taken place in a 80 cm×80 cm arena with 40 cm high walls. The open field has been the most widely used test in animal psychology. In this test, an animal (usually a rodent) is introduced into a plain and illuminated arena and its behavior is commonly regarded as a fundamental index of general behavior. In this experiment a video

camera (Gkb CC-28905S, Commat LTD.ķTĶ Ankara/Turkey) was mounted above the arena, recording behavior into the *Ethovision* videotracking system (Noldus Ethovision, Version 6, Netherland; Commat LTD.ķTĶ Ankara/Turkey) that provided a variety of behavioral measures including distance, time in the edge, time in the center, frequency in the edge, frequency in the center, mobility and velocity among the different areas of the arena. All animals were then returned to the breeding and exhibition colonies.

2.6 Elevated Plus Maze

The elevated plus maze consisted of the two open and two closed 10 cm wide arms in a plus-sign configuration 55 cm off the floor. The closed arms were enclosed by 41 cm tall black Plexiglas. All arms were covered with contact paper to prevent the animals from sliding off, and all surfaces were wiped with 70% alcohol between animals. Each animal was released into one of the closed arms and allowed to move freely on the maze for a 5-min testing period that was videotaped from above the maze. Animals that fell off the maze into compartments below were placed back on the maze for the remainder of the testing period. An observer uninformed about experimental conditions scored the videotapes with The Observer Software (EthoVision XT) (Noldus Ethovision, Version 6, Netherland; Commat LTD.ķTĶ Ankara/Turkey) for distance, duration in the open arm, frequency in the open arm, duration in the closed arms , frequency in the closed arms, mobility,and velocity. Animals were considered to have entered an arm when all four paws crossed onto the arm.

2.7 Morris Water Maze

For the spatial memory, the performances of the rats in the Morris water maze were evaluated. The experiments were carried out in a circular, galvanized steel maze (1,5 m in diameter and 60 cm in depth), which was filled with 40 cm deep water kept at 28 °C and rendered opaque by the addition of a non-toxic, water soluble dye. The maze was located in a

large quiet test room, surrounded by many visual cues external to the maze (e.g. the experimenter, ceiling lights, rack, pictures, etc.), which were visible from within the pool and could be used by the rats for spatial orientation. Locations of the cues were unchanged throughout the period of testing. A video camera fixed to the ceiling over the center of the maze was used for recording and monitoring movements of the animals. There were the four equally divided quadrants in the pool. In one of the quadrants, a platform (1.0 cm below water surface, 10 cm in diameter) was submerged centrally and fixed in position which was kept constant throughout the acquisition trials. The rats performed the four trials per day for the four consecutive days (16 trials). In the swimming trials, each individual rat was released gently into the water at a chosen quadrant except for the one that contained the hidden platform for facing an extra maze cue. The rat swam and learned how to find the hidden platform within 60 s. The escape latency which is the time elapsed before the rat reached the platform in each trial, is a measure of acquisition of spatial navigation. After reaching, the rat was allowed to stay on the platform for 15 s and was then taken back into the cage. During the inter-trial intervals, animals were kept in a dry home cage for 60 s.

A platform (10 cm diameter) was placed in one of the four maze quadrants (the target quadrant) and submerged 1.5 cm below the water surface. The platform remained in the same quadrant during the entire experiment. The rats were required to find the platform using only distal spatial cues available in the testing room. The cues were maintained constant throughout the testing. The rats received the four consecutive daily training trials. The video camera recordings were obtained on the fifth day of the experiment. The rat had to swim until it climbed onto the platform submerged underneath the water. The escape platform was kept in the same position relative to the distal cues. The time to reach the platform (latency in seconds), total distance travelled, time passed in the correct quadrant, entrance frequency to the corret quadrant, and immobility were measured as the indexes of the spatial memory. In

this experiment a video camera (Gkb CC-28905S, Commat LTD.ķTĶ Ankara/Turkey) was mounted above the arena, recording behavior into the *Ethovision* videotracking system (Noldus Ethovision, Version 6, Netherland; Commat LTD.ķTĶ Ankara/Turkey) that provided a variety of behavioral measures including distance, time in the edge, time in the center, frequency in the edge, frequency in the center, and immobility among the different areas of the arena. All animals were then returned to the breeding and exhibition colonies.

2.8 Statistical Analyses

In the first study, data were analyzed using SPSS (SPSS Statistical Software, SPSS Inc., Los Angeles, CA, USA, Ver. 15.0). Data were analyzed by 2 (pinealectomy and control) X 4 (time of the day: 06:00, 12:00, 18:00, and 24:00) ANOVA analysis with the last factor as a within subject or repeated design. Significant ANOVA results were also tested by the post test, namely Tukey test which is assumed to be a strong test for comparison of groups that has equal variance and sample size. Values were considered statistically significant at p Ò 0.05. Data are presented as MEAN ± SEM after back transforming from ANOVA results.

In the second study, data were analyzed using SPSS (SPSS Statistical Software, SPSS Inc., Los Angeles, CA, USA,Ver. 15.0). Data were examined by a means of 2 (the presence of treatment: treatment and control (sham) group) X 3 (the type of treatment groups: injection, pinealectomy, and implantation) between ANOVA design. The Post hoc test, Tukey test, was conducted to test significant effects regarding the type of treatment groups. Since each treatment group had its own control (sham) group, comparisons between treatment group and control group for each one was tested by the independent t test. Values were considered statistically significant at $p<0.05$. Data are presented as MEAN ± SEM after back transforming from ANOVA results.

3. RESULTS

STUDY 1

3.1 Open Field Measurements

Total distance travelled on the open field (TTOF)

The main effect of the measurement time was significant on the total distance travelled in the open field, F (3, 14) = 4.82, p = 0.02. There were significant differences between the time 06:00 hr (M = 1275.65) and the time 24:00 hr (M = 1862.55; t(17) = 4.00, p = 0.001), between the time 12:00 hr (M = 1304.66) and the time 24:00 hr (M = 1862.55; t (17) = 2.62, p = 0.02), between the time 18:00 hr (M = 1342.62) and the time 24:00 hr (M = 1862.55; t (17)=3.43, p = 0.003) on the total distance travelled **(Table 1 and Figure 1.A).**

No significant effects related to the pinealectomy (F (1,16) =1 .55 p = 0.23) or interaction thereof (F(3, 14) = 0.51, p = 0.68) were evident in terms of the total distance travelled in open field.

Time spent at the edge of the open field (TEOF)

No significant effects related to the measurement time (F(3, 14) = .71, p = 0.56) or pinealectomy (F(1,16) = .16, p = 0.70) or interaction thereof (F(3, 14) = 1.01, p = 0.42) were evident in amount of time spent at the edge of the open field **(Table 1)**.

Time spent at the center of the open field (TCOF)

No significant effects related to the measurement time (F(3, 14) = .71, p = 0.56) or pinealectomy (F(1,16) = .16, p = 0.70) or interaction thereof (F(3, 14) = 1.01, p = 0.42) were evident in amount of time spent at the edge of the open field **(Table 1)**.

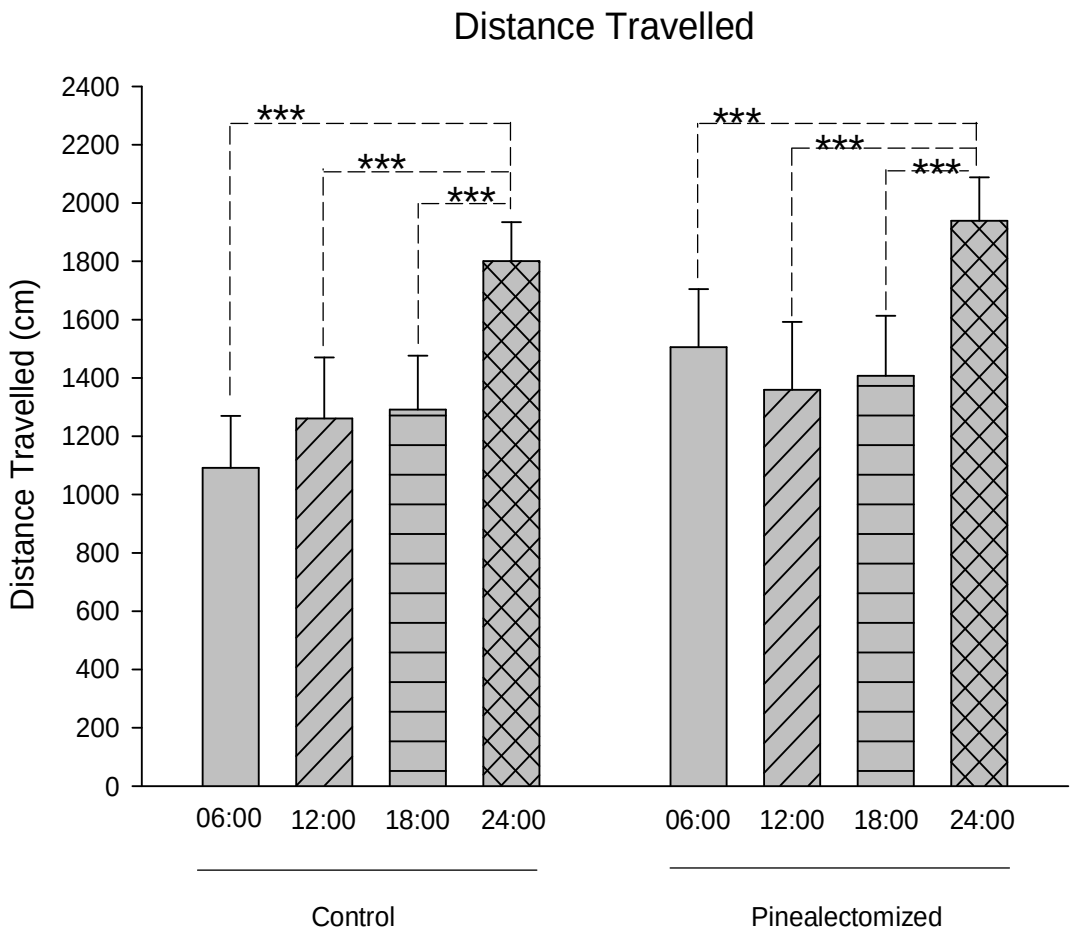

Figure 1.A: Mean (S.E.M.) Total amount distance travelled (TDT). Values at distal ends of horizontal bar differ, *p **Ò**0.05, **p **Ò** 0.01, ***p **Ò**0.005.

Total entry to the edge of the open field (TEEOF)

No significant effects related to the measurement time ($F(3, 14) = .17$, $p = 0.92$) or pinealectomy ($F(1,16) = .77$ $p = 0.39$) or interaction thereof ($F(3, 14) = .86$, $p = 0.49$) were evident in the frequency of total entries to the edge of the open field **(Table 1)**.

Total entry to the center of the open field (TECOF)

No significant effects related to the measurement time ($F(3, 14) = .14$, $p = 0.94$) or pinealectomy ($F(1,16) = .65$, $p = 0.43$) or interaction thereof ($F(3, 14) = .90$, $p = 0.47$) were evident in the frequency of total entries to the center of the open field **(Table 1)**.

Mobility in Open field

The main effect of the measurement time was significant on the mobility, $F(3, 14) = 4.01$, $p = .03$. There were significant differences between the time 6:00 hr (M = .0001 min) and the time 24:00 hr (M = .0013; $t(17) = 2.72$, $p = 0.02$), between the time 12:00 hr (M = .0001) and the time 24:00 hr (M = .0013 s; $t(17) = 2.76$, $p = 0.01$), between the time 18:00 hr (M = .0000) and the time 24:00 hr (M = .0013; $t(17)=3.20$, $p = 0.005$) on the mobility in open field **(Table 1 and Figure 1.B)**.

No significant effects related to the pinealectomy ($F(1,16) = 0.17$ $p = 0.68$) or interaction thereof ($F(3,14) = .32$, $p = 0.81$) were evident in the time (second) of mobility in open field.

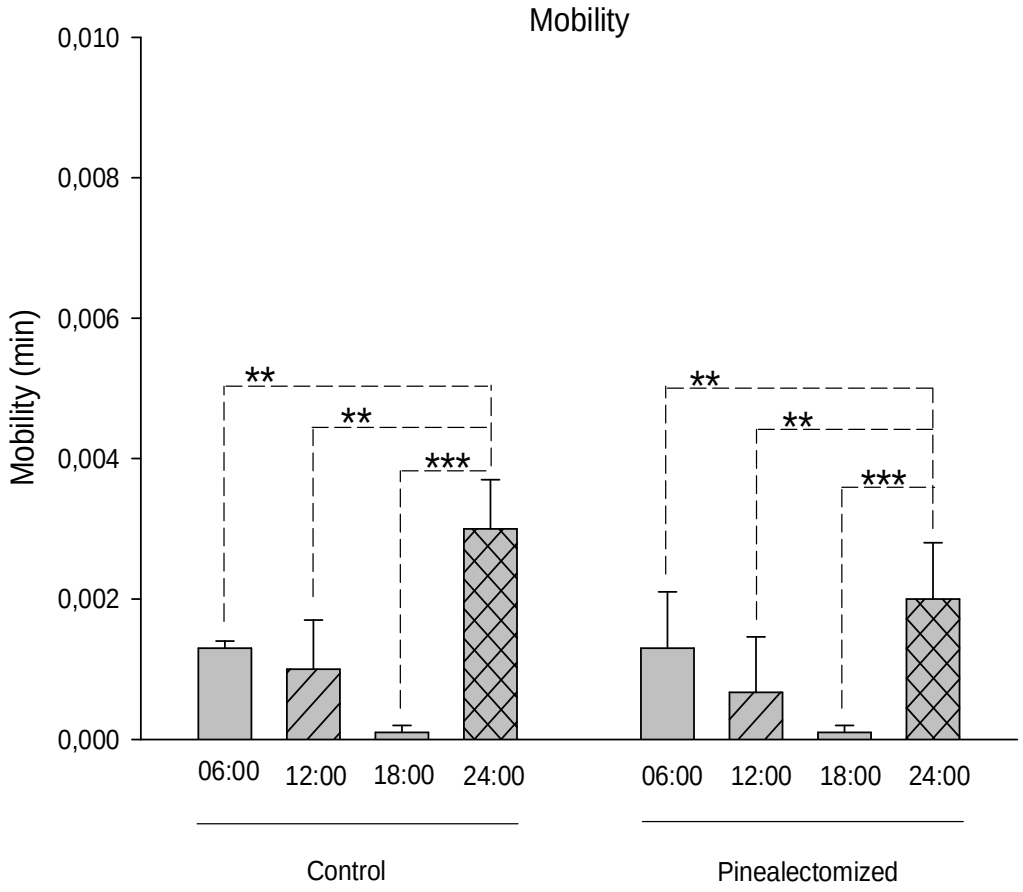

Figure 1.B: Mean (S.E.M.) The amount of mobility in the open-field behaviour apparatus (EPM) Values at distal ends of horizontal bar differ, *p **Ò**0.05, **p **Ò** 0.01, ***p **Ò**0.005.

Velocity in open field

The main effect of the measurement time was significant on the velocity in the open field, F (3, 14) = 4.85, p = .02. There were significant differences between the time 06:00 hr (M = 255.38) and the time 24:00 hr (M = 373.41; t(17) = 4.02, p = 0.001), between the time 12:00 hr (M = 261.75) and the time 24:00 hr (M = 373.41; t (17) = 2.61, p = 0.02), between the time 18:00 hr (M = 268.94) and the time 24:00 hr (M = 373.41; t (17)=3.44, p = 0.003) on the velocity in open field **(Table 1 and Figure 1.C).**

No significant effects related to the pinealectomy (F (1,16) = 1.54, p = 0.23) or interaction thereof (F(3, 14) = .51, p = 0.68) were evident in terms of the velocity of rats in open field.

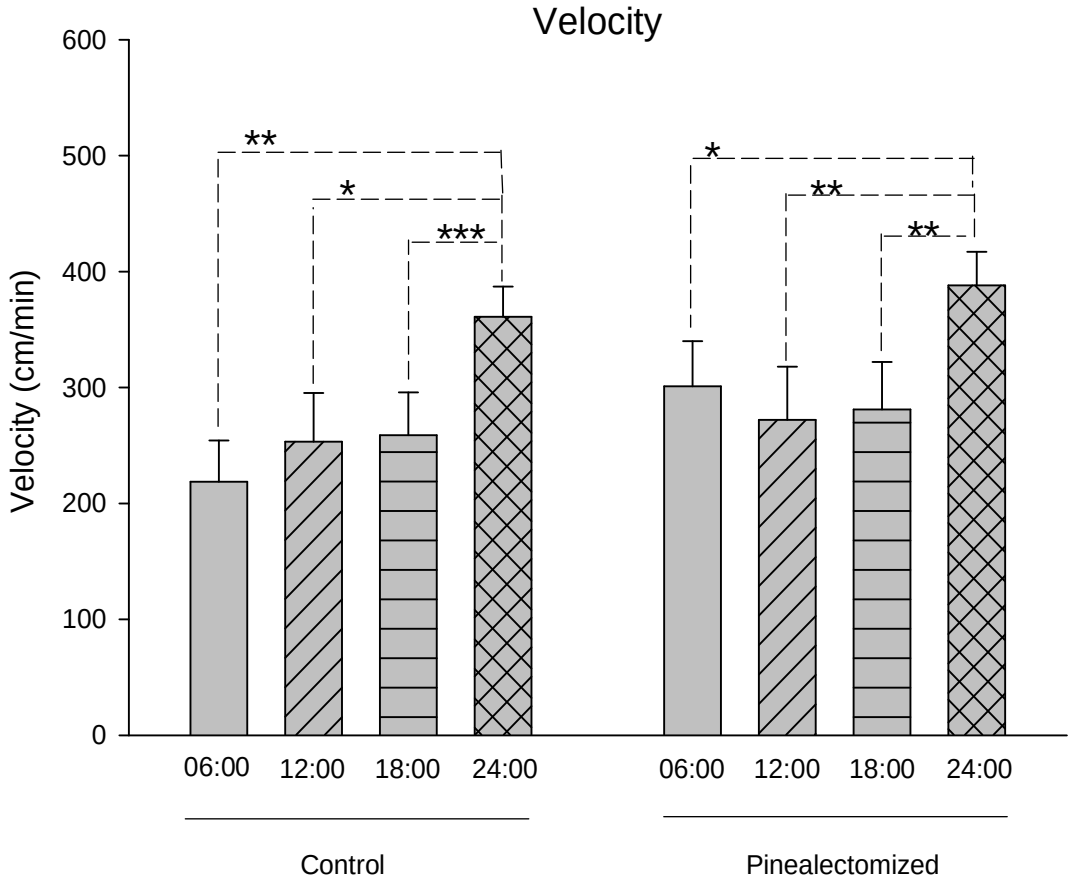

Figure 1.C: Mean (S.E.M.) The velocity of the male rats exposed to different times of the day. Values at distal ends of horizontal bar differ, *p **Ò**0.05, **p **Ò** 0.01, ***p **Ò**0.005.

3.2 Elevated Maze Measurements

Total distance travelled on the elevated arm maze (TDTEAM)

The main effect of the measurement time was significant on the total distance travelled, F (3, 13) = 3.73, p = .03. There were significant differences between the time 6:00 hr (M = 1350.68) and the time 18:00 hr (M = 1011.96; t(18) = 2.02, p = 0.05), between the time 12:00 hr (M = 1244.31) and the time 18:00 hr (M = 1011.96; t (18) = 2.03, p = 0.01), between the time 18:00 hr(M = 1011.96) and the time 24:00 hr (M = 1452.37; t (18)=2.80, p = 0.01) on the total distance travelled **(Table 2 and Figure 2.A)**.

On the other hand, no significant effects related to the pinealectomy (F(1,15) = .79 p = 0.52) or interaction thereof (F(3, 15) = .79, p = 0.52) were evident in amount of the total distance travelled on the elevated arm maze.

Time spent in open arms (TOA)

No significant effects related to the measurement time (F(3, 13) = .46, p = 0.72) or pinealectomy (F(1,15) = .67 p = 0.43) or interaction thereof (F(3, 15) = .67, p = 0.59) were evident in amount of time spent in open arms (TOA) **(Table 2).**

Total entry in open arms (TEOA)

No significant effects related to the measurement time (F(3, 13) = .39, p = 0.77) or pinealectomy (F(1,16) = .59 p = 0.45) or interaction thereof (F(3, 15) = .04, p = 0.77) were evident in the frequency of total entries to open arms **(Table 2).**

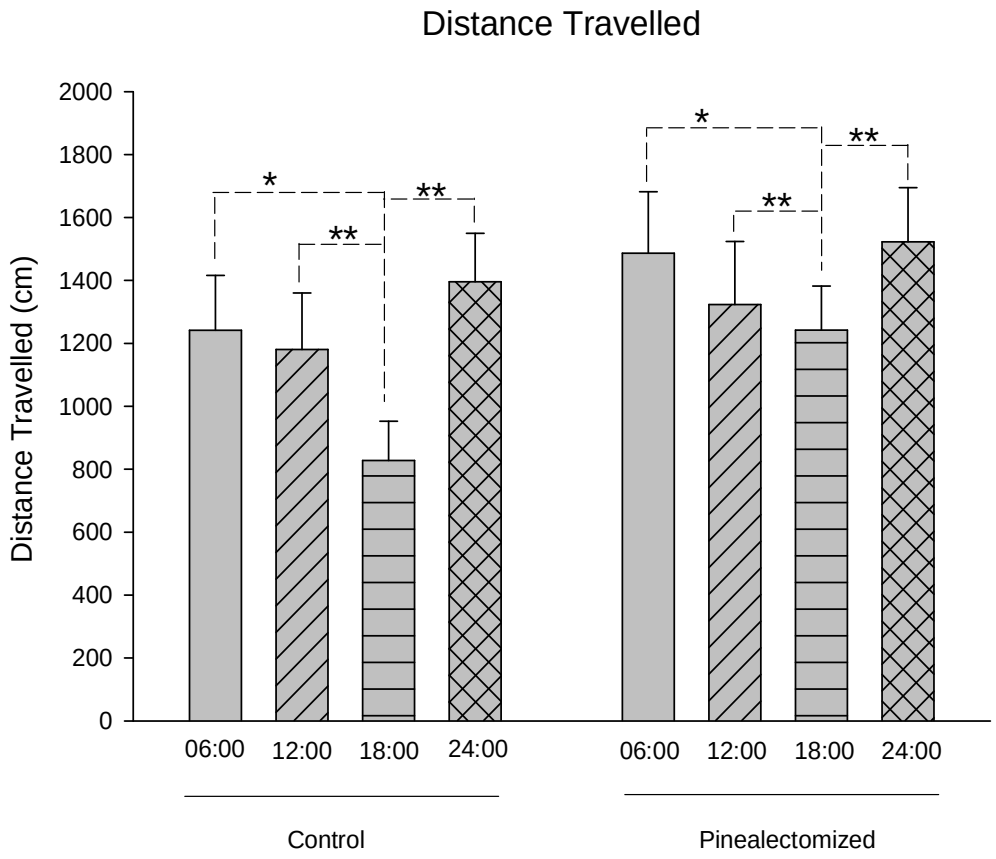

Figure 2.A: Mean (S.E.M.) Total amount distance travelled (TDT). Values at distal ends of horizontal bar differ, *p **Ò**0.05, **p **Ò** 0.01, ***p **Ò**0.005.

Time spent in closed arms (TCA)

No significant effects related to the measurement time ($F(3, 13) = 1.03$, $p = 0.41$) or pinealectomy ($F(1,16) = 1.02$ $p = 0.32$) or interaction thereof ($F(3, 14) = .28$, $p = 0.83$) were evident in amount of time spent in closed arms (TCA) **(Table 2).**

Total entry to closed arms (TECA)

The main effect of the measurement time was significant on the frequency of total entries in closed arms (TECA), $F(3, 14) = 4.24$, $p = 0.02$. There were significant differences between the time 6:00 hr (M = 3.23) and the time 24:00 hr (M = 2.22; $t(17) = 2.10$, $p = 0.05$), between the time 12:00 hr (M = 2.45) and the time 18:00 hr (M = 3.82; t (17) = 3.35, p = 0.004), between the time 18:00 hr(M = 3.82) and the time 24:00 hr (M = 2.22; t (17)=2.92, p = 0.01) on the frequency of total entries in closed arms (TECA) **(Table 2 and Figure 2.B).**

An interaction effect between the time measured and pinealectomy was also significant, $F(3, 14) = 5.37$, $p < 0.01$. This effect reflected the fact that the superiority of the control condition over pinealectomy condition in terms of the frequency of total entries in closed arms was higher in the time 6:00 hr (control (M = 3.85) and pinealectomy (M = 2.47)) and 18:00 hr (control (M = 4.14) and pinealectomy (M = 3.43)) but not significant in the time 12:00 hr (control (M = 2.76 and pinealectomy (M = 2.06)). However, the superiority of the pinealectomy condition over control condition in terms of the frequency of total entries in closed arms was higher in the time 24:00 hr (pinealectomy (M = 3.02 and control (M = 1.58)) **(Table 2 and Figure 2.B).**

No significant effects was found for the pinealectomy ($F(1,16) = 1.05$, $p = 0.32$).

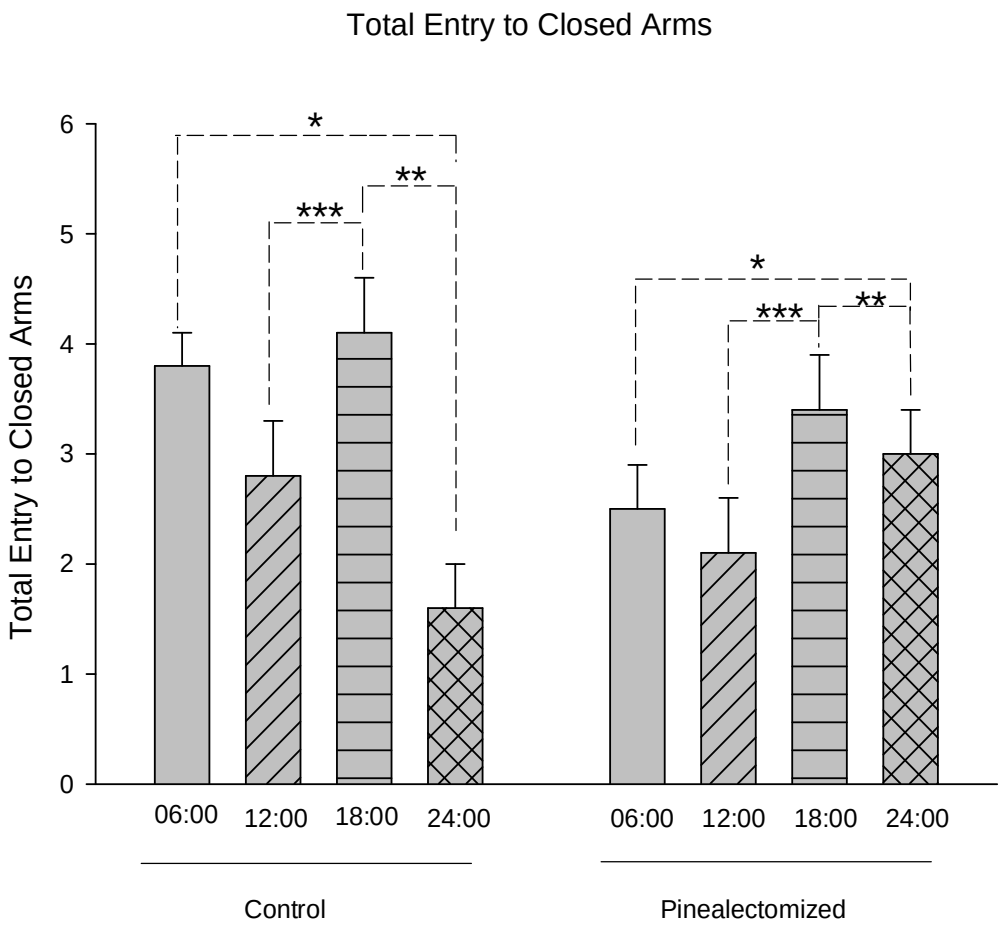

Figure 2.B: Mean (S.E.M.) number of entries into closed arms. Values at distal ends of horizontal bar differ, *p **Ò**0.05, **p **Ò** 0.01, ***p **Ò**0.005.

Mobility in Elevated maze

The main effect of the measurement time was significant on the mobility, F (3, 8) = 8.60, p = .007. There were significant differences between the time 6:00 hr (M = .96 min) and the time 24:00 hr (M = 1.63; t(13) = 3.36, p = .006), between the time 12:00 hr (M = 1.08) and the time 24:00 hr (M = 1.63 s; t (13) = 2.67, p = .02), between the time 18:00 hr(M = 1.08) and the time 24:00 hr (M = 1.63; t (13)=3.02, p = .01) on the mobility in elevated maze **(Table 2 and Figure 2.C).**

No significant effects related to the pinealectomy (F(1,10) = 3.27 p = 0.10) or interaction there of (F(3, 8) = 1.17, p = 0.38) was evident in the time of mobility in elevated maze.

Velocity in elevated maze

No significant effects related to the measurement time (F(3, 14) = 1 .48, p = 0.26) or pinealectomy (F (1,16) = .28, p = 0.61) or interaction thereof (F(3, 14) = 1.15, p = 0.36) were evident in terms of the velocity of rats in elevated maze **(Table 2)**.

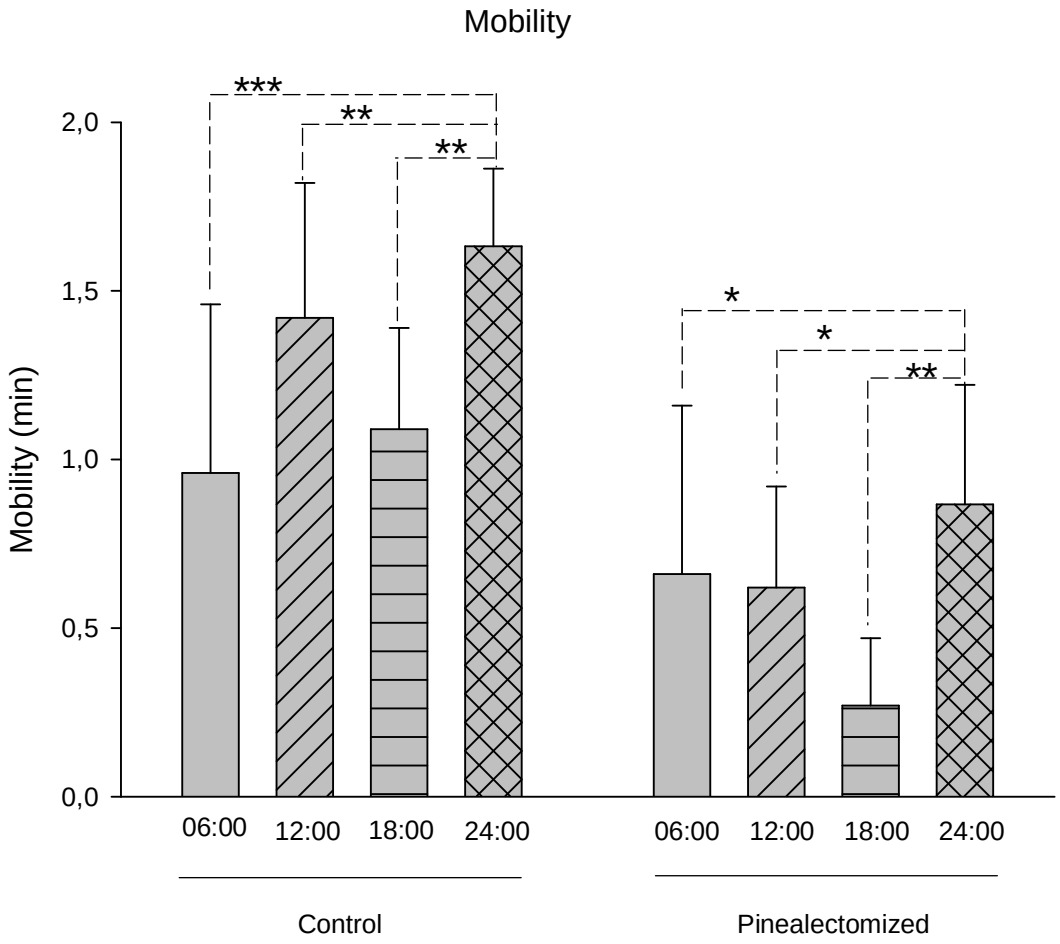

Figure 2.C: Mean (S.E.M.) the amount of mobility in the elevated plus maze apparatus (EPM), by male rats exposed to different times of the day. Values at distal ends of horizontal bar differ, *p **Ò**0.05, **p **Ò** 0.01, ***p **Ò**0.005.

STUDY 2

3.3 Water Maze Measurements

Total distance travelled on the Morris Water Maze

The main effect of the presence of treatment was significant on the total distance travelled on the Morris Water maze, F (1, 41) = 16.481, p = 0.0001, $\tilde{E}^2$ = .29. There were significant differences between (a) the injection group (M = 310.24) and control(sham) (M = 79.40; t(13) = 2.30, p = 0.04) and (b) between the pinealectomy group (M = 566.64) and control(sham) (M = 87.73; t (13) = 3.42, p = 0.005. However, there were no significant differences between the implantation group and control (sham) group, t (13) = .58, p > .57, **(Table 3 and Figure 3.A)**.

In addition, no significant effects related to the type of treatment groups, F (2, 41) = 1.48, p = 0.24, $\tilde{E}^2$ = .07, or interaction thereof (F (2, 41) = 2.74, p = 0.08, $\tilde{E}^2$ = .12) were evident in terms of the total distance travelled on the Morris Water Maze.

Time spent to find the platform

The main effect of the presence of treatment was significant on the time spent to the find the platform on the Morris Water maze, F (1, 41) = 25.66, p = 0.0001, $\tilde{E}^2$ = .39. There were significant differences between (a) the injection group (M = 14.08) and control(sham) (M = 3.39; t (13) = 2.44, p = 0.03) and (b) between the pinealectomy group (M = 25.96) and control(sham) (M = 4.17; t (13) = 4.24, p = 0.001. However, there were no significant differences between the implantation group and control (sham) group, t (13) = 1.34, p > .21, **(Table 3 and Figure 3.B)**.

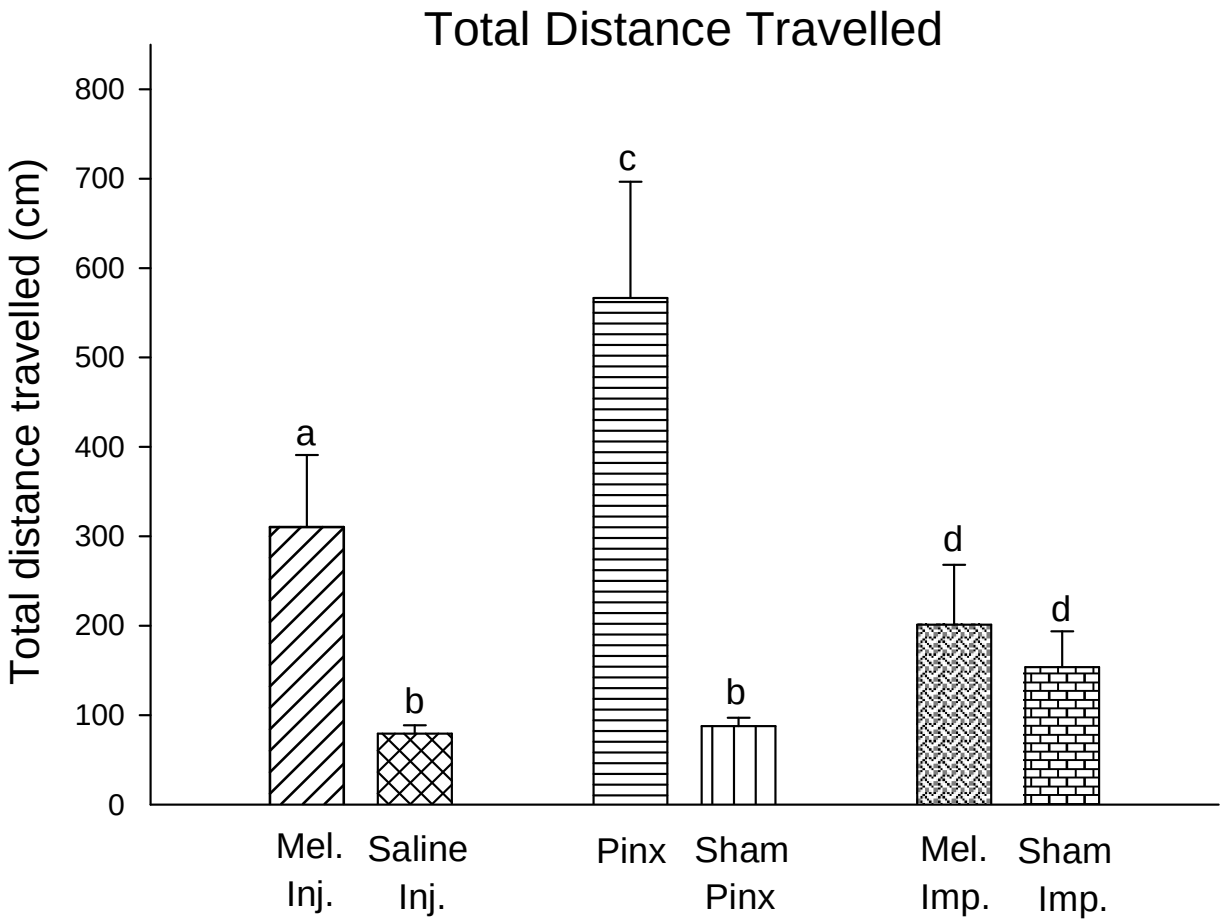

Figure 3.A: Total distance travelled (A), time spent to find the platform. Right striated bar represents Melatonin Injection (Mel. Inj.) (n:9); Cross striated bar represents Saline Injection (Saline Inj.) (n:6); Horizontally striated bar represents Pinealectomy (Pinx) (n:8); Vertically striated bar represents Sham Pinealectomy (Sham Pinx) (n:7); Herringbone striated bar represents Melatonin Implantation (Mel. Imp.) (n:8) and Bricks striated bar represents Sham Implantation (Sham Imp.) (n:7) groups. Different letters indicate the statistically different groups.

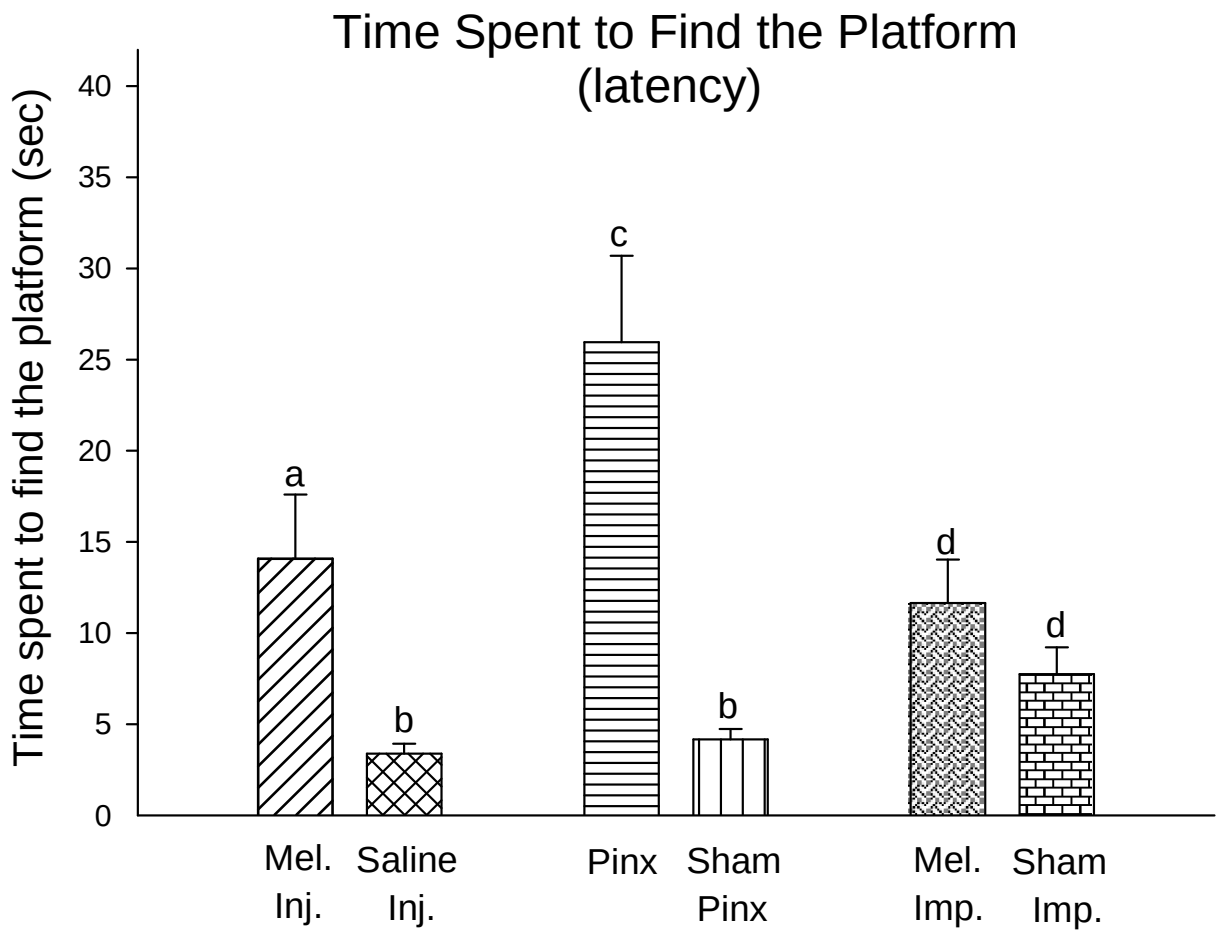

Figure 3.B: Time spent to find the platform. Right striated bar represents Melatonin Injection (Mel. Inj.) (n:9); Cross striated bar represents Saline Injection (Saline Inj.) (n:6); Horizontally striated bar represents Pinealectomy (Pinx) (n:8); Vertically striated bar represents Sham Pinealectomy (Sham Pinx) (n:7); Herringbone striated bar represents Melatonin Implantation (Mel. Imp.) (n:8) and Bricks striated bar represents Sham Implantation (Sham Imp.) (n:7) groups. Different letters indicate the statistically different groups.

In addition, no significant effects related to the type of treatment groups, F (2, 41) = 1.49, p = 0.24, $\tilde{E}^2$ = .07, or interaction thereof (F (2, 41) = 2.74, p = 0.08, $\tilde{E}^2$ = .12) were evident in terms of the total distance travelled on the Morris Water Maze.

Time spent in the quadrant of the platform

The main effect of the presence of treatment was significant on the time spent in the quadrant of the platform on the Morris Water maze, F (1, 41) = 21.72, p = 0.0001, $\tilde{E}^2$ = .35. There were significant differences between (a) the injection group (M = 0.07) and control (sham) (M = 0.03; t (13) = 2.90, p = 0.01) and (b) between the pinealectomy group (M = 0.17) and control(sham) (M = 0.04; t (13) = 3.95, p = 0.002. However, there were no significant differences between the implantation group and control (sham) group, t (13) = 0.79, p > .44, **(Table 3 and Figure 3.C)**.

Also, the main effect of the type of treatment groups was significant on the time spent in the quadrant of the platform on the Morris Water maze, F (2, 41) = 4.25, p = 0.02, $\tilde{E}^2$ = .17. The Tukey test showed that there were significant difference between the injection group (M = 0.08) and pinealectomy group (M = 0.17). However, no significant differences were detected between the injection group and implantation and between the implantation group and pinealectomy group **(Table 3)**. In addition, interaction thereof (F (2, 41) = 3.86, p = 0.03, $\tilde{E}^2$ = .16) was evident in terms of the time spent in the quadrant of the platform on the Morris Water Maze. This interaction indicated that the difference between treatment and control group in the pinealectomy (.13) in terms of the time spent in the quadrant of the platform was greater than those differences between these groups in injection(.06) and implantation(.03) **(Table 3)**.

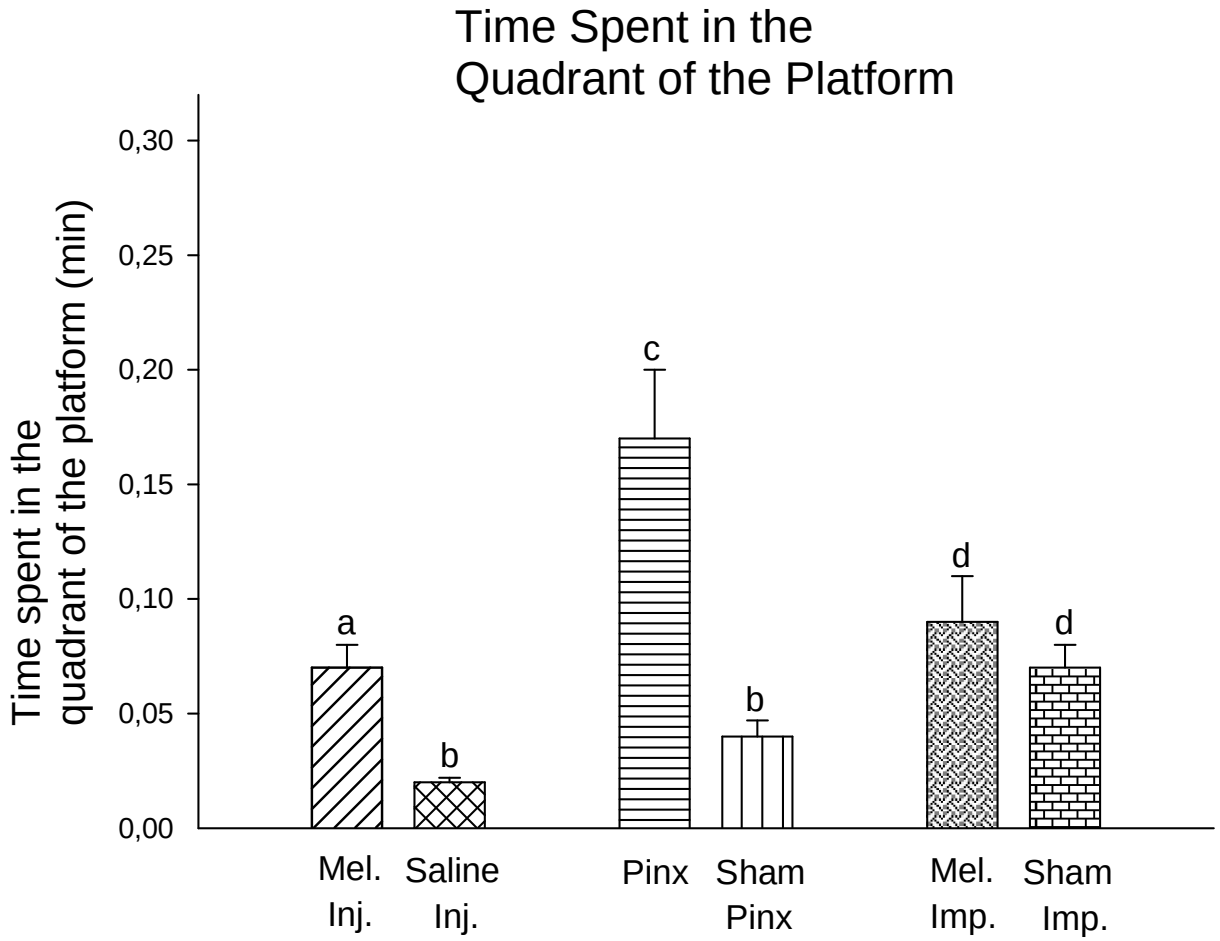

Figure 3.C: Time spent in the quadrant of the platform (C) in the Morris water maze are represented. Right striated bar represents Melatonin Injection (Mel. Inj.) (n:9); Cross striated bar represents Saline Injection (Saline Inj.) (n:6); Horizontally striated bar represents Pinealectomy (Pinx) (n:8); Vertically striated bar represents Sham Pinealectomy (Sham Pinx) (n:7); Herringbone striated bar represents Melatonin Implantation (Mel. Imp.) (n:8) and Bricks striated bar represents Sham Implantation (Sham Imp.) (n:7) groups. Different letters indicate the statistically different groups.

The entrance frequency to the quadrant of the platform

The main effect of the presence of treatment was significant on the entrance frequency to the quadrant of the platform on the Morris Water maze, F (1, 41) = 13.21, p = 0.001, $\tilde{E}^2$ = .24. There were significant differences between (a) the injection group (M = 2.44) and control(sham) (M = 1.00; t(13) = 2.20, p = 0.05) and (b) between the pinealectomy group (M = 2.50) and control(sham) (M = 1.14; t (13) = 2.30, p = 0.04. However, there were no significant differences between the implantation group and control (sham) group, t (13) = 1.14, p > .28, **(Table 3 and Figure 4.A)**.

In addition, no significant effects related to the type of treatment groups, F (2, 41) = .32, p = 0.73, $\tilde{E}^2$ = .02, or interaction thereof (F(2, 41) = .50, p = 0.61, $\tilde{E}^2$ = .02) were evident in terms of the entrance frequency to the quadrant of the platform on the Morris Water Maze.

Mobility Time

The main effect of the presence of treatment was significant on the mobility time on the Morris Water maze, F (1, 41) = 25.85, p = 0.0001, $\tilde{E}^2$ = .39. There were significant differences between (a) the injection group (M = 0.34) and control (sham) (M = 0.06; t(13) = 4.51, p = 0.001) and (b) between the pinealectomy group (M = 0.54) and control(sham) (M = 0.11; t (13) = 3.16, p = 0.007. However, there were no significant differences between the implantation group and control (sham) group, t (13) = 1.15, p > .26, **(Table 3 and Figure 4.B)**.

Also, the main effect of the type of treatment groups was significant on the mobility time on the Morris Water maze, F (2, 41) = 4.74, p = 0.01, $\tilde{E}^2$ = .19. The Tukey test showed that there were significant difference between the implantation group (M = 0.16) and pinealectomy group (M = 0.54). However, no significant differences were detected between the injection group and implantation and between the injection group and pinealectomy group

(Table). In addition, interaction thereof (F (2, 41) = 5.77, p = 0.006, $\tilde{E}^2$ = .22) was evident in terms of the mobility time on the Morris Water Maze. This interaction indicated that the difference between treatment and control group in the pinealectomy (.43) and injection (.30) in terms of the mobility time was greater than that difference between these groups in implantation (.03) **(Table 3)**.

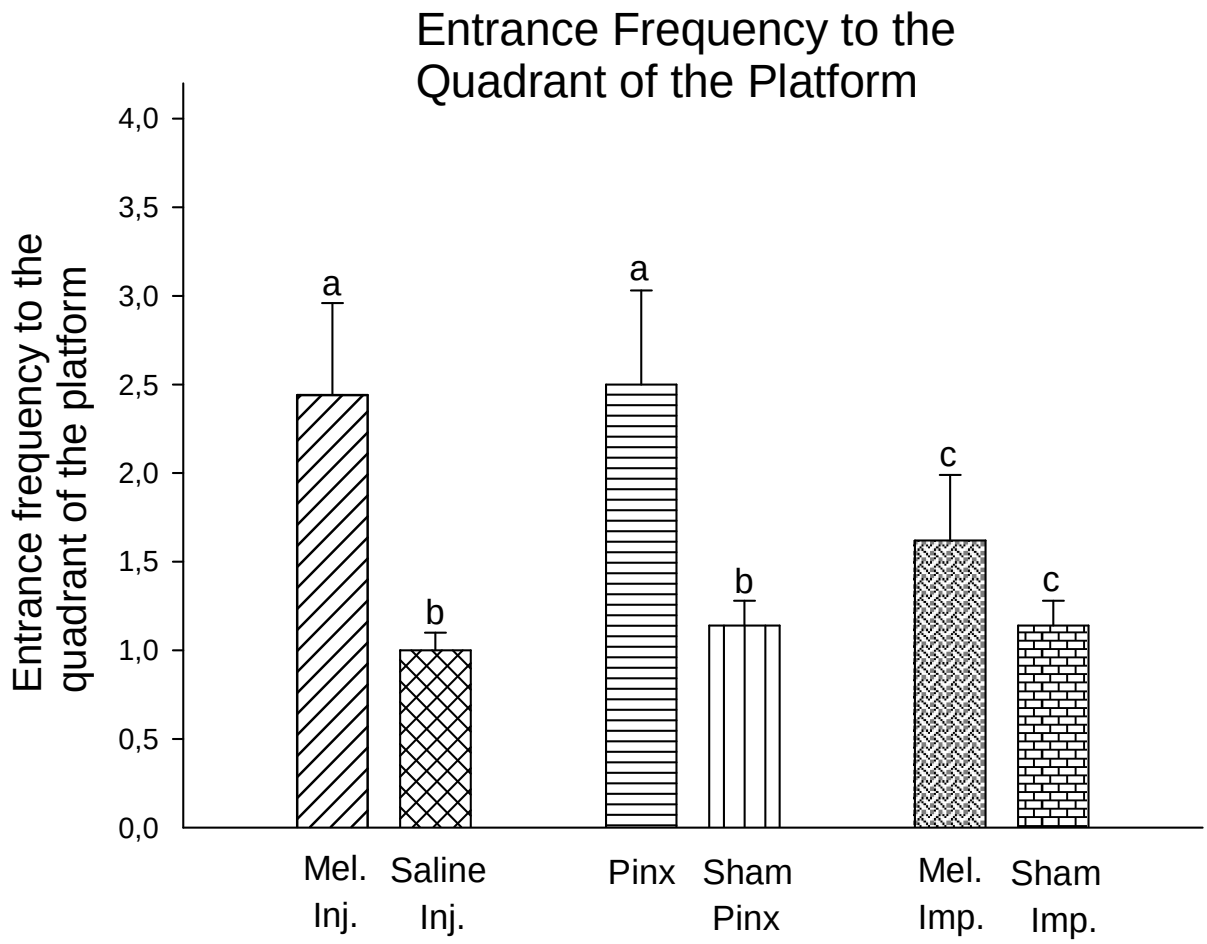

Figure 4.A: The entrance frequency to the quadrant of the platform. Right striated bar represents Melatonin Injection (Mel. Inj) (n:9).; Cross striated bar represents Saline Injection (Saline Inj.) (n:6); Horizontally striated bar represents Pinealectomy (Pinx) (n:8); Vertically striated bar represents Sham Pinealectomy (Sham Pinx) (n:7); Herringbone striated bar represents Melatonin Implantation (Mel. Imp.) (n:8) and Bricks striated bar represents Sham Implantation (Sham Imp.) (n:7) groups. Different letters indicate the statistically different groups.

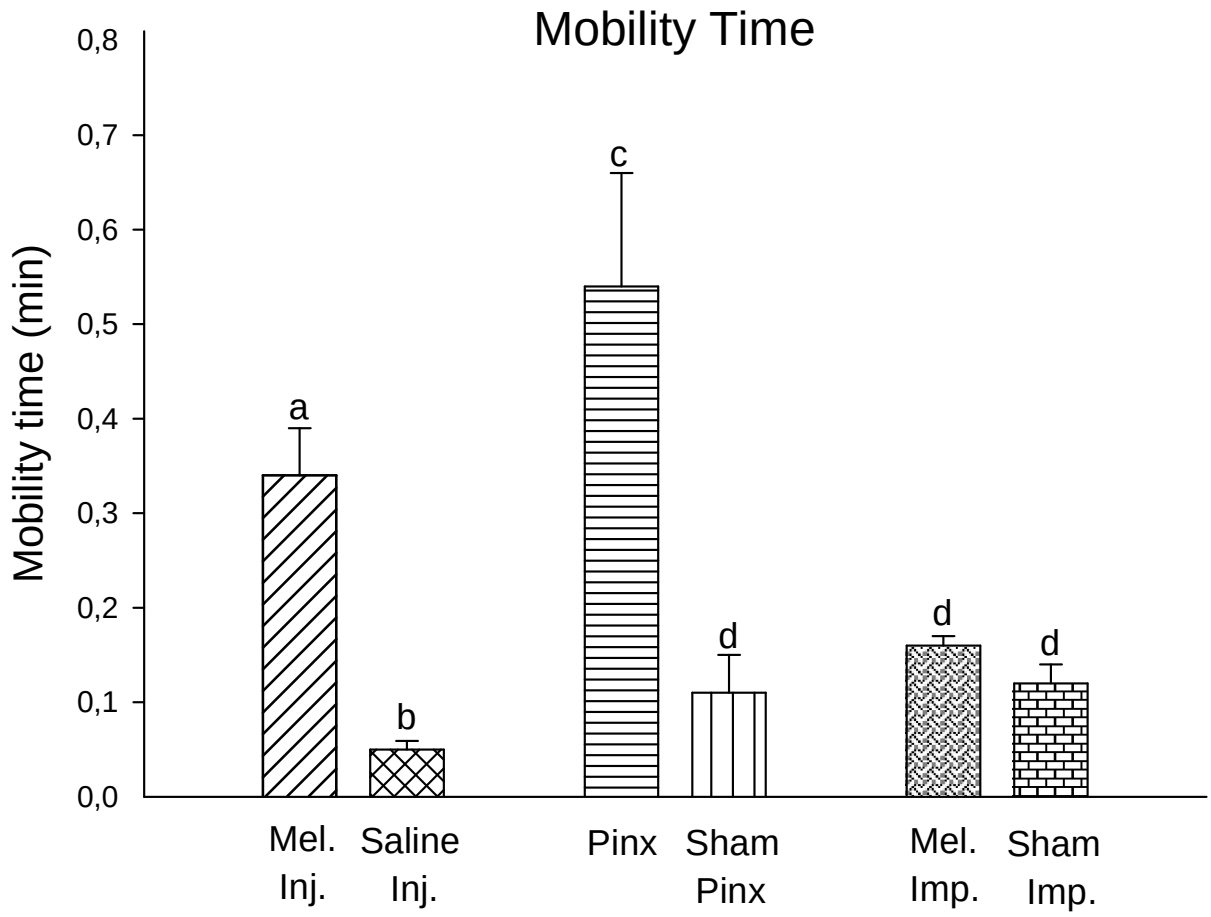

Figure 4.B: Mobility time in the Morris water maze are represented. Right striated bar represents Melatonin Injection (Mel. Inj) (n:9).; Cross striated bar represents Saline Injection (Saline Inj.) (n:6); Horizontally striated bar represents Pinealectomy (Pinx) (n:8); Vertically striated bar represents Sham Pinealectomy (Sham Pinx) (n:7); Herringbone striated bar represents Melatonin Implantation (Mel. Imp.) (n:8) and Bricks striated bar represents Sham Implantation (Sham Imp.) (n:7) groups. Different letters indicate the statistically different groups.

4. DISCUSSION

STUDY 1

The first study was mainly conducted to shed on lights on one important methodological flaw concerning the effects of different times of the day on anxiety like behaviors of Wistar rats because some of the previous studies were conducted when the animals were in their active period (Ashkenazy et al. 2009; Ashkenazy-Frolinger et al. 2009; Einat et al. 2006), while others were not (Benabid et al. 2008; Nava and Carta, 2001; Prendergast and Nelson, 2005; Pyter and Nelson, 2006) in the literature. The results of the present study, which provided an extensive research paradigm with regard to the effects of different times of day (06:00, 12:00, 18:00, and 24:00h), emerged the three principal findings that: 1) some of the parameters measured in both elevated plus maze and open field tended to show variations depending on the measurement time of the day; 2) pinealectomy was found to be ineffective on all of the measured parameters except for the total entry to closed arms in elevated plus maze; 3) the measured parameters in elevated plus maze were more sensitive to the measurement time effect than in open field.

Motor functions such as spontaneous activity is measured by the open field. Open field test is also used to measure the anxiety like behavior in rodents (Benabid et al. 2008). The total distance traveled, the total number of entries to the center and the edge of the open field, the time spent in the center of the open field versus time spent at the edge of the open field and the mobility are frequently used parameters measured in open field test in the literature (Pyter and Nelson, 2006). In this maze, if the anxiety of the animal is high, the number of the entries to the edge of the open field is increasing and the total distance traveled is decreasing. The total number of the entries into the center and the edges provides a built-in control measure for general hyperactivity or sedation.

In this study, the time points for measurement were 06:00, 12:00, 18:00 and 24:00. According to the time schedule, 06:00 was the time when the lights were turned on and 18:00 was the time when the lights were turned off. 12:00 is the midpoint of the light period and the 24:00 was the midpoint of the dark period. The present experiment provided some consistent results in both open field and elevated plus maze. Regarding open field test, the results of the present experiment have shown that rats are more relaxed during the dark phase but not at the beginning of the dark period (18:00). The measurement time of the day was found to be effective on the total distance traveled and the mobility of the rats in open field. The longest distance traveled was at the 24:00 h at night and the shortest distance traveled was at 06:00 h early in the morning. The highest mobility was recorded at 24:00 h. However, the measurement time was not found to be effective on time spent at the center and the edges of the open field and the total entries to the center and the edge of the open field. None of the parameters measured in the open field were affected by the removal of the pineal gland.

The elevated plus maze has been one of popular or widely used test to measure the anxiety like behaviours (Dawson and Tricklebank, 1995). In this maze, if the anxiety of the animal is high, the number of the entries to closed arms is increasing and the total distance traveled is decreasing. The total number of the entries into all arms provides a built-in control measure for general hyperactivity or sedation. Regarding elevated plus maze test, in the present study, the measurement time of the day was found to be effective on the total distance traveled. The longest distance traveled was at the 24:00 hour and the shortest was at 18:00 hour. Therefore, the biggest difference was in between the times 18:00 and 24:00. This outcome could be due to the fact that since the rats are nocturnal animals, they are more active at night which possibly explains the highest activity at 24:00 hour (Illnerova et al. 1983). The removal of the pineal gland did not affect the parameters measured since the results were not statistically different in control and pinealectomized animals at the same measurement time

point. The time spent in open and closed arms and total entries to open arms were not measurement time dependent. However, the total entries to closed arms were showing differences at different time points. The total entry was the highest at 18:00 hour. This may be due to the higher anxiety like behaviour of the rats at that time point. There was a significant interaction effect between the time measured and treatment. The total entrance number to the closed arm was greater in controls than pinealectomized rats. Mobility of the rats in elevated plus maze has shown some differences depending on the measurement time. The highest mobility was recorded at 24:00 h whereas the lowest one was at 06:00 h. The results of the distance traveled and the mobility of the rats were consistent to each other.

Pineal melatonin is important in the mammalian circadian organization. It has also been shown that pineal gland plays important roles in the regulation of the seasonal variation of the anxiety like behaviour (Naranjo-Rodriguez et al. 2000; Papp et al. 2006). Although, pinealectomy did not change the circadian rhythms such as locomotor activity rhythm in both free-running and light-dark cycle conditions in rats (Quay, 1970), several studies have shown the influence of melatonin on the regulation of the circadian rhythms in mammals (Laakso et al. 1992; Marumoto et al. 1996; Marumoto et al. 1996; Sharma, 2003). Our results, which showed that the anxiety like behaviour was not significantly affected by the pinealectomy in rats, are in good agreement with the findings of the previous studies (Quay, 1970; Finkelstein, 1978) that suggest pinealectomy does not make any significant effect on the circadian rhythms. The finding that pinealectomy alone did not have a significant effect on anxiety behavior is consistent with other research findings (Kovács et al. 1974; Juszcak et al. 1996), suggesting that the pineal gland is not basically involved in the behavioral performance.

There is evidence in the literature that suggesting the interaction of melatonin with central gama aminobutyric acid (GABA) neurotransmission. Melatonin has been shown to enhance the GABA in rat brain tissue *in vitro* (Niles et al. 1987; Coloma and Niles, 1988).

When melatonin was applied in vivo, it increased the GABA levels in several brain regions in rats (Rosenstein and Cardinali, 1986; Xu et al. 1995). In the present experiment we did not find a significant difference in between control and pinealectomy groups. This was expected because circadian rhythms have a continuous function. The release of the melatonin hormone in rats shows a diurnal pattern which is high throughout the darkness (Illnerova et al. 1983). However, in pinealectomy the rhythm of melatonin is abolished (Hoffman and Reiter, 1965). Consistent with this explanation, our result did not provide evidence of any significant effect of pinealectomy on anxiety like behaviors. On the other hand, there is a reciprocal relationship between SCN and melatonin. SCN is generating and controlling the circadian rhythm of melatonin while melatonin is acting on SCN as a negative feedback agent in order to control the activity of SCN. Thus the future research should examine the effects of exogenous melatonin administration to rats after pinealectomy.

The release of the melatonin hormone in mammals is basically under control of sympathetic nervous system. Environmental light is first perceived by the retina of the eye. Then it is transferred to the SCN through the retinohypothalamic tract. SCN is able to measure the ratio of light/dark periods. After SCN, light information is transferred to pineal gland by means of Para ventricular nucleus (PVN) and Superior Cervical Ganglia (SCG) of the spinal cord. The post synaptic axon terminals of the SCG are producing no epinephrine only in darkness. Because of this, melatonin is produced and released only in darkness via the control of the sympathetic nervous system (Klein et al. 1983). Both acute and chronic stress is increasing the activity of sympathetic nervous system and melatonin hormone. Depending on the GABAergic functions (Coloma and Niles, 1988; Niles et al. 1987; Rosenstein and Cardinali, 1986; Xu et al. 1995), melatonin may decrease anxiety like behavior through a negative feedback mechanism. The results of the present experiment suggest that, there may be a circadian rhythm of anxiety like behaviors. Since SCN is responsible for the generation

and the regulation of the circadian rhythms, it may also play an important role on the rhythm of anxiety like behaviors. The future studies should be conducted to examine the role of SCN on this matter.

Affective disorders may cause circadian dysfunction. Clinically depressed patients exhibit various abnormalities in circadian rhythms (Armitage et al. 2004; Crasson et al. 2004; Koenigsberg et al. 2004; Nagayama et al. 1992; Nikitopoulou and Crammer, 1976; Pflug et al. 1981; Souetre et al. 1988; Stampfer, 1998; Taillard et al. 1990; Tsujimoto et al. 1990; Wehr and Wirz-Justice, 1982; Yehuda et al. 1996). The small reduction in the amplitude of various daily rhythms (Nagayama et al. 1992) and the change in the phase of the rhythms are the common abnormalities (Crasson et al. 2004; Koenigsberg et al. 2004) Opposite to this, many studies are suggesting that the circadian dysfunction may also cause affective disorders (Ehlers et al. 1988; Goodwin et al. 1982; Kripke et al. 1978; Szuba et al. 1992; Healy and Waterhouse, 1995). For instance, it has been suggested that bipolar disorder is a result of an abnormally phase advanced clock. This suggestion is supported by the animal studies indicating that administration of many antidepressant drugs affected the circadian organization since depression is a result of circadian disorganization and the antidepressants relieve depression (Duncan et al. 1988; Duncan and Schull, 1994; Hallonquist et al. 1994; Kripke and Wyborney, 1980; Nagayama, 1996; Possidente et al. 1992). All these results support that there may also be a circadian control in anxiety like behaviour which is consistent with the results of our experiment.

In conclusion, the results of the present experiment have indicated that the data coming from the elevated plus maze and the open field are consistent to each other. However, our results have suggested that elevated plus maze measurements were more sensitive to the time of the day than open field measurements since the differences were more significant in this maze. The results of the present experiment suggest that in order to obtain more reliable

tests for measuring anxiety like behaviors and eliminating circadian variations, the experiments should be performed throughout the active period of the animals and all animals should be tested at the same measurement time. These results of the present experiment may shed lights on some inconsistencies of the findings of the previous studies with regard to the effects of measurement time of the day on the anxiety like behavior of the Wistar albino rats. Taken together, these results are unique contribution to the field of anxiety like behavior in the literature.

STUDY 2

The aim of the second study was to investigate the effect of melatonin hormone injection, pinealectomy, and melatonin implantation on the spatial memory performance of Wistar albino rats in the Morris water maze. In sum, the results of this study indicated that (a) melatonin injection impaired the spatial memory performance in terms of distance travelled, time spent to find the platform (latency), the frequency of entrance to the quadrant of the platform, and mobility (b) removal of the pineal gland reduced the spatial memory performance in terms of distance travelled, time spent to find the platform, time spend in the quadrant of platform, the frequency of entrance to the quadrant of the platform, and mobility, (c) melatonin implantation did not significantly affect the spatial memory performance in terms of distance travelled, time spent to find the platform, time spend in the quadrant of platform, and mobility.

In this study it was found that pinealectomy decreased the spatial memory capacity on the Morris Water maze. Despite the well-known fact that the removal of the pineal gland (pinealectomy) abolishes the rhythmic endogeneous melatonin release and decreases plasma levels of melatonin significantly (Hoffman and Reiter, 1965), studies on the behavioral effects of it have shown no significant or little role of pinealectomy. For instance, there is some

research evidence that pinealectomy itself did not produce detrimental effect on cognitive performance in rats but its combination with the lesion of habenula did it (Lecourtier et al., 2005). Consistent with this evidence, another research has also shown that pinealectomy did not have a significant role on the acquisition and extinction of the active avoidance behavior (Appenrodt and Schwarzberg, 2003). In a similar vein, other studies that focused on some behavioural measures such as anxiety behavior (Appenrodt and Schwarzberg, 2000), passive avoidance learning (Appenrodt and Schwarzberg, 1999), open field exploratory activity (Kovács et al., 1974), and social recognition (Appenrodt et al., 2002) did not provide evidence for the role of pinealectomy. The differences between the previous studies and the present study could be due to the test employed in these studies. In the literature the Morris water maze has been reported to be very sensitive to some exogenous modifications such as nutrition procedure, stress, infection, and etc. as well as endogenous factors such as sex, age, strain differences (Hooge and De Deyn, 2001). In addition to this test difference, some behavioral measures such as distance travelled, time spent to find the platform, time spend in the quadrant of platform, the frequency of entrance to the quadrant of the platform, and mobility could also be very sensitive to pinealectomy effects. Consistent with this explanation, the present study has also provided evidence that pinealectomized rats in contrast to its controls did spend more time in the quadrant of the platform and this trend was greater in pinealectomy than injection and implantation groups. This evidence, which indicated that pinealectomy effect was more evident in terms of time spend in the quadrant of the platform on the Morris water maze, suggests that pinealectomy interacts with the nature of test or has test specific characteristics. Another support for this explanation comes from an interaction effect indicating that pinealectomized rats did show more immobility than their counterparts in this study. Therefore the future studies should be conducted to see whether or not pinealectomy has also other test-specific effects.

The present study has also showed a detrimental effect of melatonin injection on some measures of spatial memory in the water maze. In other words, melatonin injections elongated the latency and shortened the time passed in the correct quadrant. This finding was consistent with a more recent research indicating that low dose of melatonin given from weaning did lead to learning and memory deficit in rats (Cao et al., 2009). However, this outcome was not in line with some research findings indicating that the melatonin administered group shortened the mean latency on the third and fourth days of training trials compared to the control group and spent more time in the correct quadrant compared to the control group (Gönenç et al., 2005). The plausible explanation for this difference could be found in drug dose or regimen. Gönenç et al. (2005) tested the effect of melatonin after acute ethanol treatment in rats. In our study the rats were administrated melatonin (100 µg/kg, 17:00 pm) intraperitoneally for 5 days without any acute ethanol treatment in spatial memory. Another difference is that in our study drug regimen (100 µg/kg) is different from that of other research (10 mg/kg: Gönenç et al. 2005). In Cao et al study (2009) the low doses of melatonin injection (i.e., 3 mg/kg) was implemented. This suggests that physiological concentrations or doses of melatonin are more effective than pharmacological concentrations of it. We concluded that there may be a linear relationship between melatonin doses and its affects on spatial memory. It is a well known fact that melatonin readily passes all cell membranes, including the blood-brain barrier (Reiter et al., 1993). Melatonin binding sites exist in various brain structures such as the hippocampus and prefrontal cortex are considered to involve in memory function (Brzezinski, 1997; Ekmekcioglu, 2006; Mazzucchelli et al., 1996; Savaskan et al., 2001; Savaskanet al., 2005). Moreover, considering that melatonin is a potent sleep inducing enhanced consolidation of hippocampus-dependent memories (Jern et al., 1991; Rasch et al., 2007), it is possible that ósleep-likeô melatonin effects on consolidation in the aftermath of encoding added to its

effects on encoding. Despite this evidence, exact mechanism of melatonin concerning cognitive performance is still not known and there are some plausible explanations. One explanation deals with its pathway. Melatonin could have direct or indirect effect on memory. Some studies have provided evidence for its direct effect. For instance, a research has suggested that melatonin could be involved in structural remodeling of synaptic connections during memory and learning processes (Baydas etal., 2002). Other research has also suggested that melatonin may influence memory formation in the hippocampus (El Sherif et al., 2003). In addition to its direct action, indirectly, melatonin may act as an antioxidant to reduce oxidative damage to the synapses in hippocampus and therefore improves learning and memory deficits. Tuzcu and Baydas (2006) have found an evidence indicating that melatonin significantly ameliorated the cognitive impairment, reduced lipid peroxidation, and increased glutathione levels in diabetic rats. In conclusion, the effect of melatonin on learning performance could be in both ways. Even though the present study was not aimed to directly test this explanation, its results suggest that melatonin injection seems to have direct effect on spatial memory that has been related to limbic system of rat brain. Melatonin may also have an indirect effect on learning performance via some neurotransmitter such as gama aminobutyric acid (GABA), which was discussed later in this section. In addition, melatonin may also show its effects through its reciprocal relationship with some parts of rat brain such as suprachiasmatic nucleus (SCN), discussed later as well.

The other explanation for the effects of melatonin on learning performance is related with the circadian effects of melatonin. Several studies have shown the regulatory roles of melatonin in circadian rhythms (Brzezinzki, 1997; Borjigin et al., 1999; Arendt, 2000). For instance, our recent experiment has shown that daily injections of melatonin can entrain the activity rhythms of the pinealectomized Mongolian gerbils (*Meriones unguiculatus*) (unpublished data). This effect of melatonin might be due to the direct inhibition of

locomotor activity, rather than an effect on the circadian clock. Although, it was reported that the entraining effect was not distinguishable between intact and pinealectomized rats (Marumoto et al., 1996), it is still possible that the lack of effect of the melatonin implants in intact rats may have been due to maintenance of the endogenous melatonin rhythm from the pineal gland. These findings suggest that the effect of melatonin implants and pinealectomy differs among rodents.

The nature of the endocrine message communicated via the melatonin rhythm has been the subject of much investigation. The two different possibilities have been proposed to explain how melatonin conveys the photoperiod information to the reproductive axis (Reiter, 1991; Reiter, 1993). The duration hypothesis implies that the photoperiod induced modification in the length of the nocturnal melatonin rise, is the message that synchronizes biological and reproductive functions to the different seasons of the year (Reiter, 1987; Wade etal., 1997; Carter and Goldman 1983; Goldman et al., 1984; Maywood et al., 1990). The internal coincidence hypothesis implies that melatonin exerts an effect only when its circadian secretion is coincident with target tissue sensitivity. This hypothesis supposes that the time of presence of melatonin is important. (Stetson and Tan, 1983; Hong and Stetson, 1987; Stetson et al., 1986; . Stetson and Watson-Whitmyre, 1986; Watson-Whitmyre and Stetson, 1985; Gündüz and Stetson 2001a; Gündüz, & Stetson 2001b). In the present study, pinealectomy and only admistration of melatonin via timed injections caused impairment of the learning performance of the rats. Although the given examples above stand satisfactory explanations for the effects of melatonin on reproductive system, this may be helpful for the explanation of the endocrine message communicated via the melatonin hormone in learning and memory processes.

In the present experiment we found significant differences in between melatonin injection and their control (sham injection) animals administered saline via injections. This

was expected because evidence was existed for the interaction of melatonin with central gama aminobutyric acid (GABA) neurotransmission. There is some evidence that the GABA in rat brain tissue *in vitro* was enhanced by melatonin (Niles et al. 1987; Coloma and Niles, 1988). Melatonin has also shown to increase the GABA levels in several brain regions in rats when it was applied in vivo (Rosenstein and Cardinali, 1986; Xu et al. 1995). In conclusion, an increase in melatonin level via injection may also affect the GABA, an inhibitory neurotransmitter, which in turn may decrease the neural transmission in the limbic system. Through this way, melatonin injection may show its impairing effect on learning and memory processes. Melatonin hormone may also show its effect through an interaction with suprachiasmatic nucleus (SCN). While SCN is generating and controlling the circadian rhythm of melatonin, melatonin hormone is also acting on SCN as a negative feedback agent in order to control the activity of the SCN. It is well known fact that the release of the melatonin hormone in rats shows a diurnal pattern which is high throughout the darkness (Pickavance et al., 1998). However, in pinealectomy the blood melatonin levels drop significantly and the rhythm of melatonin is abolished (Chapmann, 1970). Thus the future research on learning behaviour should examine the effects of exogenous melatonin administration to rats after pinealectomy and SCN lesions.

Melatonin implants did not have significant effects on the spatial memory performance of the rats in the Morris water maze. The rats implanted with the capsules containing melatonin showed the similar performances on the water maze with their controls implanted with the capsules containing only beeswax. These results may suggest that the continuous exposure to melatonin decreases the sensitivity of the brain regions comprising the limbic system rendering the learning performance. The timed administration of melatonin via single daily injections was more effective than implantations since injections impaired the spatial memory performance in the present study. This suggests that the perception of the

endogenous melatonin rhythm may be required for the animals to respond to the treatments. Our study stands a single one that has touched the issue of the relationship between melatonin implants and spatial memory. As far as it is known, no study has been reported about the effect of melatonin implants on spatial memory except for our present study. Consistent with the research finding on reproductive system (Karakaк and Gündüz, 2003), the present study has indicated no effect of melatonin implantation on spatial memory.

In conclusion, the results suggest that while the removal of the pineal gland and exogenous administration of melatonin via injections are causing impairment, constant release melatonin administration via implantation does not affect the learning performance and the spatial memory.

5. CONCLUSION

The results of the first experiment have indicated that the data coming from the elevated plus maze and the open field are consistent to each other. However, our results have suggested that elevated plus maze measurements were more sensitive to the time of the day than open field measurements since the differences were more significant in this maze. The results of the present experiment suggest that in order to obtain more reliable tests for measuring anxiety like behaviors and eliminating circadian variations, the experiments should be performed throughout the active period of the animals and all animals should be tested at the same measurement time. These results of the present experiment may shed lights on some inconsistencies of the findings of the previous studies with regard to the effects of measurement time of the day on the anxiety like behavior of the Wistar albino rats. Taken together, these results are unique contribution to the field of anxiety like behavior in the literature.

The results of the second experiment suggest that while the removal of the pineal gland and exogenous administration of melatonin via injections are causing impairment, constant release melatonin administration via implantation does not affect the learning performance and the spatial memory.

Table 1. Mean (SEM) performance values on open field maze text of male rats.

Open field	CONTROL				PINEALECTOMIZED			
	06:00	**12:00**	**18:00**	**24:00**	**06:00**	**12:00**	**18:00**	**24:00**
TDT	1091.7±177.8	1261.02±209	1291.35±185	1801.1±133	1505.5±199	1359.233±233.4	1406.7±206.4	1939±149
ED	4.9151±0.071	4.85893±0.07	4.89407±0.05	4.8272±0.07	4.7865±0.08	4.927333±0.079	4.9445±0.059	4.926±0.08
CD	0.2851±0.154	0.28347±0.05	0.2132±0.13	0.2478±0.07	0.2856±0.15	0.097778±0.05	0.1498±0.126	0.1±0.07
EE	3.2±1.644	3.8±1.21	5.2±1.74	4±0.95	4.875±1.84	2.25±1.349	2.125±1.941	2.25±1.06
CE	2.2±1.646	2.8±1.22	4.2±1.74	3.1±0.93	4±1.84	1.375±1.362	1.125±1.941	1.5±1.04
MOB	0.0013±0.071	0.001±0.07	0.0060±0.05	0.003±0.07	0.0013±0.08	0.000667±0.079	0.0007±0.059	0.002±0.08
VEL	218.66±35.54	253.287±42	258.816±37	361±26.6	301.28±39.7	272.3353±46.91	281.58±41.33	388.9±29.8
ROT	2.4±0.538	2.5±0.72	2.5±0.54	4.5±0.66	2.625±0.6	2±0.803	1.75±0.599	2.75±0.74

TDT: Total distance travelled (cm). ED: Edge duration (min). CD: Centre duration (min). EE: Edge entry. CE: Centre entry. MOB: Mobility (min). VEL: Velocity (cm min^{-1}). ROT: Rotation.

Table 2: Mean (SEM) performance values on elevated plus maze test of male rats.

Elevated plus maze	CONTROL				PINEALECTOMIZED			
	06:00	12:00	18:00	24:00	06:00	12:00	18:00	24:00
TDT	1241.8±174.3	1180.86±179	827.575±125	1395.8±154	1486.8±195	1323.622±200.1	1242.4±140.1	1523±172
OAD	6.46±2.142	6.29233±2.22	4.59187±1.92	6.8507±1.94	9.8468±2.56	6.679667±2.658	6.2382±2.295	7.749±2.32
OAE	24±6.228	21.1±5.74	17.8±6.89	24.1±5.85	30.125±6.96	22.5±6.418	18.375±7.707	29.75±6.54
CAD	0.94±0.236	1.23927±0.34	0.94707±0.41	1.2823±0.38	0.3673±0.26	0.875333±0.382	0.9043±0.456	1.218±0.43
CAE	3.8465±0.362	2.759±0.48	4.1372±0.46	1.5776±0.37	2.4653±0.41	2.065083±0.538	3.4327±0.515	3.015±0.42
MOB	0.9663±0.5	1.4204±0.36	1.08527±0.26	1.6323±0.23	0.656±0.5	0.622222±0.356	0.2646±0.263	0.867±0.35
ROT	5.8±0.803	5.4±0.95	2.9±0.76	3.5±0.77	4.75±0.9	4.875±1.058	3.25±0.85	4.75±0.86
VEL	350.38±23.88	334.498±28.4	331.338±39.1	280.09±30.8	354.12±26.7	320.1146±31.77	256.51±43.69	305.3±34.5

TDT: Total distance travelled (cm). OAD: Open arm duration (min). OAE: Open arm entry. CAD: Closed arm duration (min). CAE: Closed arm entry. MOB: Mobility (min). ROT: Rotation. VEL: Velocity (cm/min).

Table 3. Mean±SEM performance values of the rats in Morris water maze test.

Morris water maze	Melatonin injection	Saline injection	pinealectomy	Sham pinealectomy	Melatonin implantation	Sham implantation
TDT	310.24±80.748	79.403±9.2	566.637±130	87.727±9.377	201.27±66.717	153.702±40.03
TSFP	14.084±3.524	3.387±0.55	25.955±4.75	4.171±0.571	11.65±2.381	7.754±1.47
TSQP	0.073±0.013	0.025±0	0.173±0.03	0.04±0.007	0.099±0.023	0.077±0.011
EFQP	2.444±0.53	1±0	2.5±0.53	1.143±0.143	1.63±0.375	1.143±0.143
MT	0.344±0.051	0.056±0.01	0.549±0.12	0.11±0.045	0.164±0.02	0.128±0.024

TDT: Total distance travelled (cm). TSFP: Time spent to find the platform (sec). TSQP: Time spent in the quadrant of the platform (min). EFQP: Entrance frequency to the quadrant of the platform. MT: Mobility time (min).

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