Chapter 2

Intraamygdalar Melatonin Administration and Pinealectomy Affect Anxiety Like Behavior and Spatial Memory

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Additional information is available at the end of the chapter

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1. Introduction

1.1.The pineal gland

The pineal gland, which is called as "seat of the rational soul" by Descartes, is a pine shaped, unpaired organ located at the epithalamus of the brain. The invagination of the diencephalon develops the pineal gland and it is connected to the habenular commissure with a stalk. There is a close link between the pineal gland and the third ventricle of the brain and the area of the third ventricle receiving the pineal stalk is known as the pineal recess. The pineal gland has an endogenous, circadian(around 24 hours) rhythmic pattern in its metabolic and/or neural activity. The weight and the volume of the pineal gland show big differences within and between the species depending on the time of year, age and the physiological status of the animal. The volume of the pineal gland tends to increase in line with increasing body weight (Binkley, 1988).

The mammalian pineal is specialized for only secretion whereas fish and amphibian pineal glands acting as a photoreceptive organ and in reptiles and in birds, pineal gland is both receiving the light and has secretory function. In some birds and lower vertebrates, pineal gland also works as a rhythm generator but in mammals it is working in the coordination of rhythm physiology. In mammals, the rhythm generator is located in the suprachiasmatic nuclei of the hypothalamus (Refinetti et al, 1994). Some fish, amphibians and reptiles have a pineal gland with the two components, namely, the extracranial parietal organ and the intracranial pineal organ (Arendt, 1995).

The neuronal innervation of the pineal gland in lower vertebrates and mammals is not alike because of the lost of the efferent innervation during the phylogenesis in lower vertebrates.

The post-ganglionic sympathetic fibers arising from the superior cervical ganglion innervates mainly pineal gland of the mammals. Postganglionic fibers reaching the pineal organ via the nervi conarii release norepinephrine at night. This neurotransmitter then activates adenylate cyclase, stimulating production of the second messenger cyclic adenosine monophosphate (cAMP), which accelerates melatonin synthesis. The vascular supply of the pineal gland is very rich. The arterial supply of the pineal gland is provided by the branches of the posterior choroidal arteries. There is also a well-developed internal capillary network in pineal gland (Quay, 1974).

1.2. Melatonin hormone

1.2.1. General information about melatonin hormone

Melatonin was first identified by Lerner and colleagues in 1958, as the constituent of bovine pineal glands that lightens isolated frog skin. After it's discovery, many studies focused on the physiologic roles of melatonin on pigmentation in lower vertebrates and gonodal maturation in mammals.

Pineal gland functions as a chemical neurotransducer which converts the neural stimuli to a hormonal product as melatonin. This gland regulates many physiological functions by secreting and releasing melatonin. In the secretion of melatonin, the time of the day, age of the animal and in some photoperiodic species, time of year may be important determinant. Melatonin is secreted and released in a circadian fashion, high levels at night and very low levels at day time (Arendt, 1988). The circadian rhythm of melatonin release persists in constant darkness. However, this rhythm can be altered by nighttime light exposure, because light can suppress melatonin production. Many physiological rhythms are synchronized by the normal daily variations of the melatonin secretion. The nighttime sleep initiation and maintenance in diurnal species is also controlled by the melatonin secretion.

Light information is first received by the retina of the eye. This information is transferred to Suprachiasmatic nuclei (SCN) by the retinohypothalamic tract. SCN is capable of measuring the length of the dark/light. The information of light is then transferred to Superior Cervical Ganglia (SCG) of the spinal cord. Pineal gland receives the projections from postganglionic sympathetic nerve endings emerging from the SCG which release norepinephrine (NE). The secretion of melatonin determined by the NE since the release of NE is associated with darkness. 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. Some other factors such as the species and tissues may influence the rate and the pattern of the nocturnal increase in melatonin production (Sugden, 1991; Klein, 1993).

Melatonin has a half life of nearly 20-40 minutes. It does not remain in the blood very long. Unless the pineal gland continues to produce and secrete melatonin, blood levels of the hormone drop quickly. Melatonin is removed from the blood in at least four ways. 1) It is enzymatically degraded primarly to 6-hydroxy melatonin by the liver. 2) Melatonin that is

taken up by other cells is non enzymatical degraded when it scavenges hydroxyl radicals. 3) Also, melatonin in the blood rapidly escapes into other body fluids. 4) Finally, melatonin attaches to specific receptors or binding sites located at various locations in the organism (Panke et al, 1979; Steinlechner, 1996).

The melatonin receptors involved in mediating the effects of melatonin on the reproductive and endocrine systems are presumed to be those located in the pars tuberalis of the anterior pituitary gland (Stankov et al, 1991). These cells are in close proximity to the primary portal plexus and the terminals of the hypothalamic releasing hormone neurosecretory cells in the median eminence. Melatonin theoretically controls the release of substances, e.g., gonadotropins or other factors, that act in a paracrine manner in the nearby median eminence thereby regulating the release of the hypothalamic releasing hormones, e.g., gonadotropin releasing hormone (GnRH). In this manner melatonin can obviously regulate the functional status of the gonads and control the reproductive capability of an animal on a seasonal basis.

Melatonin modulates many physiological functions such as sleep, circadian, visual, cerebrovascular, reproductive, neuroendocrine, and neuroimmunological functions (Arendt, 2000; Wirz-Justice, 2001; Borjigin et al., 1999; Brzezinzki, 1997; Masana and Dubocovich, 2001; Vanecek, 1999; Hardeland et al., 2006). 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 influences the behavioural processes such as learning, stress, anxiety like behaviors, and depression (Krause and Dubocovich, 1990; Mantovani et al, 2003; Naranjo-Rodriguez et al., 2000; Loiseau et al., 2006). 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). The 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]. Taken together, these findings suggest the beneficial effect of melatonin on cognition and memory.

Melatonin receptors represent saturation by the melatonin concentrations, which are close to physiologic nighttime melatonin levels. Because of this reason, these receptors show a dosage dependent activity. The sleep-promoting and activity-inhibiting effects of melatonin are provided by its low levels (e.g.,50 pg/mL in blood plasma) at the beginning of the night. However, the high levels of melatonin (e.g.,150 pg/mL in blood plasma) do not enhance these behavioral parameters. Some diurnal variations are also evident in the sensitivity of the melatonin receptors since melatonin receptors are more sensitive during the daytime when the time endogenous melatonin is not secreted. The circadian phase shifting effect of melatonin may be due to the enhanced sensitivity of melatonin receptors to melatonin in the morning or in the evening hours in response to small increases in melatonin secretion (Reppert, 1997).

1.2.2. The role of melatonin hormone on anxiety and learning performance

Melatonin seems to produce anxiolytic (Naranjo-Rodriguez et al., 2000; Papp et al., 2000) effects. The effect of melatonin on anxiety is suggested to be mediated by central gamma

amino butyric acid (GABA) neurotransmission (Golombek et al., 1996). The literature findings have provided evidence for an interaction between melatonin and central GABA neurotransmission. GABA release is augmented by melatonin in rat brain tissue *in vitro* (Niles et al., 1987; Coloma and Niles, 1988). Also, when melatonin was applied *in vivo*, GABA levels increased in several brain regions in rats (Rosenstein and Cardinali, 1986; Xu et al., 1995). These findings mean that melatonin increases GABA levels, which in turn may affect anxiety of animals.

It has been shown that melatonin affects passive and active avoidance learning. Melatonin that decreases recognition time, leads to a facilitation of short-term memory. We have previously shown that melatonin implementations have some effects on learning performance depending on treatment. We investigated the effects of pinealectomy, constant release melatonin implants, and timed melatonin injections on spatial memory in male rats by using Morris water maze. Our findings showed that 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 change significantly the spatial memory performance of the rats. This outcome suggests that while the removal of the pineal gland and exogenous administration of melatonin via injections did impair learning performance, constant release melatonin administration via implantation did not affect the spatial memory in Wistar albino rats. There is also consistent research evidence that melatonin given from weaning did lead to learning and memory deficit in rats (Cao et al., 2009). Despite this new emerging evidence in the literature, there is more research needed for illuminating the role of the implementations on the various areas of the rat's brain. For instance, the effect of intraamygdalar melatonin administration on anxiety-like behaviors and spatial learning has not been investigated yet.

1.2.3. Pinealectomy

Pinealectomy is one of the methods to investigate the effect of melatonin in animals. It eliminates the melatonin hormone from blood circulation. 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). It prevents the animal from responding against the changes in day length (Hoffmann and Reiter, 1965; Hoffmann, 1974).

The effects of pinealectomy have been mostly studied on the reproductive system. The reproductive cycle desynchronizes from the environmental photoperiodic cycle by the pinealectomy. The effects of pinealectomy on reproductive system have been well documented in some hamster species. Pinealectomy prevents the regression effect of short photoperiods while gonadal maintenance on long photoperiods is not affected in Syrian hamster (Hoffmann and Reiter, 1965). Pinealectomy blocks short photoperiod induced gonadal regression of hamsters previously housed on long photoperiod (Hoffmann, 1974).

In addition to studies on the effects of regulatory function of pinealectomy on the reproductive system, it has been received a research attention in the behavioral studies.

However, these studies have provided rather inconsistent findings. For instance, while the pinealectomy itself did not have a detrimental effect on cognitive performance in rats, the interaction of it with the other lesion (i.,e, lesion on habenula) impaired such performance (Lecourtier et al., 2005). Many studies have shown that pinealectomy did not have a significant effect 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).

1.3. The amygdala regulate the behaviours related to the anxiety and memory

In this section, the general features of the amygdala and its role on anxiety like behaviors and learning performance will be examined.

1.3.1. General features of amygdala

The amygdala, a complex mass of gray matter, is located within the anterior-medial portion of the temporal lobe, just rostral to the hippocampus. The subnuclei and cortical regions of the amygdala are connected to other nearby cortical areas on the ventral and medial aspect of the hemispheric surface. The amygdala has three major functional and anatomical subdivisions, each of which are connected to the other parts of the brain. The first subdivision, namely the medial group of subnuclei, is connected to the olfactory bulb and the olfactory cortex. The second one, the basal-lateral group, has major projections with the cerebral cortex. The third one, the central and anterior group of nuclei, makes connections with the hypothalamus and brainstem which process sensory information with hypothalamic and brainstem effector systems. The visual, somatic sensory, visceral sensory, and auditory stimuli information are provided by the cortical inputs. The amygdala and the hypothalamus are separated from each other by the pathways from sensory cortical areas (Gilman and Newman, 1992.

The amygdala receives some projections directly from thalamic nuclei, the olfactory bulb, and visceral sensory relays in the brainstem. There is evidence for this convergence of sensory information. For instance, many neurons in the amygdala are sensitive to visual, auditory, somatic, sensory, visceral sensory, gustatory, and olfactory stimuli. In addition to sensory inputs, the prefrontal and temporal cortical connections of the amygdala also make connections with cognitive neocortical circuits or integrative areas, especially for integration of the emotional significance of sensory stimuli with guide complex behavior, or vice versa. Moreover, projections from the amygdala to the hypothalamus and brainstem involve in the processing of emotions such as fear, anger, and pleasure (Gilman and Newman, 1992).

1.3.2. The role of amygdala on anxiety like behavior and learning performance

It has been demonstrated that amygdala plays a regulatory role for behaviors related to anxiety and depression (Hale et al., 2006; Blackshear et al., 2007; Martinez et al., 2007). Serotonergic activity is especially high in amygdala (Abrams et al., 2004 a, 2004b). For

instance, a research has indicated that mCPP (a serotonin receptor agonist) microinjections to amygdala increased behavioral indices of anxiety without altering general activity level. In other words, it decreased open arm time and entries, but increased the closed arm ones (Cornelio and Nunes de Souza, 2007). In another study, Herdade et al. (2006) injected locally muscimol (a $GABA_A$ receptor agonist) to the medial nucleus of the amygdala and found that such treatment inhibited escape behavior in elevated T maze.

In addition to the regulatory role of amygdala in anxiety, amygdala is of great importance in regulating memory and learning functions. The amygdala is responsible for determining what memories are stored and where the memories are stored in the brain. The removal of the temporal lobe in animals leads to an impairment in memory and this impairment is global and thus none of the sensory memory is developed. For instance, the subjects experience difficulties in learning new material (i.e., anterograde amnesia) after the removal of amygdala (Almonte et al., 2007). One research has shown that amygdala damage leads to an impairment of learning an association between an auditory cue and food reward. When scopolamine, the muscarinic receptor antagonist, was injected to amygdala, it impaired performance on conditioned place preference task but not a spatial radial maze task (McIntyre et al., 1998). Moreover, the infusion of nicotinic receptor antagonists methyllycaconitine (MLA) or dihydro-b-erythroidine (DHbE) impaired working memory (Addy et al., 2003). Taken together, these findings suggest that amygdala damage has detrimental effect on the cognitive performance. However, the effect of melatonin administration to amygdala was not well known prior to the research mentioned below. The administration of melatonin to amygdala with the abolishment of melatonin hormone via pinealectomy might produce different effects on anxiety-like and learning behaviors. In other words, the endogeneous melatonin concentration and the rhythm of melatonin release might affect the effects of exogeneous melatonin administration on such behaviors.

2. Materials and methods

2.1. Animal care

A total of forty seven adult male Wistar rats (200 – 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. The ambient temperatures in the animal facilities were held constant at 22 ± 2 °C in air-ventilated rooms.

2.2. Experimental protocol

A total of the forty seven male adult rats were used and were randomly divided into two groups as control (sham –pinealectomy) and pinealectomy in this study. In the control

group, animals were exposed to the same surgical procedure with the experimental group except for the removal of the pineal gland. We performed the four subgroups as Melatonin (1 and 100 μg/kg) (n:14), Saline (0.9%NaCl) (n:5) and Diazepam (2mg/kg) (n:5) under control and pinealectomy groups. All pinealectomies and cannulation surgeries were applied before starting the experiment. The experiments were started after a week of the pinealectomies and implantations, when surgery wounds healed up completely. The anxiety-like behaviour of animals were tested by open field and elevated plus maze tests, and spatial memory was tested by means of the Morris water maze test. All animals were exposed to these behavioral tests after 30 minutes of melatonin, saline, and diazepam administrations.

2.3. Anesthesia

Before surgery, rats were anesthetized subcutaneously with Ketamine (20 mg/kg BW, Sigma Chemical Company, MO, USA) and intraperiotoneally with pentobarbitol (32.5 mg/kg BW). The depth of anesthesia was monitored by frequent testing for the presence of leg flexion reflexes and active muscle tonus. After awaking from anesthesia, the animals were placed in their cages.

2.4. Cannulation

Cannula was implanted into the amygdala. The rats were anesthetized and fixed in a stereotaxic instrument (Stoelting Co., IL, USA) and a hole was opened at the skull by a dental drill; a 22-gauge stainless steel guide cannula 313-G/Spc (Plastics One Inc., VA, USA) was implanted aseptically into amygdala region (coordinates: - 2.6 mm posterior to the bregma; + 4.3 mm lateral to the midline and -8.4 mm ventral according to the skull). The guide cannula was secured in place by dental cement (Dental Products of Turkey, Istanbul) affixed to two mounting screws. A stainless steel dummy cannula was used to occlude the guide cannula when not in use. Each cannulated rat was then kept individually for a week to recover from surgery.

2.5. Pinealectomy

The pinealectomy of Wistar rats was performed according to the method of Hoffmann and Reiter [26]; aspiration was used to control the hemorraging. The 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 were used to extend into the confluence of the sinuses to grasp and remove the pineal gland. After the 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 the 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.

2.6. Melatonin administrations

First, melatonin was dissolved in 100% ethanol (1/10 μl) and then diluted in saline (0.9 % NaCl) (9/10 μl) to the desired concentrations. Stock solutions were kept at 4 °C prior to use. The stock was diluted with sterile saline to the desired concentrations in order to make fresh working melatonin solutions. Vehicle solutions were made in the ratio of one part absolute ethanol to 1000 parts sterile saline. Melatonin was injected in a dose of either 1 or 100 μg/kg (15:00 pm).

2.7. Open field

Open-field test was 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.ŞTİ. Ankara/Turkey) was mounted above the arena, recording behavior into the *Ethovision* videotracking system (Noldus Ethovision, Version 6, Netherland; Commat LTD.ŞTİ. 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.

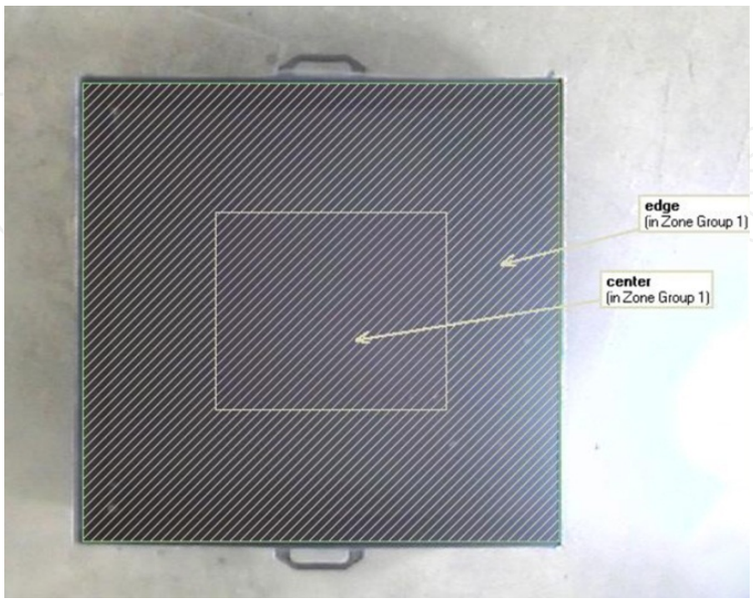

Figure 1. The edge and the center of the open field are seen.

Figure 2. Rat is seen in the open field.

Figure 3. Total distance travelled is high. The anxiety-like behaviour is low.

2.8. 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.ŞTİ. 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.

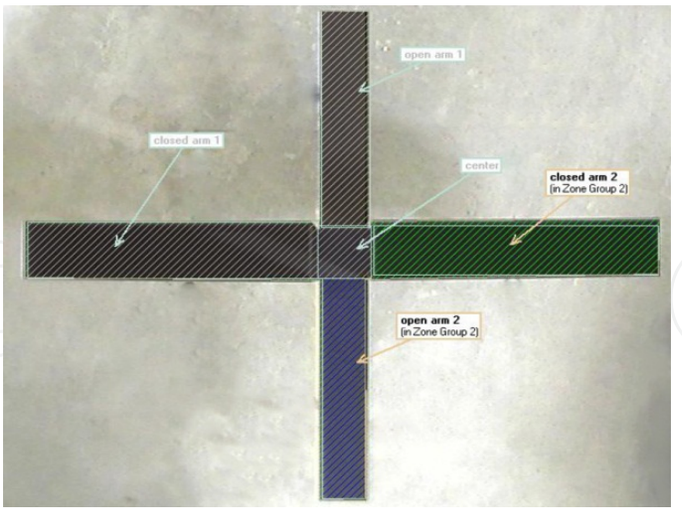

Figure 4. Open and closed arms of the elevated plus maze are seen.

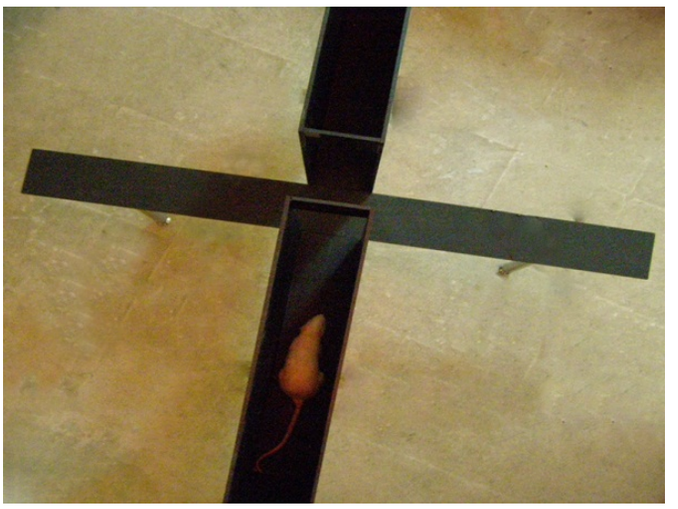

Figure 5. Rat is seen in the closed arm of the elevated plus maze.

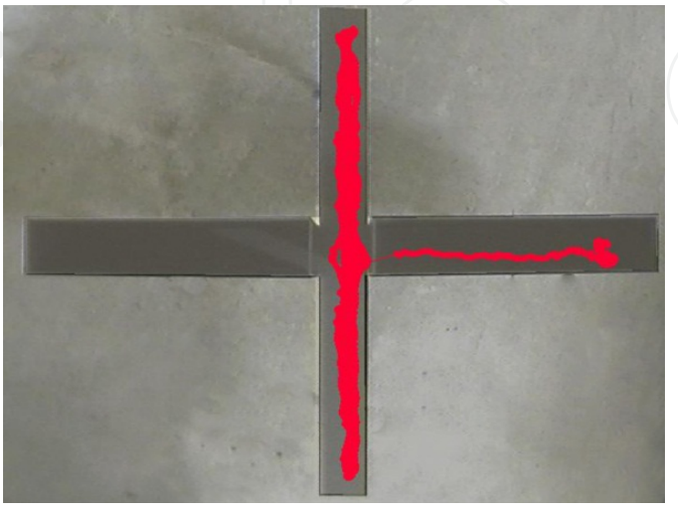

Figure 6. Time spent in open arm is high. The anxiety is low.

2.9. Morris water maze

For the spatial memory, the performance in the Morris water maze was 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. The 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 or probe trials. The rats performed the five trials per day for the four consecutive days (20 trials). In the swimming trials each individual rat was released gently into the water at a randomly 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. 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, the rats were kept in a dry home cage for 60 s.

In order to assess the spatial memory, the platform was kept away from the maze for 24 hours in the final trial. Each rat was placed into the water as in the training trials and the time in seconds spent in the quadrant formerly occupied by the platform (correct quadrant) was recorded. The platform remained in the same quadrant during the entire experiment. The rats were required to find the platform using only the distal spatial cues available in the testing room. The cues were kept constant throughout the testing.

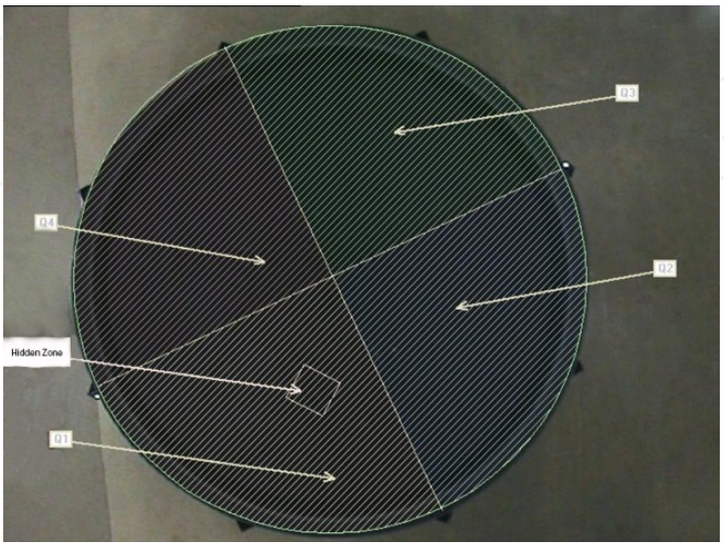

Figure 7. The quarters of the water maze and the hidden platform are seen.

Figure 8. Rat is on the platform after it found the hidden platform.

Figure 9. Total distance travelled is high. Time to find the platform is long.

2.10. Statistical analyses

Data were analyzed using SPSS (SPSS Statistical Software, SPSS Inc., Los Angeles, CA, USA, Ver. 15.0). A 2 (pinealectomy and control) X 4 (treatments: saline, diazepam, 1 μg/kg melatonin and 100 μg/kg melatonin) ANOVA analyses on data were performed with the last factor as a within subject or repeated design. Significant ANOVA results were also tested by the post test, namely the 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 $p \leq 0.05$. Data are presented as MEAN ± SEM after back transforming from ANOVA results.

3. Results

3.1. Anxiety measures

3.1.1. Open field measurements

3.1.1.1. Total distance travelled

An interaction effect between the group and the treatment was significant on the total distance travelled on the open field, $F(3, 36) = 6.15$, $p < 0.002$, $\eta^2 = .34$. This effect reflected the fact that in control condition subjects received 100 μg/kg melatonin (M = 699.65) and 1 μg/kg melatonin (M = 690.46) treatments travelled less distance than those received diazepam (M = 1400.04) and saline (M = 1214.95), whereas in the pinealectomy condition, the subjects received diazem (M = 643.75) travelled less distance than those received 100 μg/kg melatonin (M = 1070.22), 1 μg/kg melatonin (M = 914.38) and saline (M = 902.11) treatments.

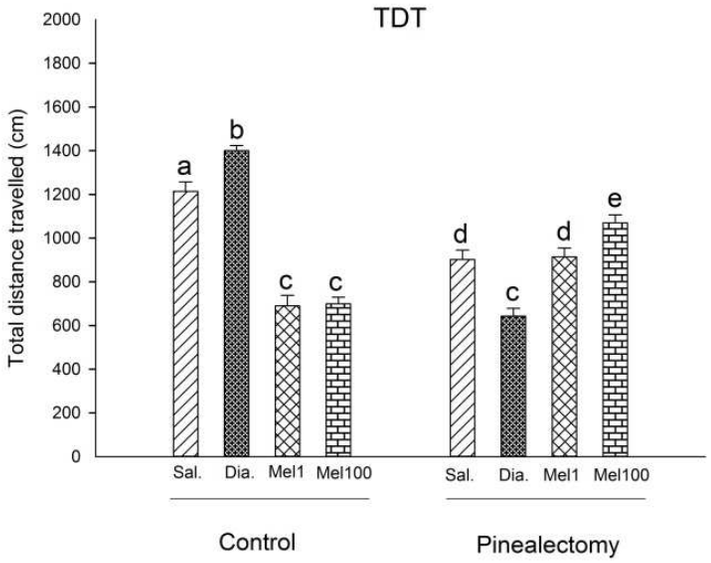

Figure 10. The total distance travelled. Right striated bar represents the saline injection and black bar represents diazepam injections, cross striated bar represents 1 μg/kg melatonin injection and bricks striated bar represents 100 μg/kg melatonin injection for both control and pinealectomy groups. Data are presented as means (± S.E.M.). Different letters indicate the statistically different groups. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.1.2. Time spent at the edge of the open field (Edge duration)

An interaction effect between the group and the treatment was also significant, F (3, 36) = 5.38, $p = .004$, $\eta^2 = .31$. Indicating that in control condition subjects received diazepam spent less time than the other treatments whereas, in pinealectomy condition the subjects were not significantly different from each other.

3.1.1.3. Time spent at the center of the open field (Center duration)

The interaction effect between the group and the treatment was also significant, $F(3, 36) = 5.29$, $p < 0.004$, $\eta^2 = .31$. Indicating that in control condition subjects received diazepam spent

more time than the other treatments whereas, in pinealectomy condition the subjects were not significantly different from each other.

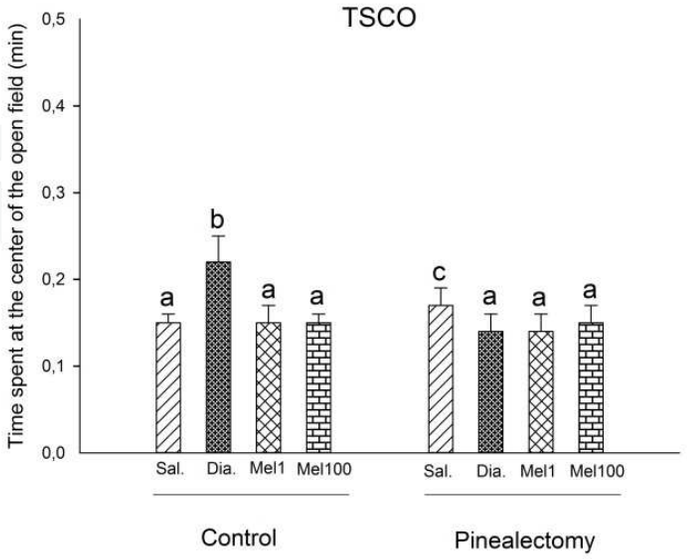

Figure 11. The time spent at the center of the open field. The bar explanations can be seen in Figure 10. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.1.4. Entrance frequency to the edge of the open field (Edge frequency)

The interaction effect between the group and the treatment was significant, $F(3, 36) = 3.02$, $p < 0.04$, $\eta^2 = .20$, reflecting the fact that in control condition subjects received diazepam entered more frequently to the edge of the open field than the other treatments, whereas in pinealectomy condition the subjects who received saline treatment entered more frequently than the other treatments.

3.1.1.5. Entrance frequency to the center of the open field (Center frequency)

The interaction effect between the group and the treatment was significant, $F(3, 36) = 3.02$, $p < 0.04$, $\eta^2 = .20$, reflecting the fact that in control condition subjects received diazepam entered more frequently to the center of the open field than the other treatments, whereas in pinealectomy condition the subjects who received saline treatment entered more frequently than the other treatments.

3.1.1.6. Mobility

The main effect of the group was significant, $F(1, 36) = 6.89$, $p = .01$, $\eta^2 = .16$. Control group was more mobile than pinealectomy group. The main effect of the treatment was also significant, $F(3, 36) = 6.73$, $p = .001$, $\eta^2 = .36$. The subjects who received saline were more mobile on the open field than the other subjects with each being not significantly different from each other.

In addition, the interaction effect between the group and the treatment was significant, $F(3, 36) = 7.08$, $p < 0.001$, $\eta^2 = .37$. This reflected the fact that in control condition subjects received

saline was more mobile than the other treatments, whereas in pinealectomy condition the subjects who received 100 μg/kg melatonin treatment were more mobile than the other treatments.

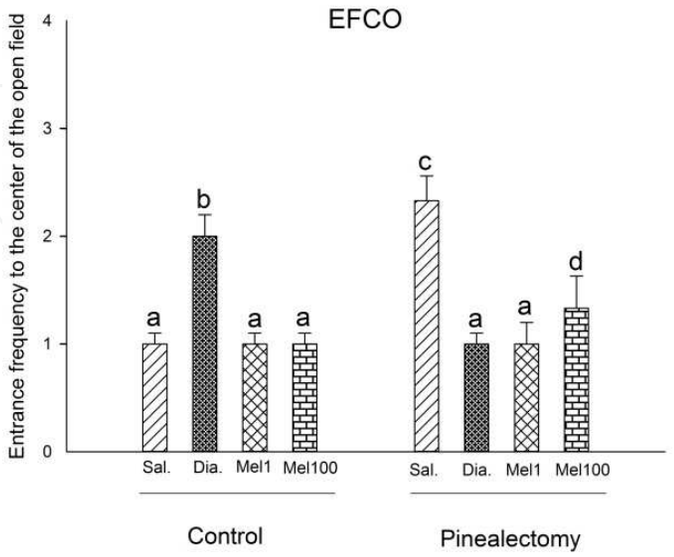

Figure 12. The entrance frequency to the center of the open field. The bar explanations can be seen in Figure 10. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

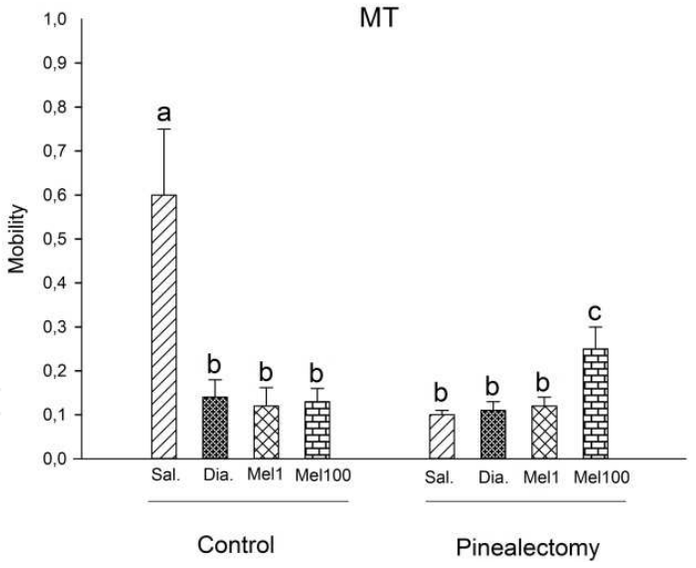

Figure 13. The mobility time . The bar explanations can be seen in Figure 10. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.1.7. Velocity

The interaction effect between the group and the treatment was significant, F (3, 36) = 6.52, p < 0.001, η^2 = .35, indicating that in control condition subjects received saline and diazepam were faster than the other treatments, whereas in pinealectomy condition the subjects who received 100 μg/kg melatonin treatment were faster than the other treatments.

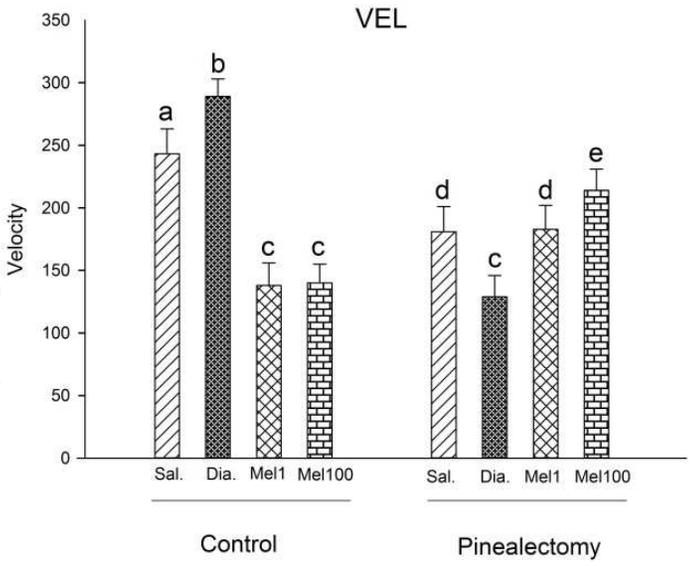

Figure 14. The velocity. The bar explanations can be seen in Figure 10. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.2. Elevated plus maze measurements

3.1.2.1. Total distance travelled

The main effect of the treatment was significant, F (3, 39) = 3.06, p = .04, η^2 = .19. The groups who received diazepam travelled less distance than those who received 100 μg/kg melatonin treatments. No other differences were found to be significant.

The interaction effect between the group and the treatment was also significant on the total distance travelled on elevated plus maze, F (3, 39) = 6.52, p = 0.001, η^2 = .33. This interaction effect reflected the fact that in control condition, the subjects received diazepam travelled less distance than the other treatments, whereas in pinealectomy condition 100 μg/kg melatonin treatments travelled less distance than other treatments, whereas in the pinealectomy condition, the subjects received 100 μg/kg melatonin travelled less distance than the other treatments.

3.1.2.2. Time spent in open arms (Open arm duration)

The main effect of the treatment was significant, F (3, 39) = 6.53, p = .001, η^2 = .33. The subjects who received 100 μg/kg melatonin treatment spent more time in open arms than those who received other treatments.

An interaction effect between the group and the treatment was also significant, F (3, 39) = 6.87, p < 0.001, η^2 = .35. This effect indicated that in control condition subjects received 100 μg/kg melatonin treatment spent more time than those receiving the other treatments, whereas in pinealectomy condition the subjects who received saline, 100 μg/kg melatonin and diazepam treatments spent more time than those who received 1 μg/kg melatonin treatment.

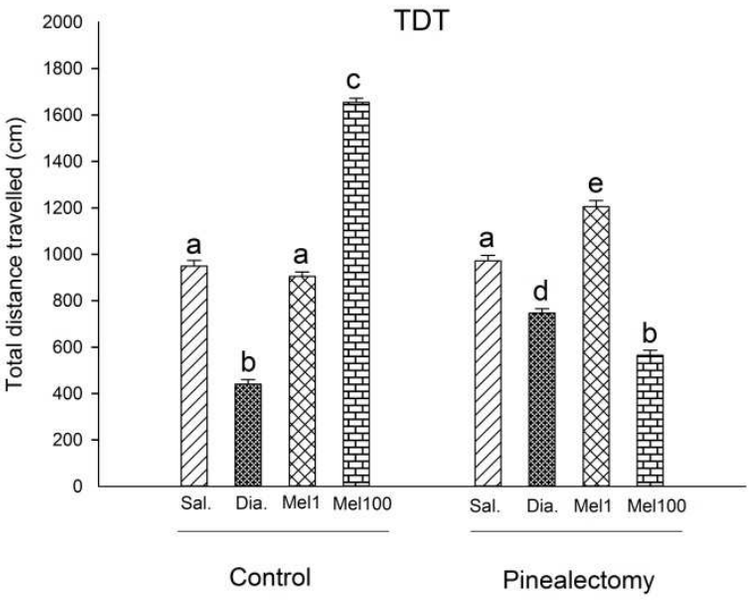

Figure 15. The total distance travelled. Right striated bar represents the saline injection and black bar represents diazepam injections, cross striated bar represents 1 μg/kg melatonin injection and bricks striated bar represents 100 μg/kg melatonin injection for both control and pinealectomy groups. Data are presented as means (± S.E.M.). Different letters indicate the statistically different groups. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

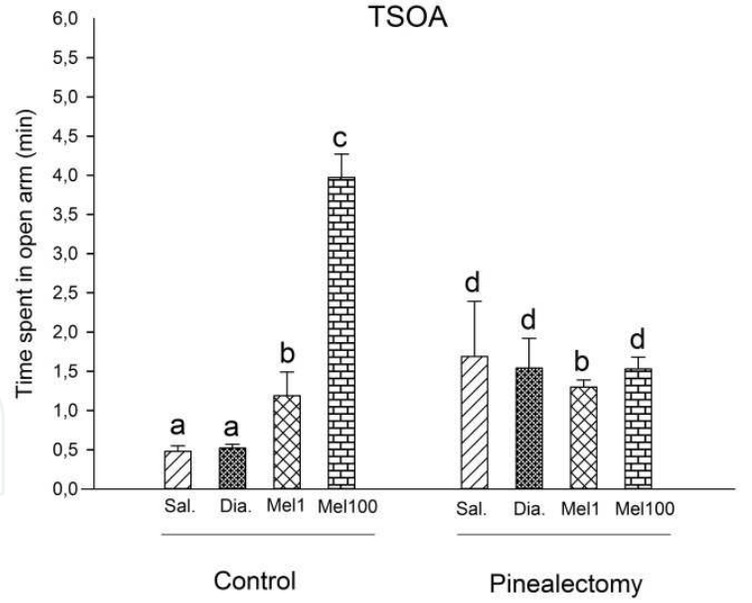

Figure 16. The time spent in open arms (TSOA). The bar explanations can be seen in Figure 15. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.2.3. Time spent in closed arms (Closed arm duration)

The main effect of the treatment was significant, F (3, 39) = 6.56, p = .001, η^2 = .34. The subjects who received 100 μg/kg melatonin treatment spent less time in closed arms than those who received other treatments.

An interaction effect between the group and the treatment was also significant, F (3, 39) = 7.30, $p < 0.001$, $\eta^2 = .36$. This interaction effect reflected the fact that in control condition subjects received 100 μg/kg melatonin treatment spent less time than those receiving the other treatments, but there were no significant differences between treatment conditions in pinealectomy.

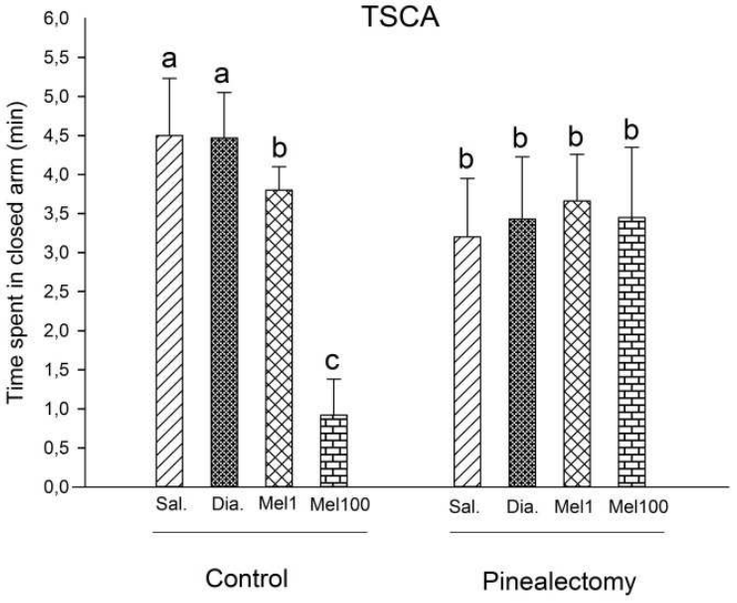

Figure 17. The time spent in closed arms (TSCA). The bar explanations can be seen in Figure 15. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.2.4. Entrance frequency to open arms

The main effect of the group was significant, F (1, 39) = 14.40, $p = .001$, $\eta^2 = .27$. The subjects in the control condition entered more frequently to open arm than those in the pinealectomy.

The main effect of the treatment was also significant, F (3, 39) = 19.39, $p = .0001$, $\eta^2 = .60$. The subjects who received 100 μg/kg melatonin treatment entered more frequently to the open arm than those who received other treatments.

In addition, the interaction effect between the group and the treatment was significant, F (3, 39) = 37.65, $p = 0.0001$, $\eta^2 = .74$. This reflected the fact that in control condition subjects who received 100 μg/kg melatonin treatment entered more frequently than those who received the other treatments, whereas in pinealectomy condition the subjects who received saline, 1 μg/kg melatonin and diazepam treatments entered more frequently than those who received 100 μg/kg melatonin treatment.

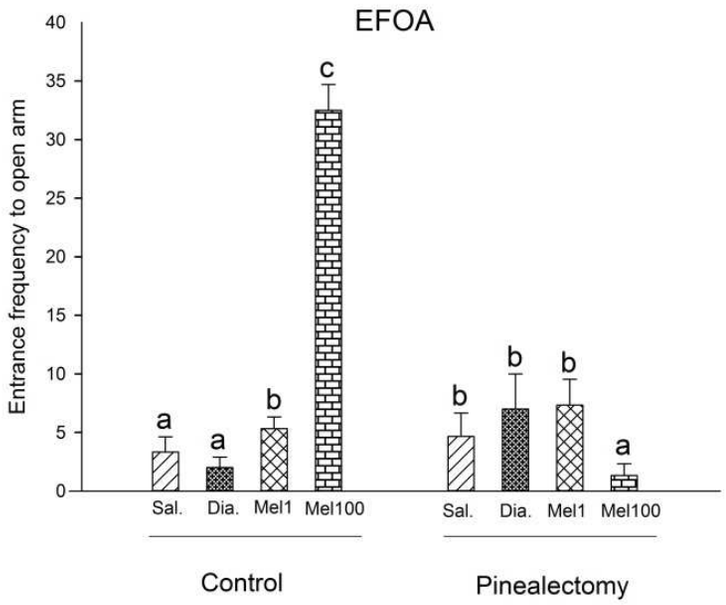

Figure 18. The entrance frequency to the open arms (EFOA). The bar explanations can be seen in Figure 15. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.2.5. Entrance frequency to closed arms

No significant effects were found with regard to the total entrance to the closed arm of the elevated plus maze.

3.1.2.6. Mobility

The main effect of the group was significant, F (1, 39) = 6.95, p = .01, η^2 = .15. The subjects in the pinealectomy condition were more mobile than those in the control condition.

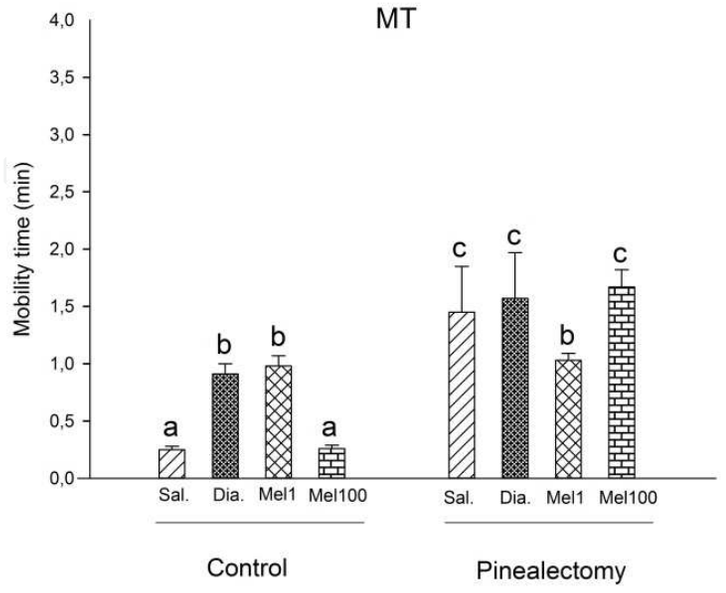

Figure 19. The mobility time (MT). The bar explanations can be seen in Figure 15. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.2.7. Velocity

No significant effects were found with regard to the total entrance to the closed arm of the elevated plus maze.

3.1.3. Spatial memory measures (Morris water maze measures)

3.1.3.1. Total distance travelled

The interaction effect between the group and the treatment was significant on the total distance travelled on elevated plus maze, $F(3, 40) = 4.84$, $p = 0.006$, $\eta^2 = .27$. This reflected the fact that in control condition, the subjects received diazepam travelled more distance than the other treatments, whereas in pinealectomy condition subjects who reveived 100 μg/kg melatonin and the saline treatments travelled more distance than those who received 1 μg/kg melatonin and diazepam treatments.

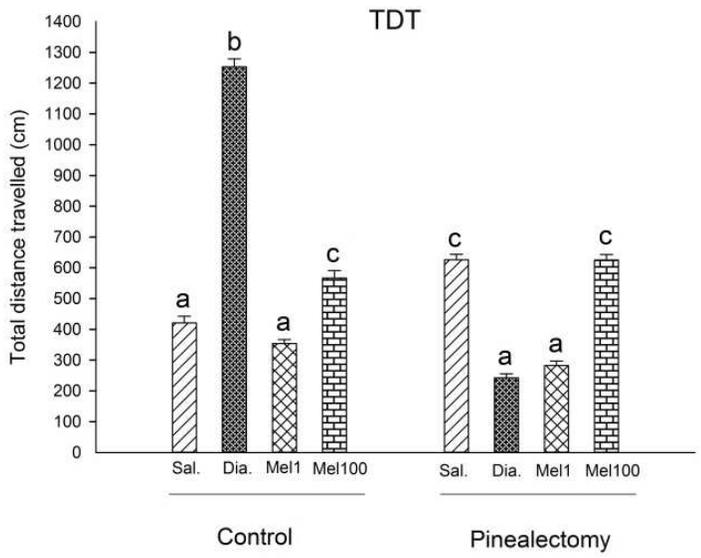

Figure 20. The total distance travelled (TDT). Right striated bar represents the saline injection and black bar represents diazepam injections, cross striated bar represents 1 μg/kg melatonin injection and bricks striated bar represents 100 μg/kg melatonin injection for both control and pinealectomy groups. Data are presented as means (± S.E.M.). Different letters indicate the statistically different groups. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.3.2. Time spent to find the platform (Latency)

The main effect of the treatment was significant, F (3, 40) = 3.02, p = .04, η^2 = .19. The subjects who received diazepam treatment spent more time than those who received 1 μg/kg melatonin treatment.

In addition, the interaction effect between the group and the treatment was also significant, F (3, 40) = 4.90, p = 0.005, η^2 = .41. This interaction effect reflected the fact that there were no significant differences between control and pinealectomy groups in 100 μg/kg and 1 μg/kg melatonin treatments but was significant differences between these groups in saline and diazepam treatments.

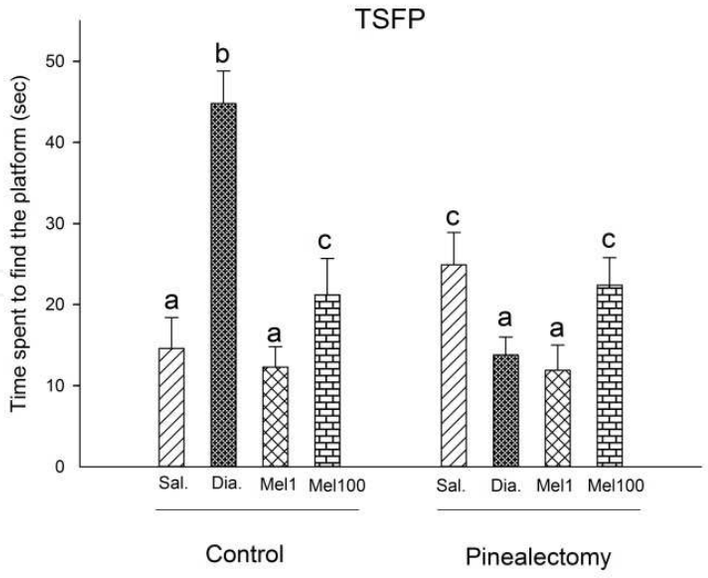

Figure 21. The time spent to find the platform (TSFP) The bar explanations can be seen in Figure 20. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

3.1.3.3. Time spent in the correct quadrant

The main effect of the treatment was also significant, F (3, 40) = 4.11, p = .01, η^2 = .24. The subjects who received 1 μg/kg melatonin treatment spent less time than those who received other treatments with each being not significant from each other.

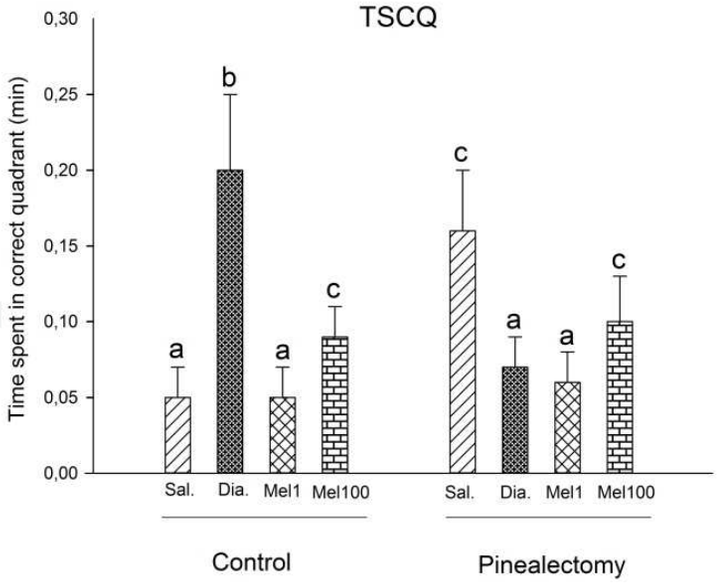

Figure 22. The time spent in the correct quadrant (TSCQ). The bar explanations can be seen in Figure 20. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

In addition, the interaction effect between the group and the treatment was also significant, F (3, 40) = 9.29, p = 0.001, η^2 = .41, indicating that in control condition subjects who received diazepam treatment spent more time than those who received the other treatments, whereas

in pinealectomy condition the subjects who received saline treatments spent more time than those who received other treatments.

3.1.3.4. The entrance frequency to the correct quadrant

The interaction effect between the group and the treatment was significant, $F(3, 40) = 6.72$, $p = 0.001$, $\eta^2 = .34$, indicating that in control condition subjects who received diazepam entered more frequently those who received the other treatments, whereas in pinealectomy condition the subjects who received saline treatments entered more frequently than those who received other treatments.

3.1.3.5. Mobility

No significant effects were found with regard to the mobility in the Morris water maze.

3.1.3.6. Velocity

The main effect of the group was significant, $F(1, 40) = 11.31$, $p = .002$, $\eta^2 = .22$. The subjects in the control condition were faster than those in the pinealectomy condition.

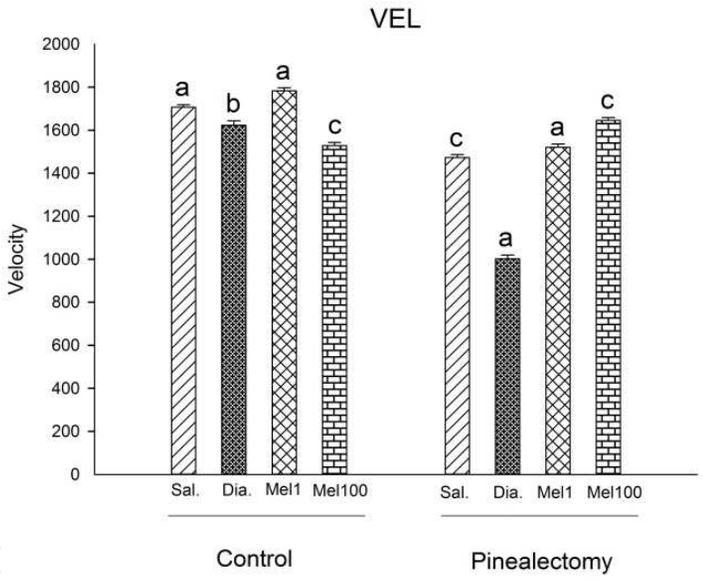

Figure 23. The velocity (VEL) are represented for the Morris water maze. The bar explanations can be seen in Figure 20. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

The main effect of the treatment was also significant, $F(3, 40) = 4.16$, $p = .01$, $\eta^2 = .24$. The subjects who received diazepam treatment were slower than those who received other treatments.

In addition, the interaction effect between the group and the treatment was significant, $F(3, 40) = 4.13$, $p = 0.01$, $\eta^2 = .24$. This interaction effect reflected the fact that in control condition subjects who received 100 μg/kg melatonin treatment were slower than those who received the other treatments, whereas in pinealectomy condition, the subjects who received diazepam were slower than those who received other treatments.

3.2. Evaluation of the correct placement of the cannula

After all experiments finished, animals were decapitated and the brains were removed. We checked the placement of the cannulas histologically whether they were placed to the amygdala of the brain or not. Figure 24 represents a histological section of a brain which the cannula was placed correctly.

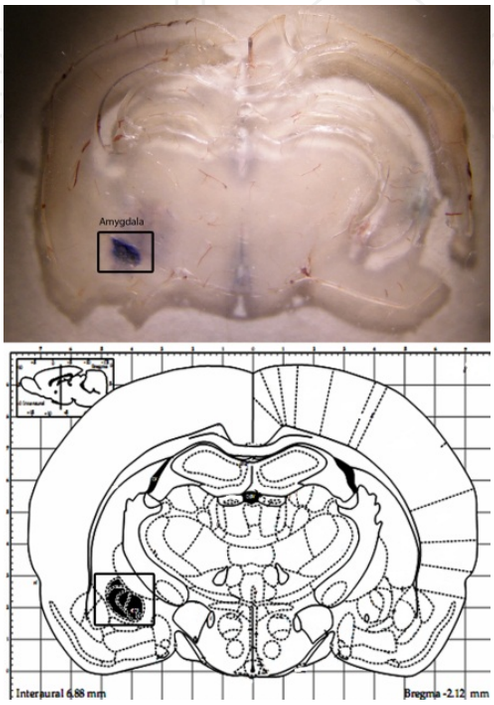

Figure 24. The figure represents the amydala region of the brain where the injections were applied. Blue colored region of the brain represents the amygdala region. (Reproduced with permission from Karakas et al.; published by Elsevier, 2011a.)

4. Discussion

The results of the present study can be described under the two main headings: anxiety-like behaviour (in open field apparatus and elevated plus maze) and spatial memory performance (in Morris water maze).

4.1. Anxiety-like behaviours

Open field test is used to measure the anxiety like behaviors 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, (a) the number of the entries to the edge of the open field tends to increase, whereas that of entries to the center of the open field decrease, (b) the time passed at the edge of the open field increases, while the time passed at the center of the open field decreases, and (c) the total

distance traveled, mobility and velocity in the open field decrease. The total number of the entries into the center and the edges provides a built-in control measure for general hyperactivity or sedation.

In sum, our findings were in open field that a) diazepam was more effective in reducing the anxiety since the time passed at the center of the open field was longer especially than those the 0,1 melatonin administration treatments, b) the control subjects were more mobile than the pinealectomized ones, and c) 100 μg/kg melatonin administration in contrast to other treatment conditions reduced the velocity of the animals.

Our findings reflected the fact that diazepam was more effective in reducing the anxiety. This effect was expected since the diazepam inhibits the serotonergic activity via GABAergic system. Benzodiazepines are widely used in reducing the anxiety-like behaviours. They are preferred because of their effectiveness and wide therapeutic index. They make their effect by binding their receptors which are found near the GABA receptors and by making an allosteric effect. By this way, they increase the affinity of these GABA receptors to benzodiazepines (Sinclair and Nutt, 2007). There is also possibility that amygdala also plays an important role on anxiety. The high serotonergic activity in amygdala may be one plausible explanation for this important role. This role of amygdala is supported by some research evidence that (a) a serotonin receptor agonist increased behavioral indices of anxiety without altering general activity level, and (b) a $GABA_A$ receptor agonist treatment to the medial nucleus of the amygdala inhibited escape behavior in elevated T maze.

The second finding was that there was the biggest difference in between the controls and the pinealectomies in mobility. This means that mobility measurement is more sensitive to the removal of pineal gland. It should be kept in mind that such effect was not observed in terms of other indices of the anxiety-like behaviours in this study. This finding also suggests that the amount and the rhythm of the endogeneous melatonin release in the pinealectomized animals is abolished; however, this endogeneous rhythm in the sham pinealectomized animals is intact. Therefore, the plausible effect of external high dose of melatonin administration may not become evident.

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 indicating that pinealectomy alone did not have a significant effect on anxiety behavior [Kovacs et al., 1974; Juszcak et al., 1996). This suggests that the pineal gland is partially involved in the anxiety-like behaviours. The third finding was that the high dose of melatonin (100 μg/kg) administrations reduced the velocity of the animals. This effect of melatonin might be due to the direct inhibition of locomotor activity, rather than an effect on the circadian clock.

The elevated plus maze has been one of widely used tests to measure the anxiety like behaviours (Dawson and Tricklebank, 1995). In this test, the total distance traveled, the total number of entries to the closed and open arms, the time spent in closed and open arms, the mobility and the velocity are used parameters measured. In this maze, if the anxiety of the

animal is high, (a) the number of the entries to closed arms increases whereas those to open arms decreases, (b) the time passed in closed arm increases whereas that passed in open arms decreases and (c) the total distance traveled, mobility and velocity decrease. The total number of the entries into all arms provides a built-in control measure for general hyperactivity or sedation.

In sum, our findings were in elevated plus maze that, a) the high dose (100 μg/kg) of melatonin increased the distance totally travelled, whereas such dose after pinealectomy decreased it, b) 100 μg/kg melatonin increased the time spent in open arms; however, after the pinealectomy, the low dose of melatonin (1 μg/kg) decreased it and c) pinealectomized animals were more mobile than control ones.

The increase in travelled distance induced by high dose of melatonin administration was reversed by the pinealectomy. This suggests that internal melatonin concentrations and rhythm may be more likely to change the effects of exogeneous melatonin administration in the anxiety like behaviors. It is well known fact that pinealectomy abolishes the rhythmic endogenous melatonin release and decreases the plasma levels of melatonin significantly (Hoffman and Reiter, 1965). Thus, after the removal of the pineal gland a high dose of melatonin could show its effect on anxiety-like behavior. The second finding in the elevated plus maze was that the high dose of melatonin increased the time spent in open arms, while, after the pinealectomy, the low dose of melatonin decreased it. In the literature, there is evidence for the interaction of melatonin with central gama aminobutyric acid (GABA) neurotransmission. Melatonin has been shown to increase the GABA levels 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 conclusion, our findings can be attributed the fact that high dose of melatonin increased the GABA levels, which in turns reduce anxiety like behaviors. Through this mechanism, the high dose of melatonin administered subjects spent more time in open arms than the others.

The third finding in the elevated plus maze was that pinealectomy increased the mobility time in compared to controls. This finding suggests that mobility measurement is more sensitive to the removal of pineal gland. One can see that this effect was opposite of what was found in open arms. This difference may be due to the task difference between open field and elevated plus maze. 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. On the other hand, the elevated plus maze has been one of popular or widely used

test to measure the anxiety like behaviors (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 and open field tests, the present study represent a difference in mobility, which needs a further investigation. Our findings also suggest that the elevated plus maze condition provides melatonin specific outcomes more than the open field condition.

4.2. Spatial memory performance

The Morris water maze has been one of widely used tests to measure the spatial memory performance. In this maze, the time passed to find the platform, total distance travelled, the frequency of the entrance to the correct quadrant, the time passed in correct quadrant, mobility and velocity parameters are measured (Morris, 1984).

In this study,(a) diazepam administration increased the total distance travelled more than the others in the control condition whereas, in the pinealectomy condition the high dose of melatonin and saline groups travelled more distance than the others,(b) in the pinealectomy condition, the subjects with the high dose of melatonin also travelled more distance than those with the low dose of melatonin and diazepam, (c) the subjects who received 1 μg/kg melatonin spent less time than those who received other treatments, and (d) in the control condition, the subjects with the high dose of melatonin treatment were slower than those who received the other treatments. Longer distance travelled and less time spent in the correct quadrant indicates less spatial learning in this maze. It should be especially noted that the high doses of melatonin decreased some behavioral indices of spatial memory. In line with this finding, other studies have consistently shown that amygdala damage through various implementations leads to the impairment of learning an association between an auditory cue and food reward (Sutherland and Mc Donalds, 1990), of performance on conditioned place preference task (McIntyre et al., 1998), and working memory (Addy et al., 2003). 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; Ekmekçioğlu, 2006; Mazzuchelli et al., 1996; Savaskan et al., 2001; 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 'sleep-like' 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 et al., 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 evidence indicating that melatonin significantly ameliorated the cognitive impairment, reduced lipid per oxidation, 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 amino butyric acid (GABA). 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 microinjection to amygdala may show its impairing effect on learning and memory processes. In addition to the regulatory role of amygdala in anxiety, amygdala is of great importance in regulating memory and learning functions. The removal of the temporal lobe in animals leads to an impairment in memory in a way that the subjects experience difficulties in learning new material after the removal of amygdala. Also, damage to amygdala leads to an impairment of learning an association between an auditory cue and food reward. In addition, the muscarinic receptor antagonist administration to amygdala impaired performance on conditioned place preference task (McIntyre et al., 1998). Moreover, the nicotinic receptor antagonist administrations impair working memory (Addy et al., 2003). The results of our study indicate that the administration of melatonin to amygdala with the abolishment of melatonin hormone via pinealectomy produced different effects on anxiety-like and learning behaviors.

In addition, melatonin may also show its effects through its reciprocal relationship with some parts of rat brain such as 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 circadian pattern which is high throughout the darkness (Klein, 1974). However, in pinealectomy the blood melatonin levels drop significantly and the rhythm of melatonin is abolished (Chapman, 1970).

The other explanation for the effects of melatonin on learning performance is related with the circadian effects of melatonin. Several studies have demonstrated the regulatory roles of melatonin in circadian rhythms (Brzezinski, 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.

It should be kept in mind that we implemented microinjections in the afternoon when the melatonin receptors are re-sensitive to the melatonin hormone. According to the internal

coincidence hypothesis, 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 Tay, 1983; Hong and Stetson, 1987). In line with this explanation, we found in our another study that pinealectomy and only admistration of melatonin via timed injections caused impairment of the learning performance of the rats (Karakas et al., 2011b).

4.3. Conclusion

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 melatonin microinjections to amygdala than open field measurements, since the differences were more evident in this maze. This suggests that pinealectomy treatment interacts with anxiety provoking test situations. In open field, it was assumed that the anxiety level experience by animals may be greater in elevated plus maze than open field. In open field the mobility was smaller in pinealectomized rats than controls since the anxiety level may be low compared to the elevated plus maze. This explanation requires further experimental research that illuminates differential effects of pinealectomy on testing conditions.

Taken together, these results are unique contribution to the field of anxiety like behavior and spatial learning in the literature. Further research should take multiple measures of anxiety and learning in the given consideration that melatonin injection produce different outcomes in the investigated parameters in open field, elevated plus maze and Morris water maze.

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