Title: Perinatal Music Exposure Alters Cognitive Functions in Adult Rats

Running Title: Perinatal Music and Cognitive Functions

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ABSTRACT

The Purpose of the Study: Music is one of the factors known to have an impact on brain development. Herein, the effects of perinatal exposure to different music patterns on some behavioral characteristics and their underlying molecular mechanisms were investigated.

Materials and Methods: On the first day of their gestation, Wistar rats separated into control, classical, Sufi, and rock groups. Pregnant rats and their pups were exposed to music patterns for one hour/day during pregnancy until weaning. On the 60th day, behavioral tests were performed; later the brains of rat pups were dissected for molecular analysis.

Results: At adult age, pups in rock group had lower level of anxiety, learning and memory with an increase in motor coordination. However, classical and Sufi groups had increased level of anxiety and depression due to low BDNF expression. In addition, Sufi group presented good results in object recognition while classical group showed a better performance in spatial learning due to increased neuroprotective/neurogenesis factors. In contrast, lower DCX levels in both the Sufi and the rock groups suggested a decrease in the neurogenesis. Indeed, the increased NFkB expression may explain poor performance in learning and memory of the rock group.

Conclusions: In conclusion, the type of music may be an important epigenetic factor during hippocampal development because it may have an impact on the function of higher brain areas in adults.

Keywords: Behavioral tests; Microarray; Neurogenesis; Prenatal music exposure; Rat

1. Introduction

Vision, olfaction, touch, taste, and sound are the types of environmental factors that stimulate brain (Chaudhury et al., 2013), and sound has been shown to cause physiological effects on humans (Knight & Rickard, 2001). For a long time, it is frequently discussed that music exposure during gestational period is effective in the postnatal development. Some researchers claimed that listening to relaxing music by mothers may be useful for their babies, while listening to uncomfortable music disrupts babies in a similar way with their mothers thers (Fritz et al., 2014). Recent prenatal music studies related to neurogenesis (Kim et al., 2013), neuroplasticity (Sheikhi & Saboory, 2015), and spatial learning and memory (Sanyal et al., 2013) showed that prenatal music has several influences on newborns. For example, music enhances fetal brain development, increases newborn spatial-temporal learning and allows development of motor abilities to progress faster (Sayh, 2012).

On a behavioral level, it has been reported that music pattern exposure in rodents modulates brain development and protects spatial memory (Amagdei et al., 2010). The fetal response to music was also noted in human studies as improvements in abilities such as verbal memory and general intelligence (Chelli & Chanoufi, 2008; Rabipour & Raz, 2012). In similar studies, it was concluded that music enhances learning capacity and memory consolidation, directly or indirectly (Vieillard & Gilert, 2013). In addition, listening to music for 2 weeks or longer might also reduce stress, anxiety, and sleep disturbances in pregnant women stated as reflection of prenatal benefits (Liu et al., 2016). In addition, one-hour exposure of classical music might be also used as a therapy on rats with bone cancer to reduce pain-associated behaviors (Gao et al., 2016).

At the molecular level, it was found that over 800 genes related to functionality of ion channels and hormones, neuronal development, gene transcription, neurotransmission, and cytoskeletal

activity in forebrain cortex and hippocampus were influenced (up-regulated or down-regulated) by music exposure (Meng et al., 2009). Studies have reported that after music exposure, expression levels and/or activity of BDNF, NGF, TrkB, NR2B, GluR2 genes were regulated (Xu et al., 2009; Chikahisa et al., 2006; Xu et al., 2007; Angelucci et al., 2007). "Mozart effect" (approx. 65-75 dB) enhanced cognitive and spatial abilities in rats with temporal lobe epilepsy and an increased level of spatial memory had been associated with BDNF/TrkB level of CA3 and DG regions of dorsal hippocampus (Xing etal., 2016a).

Due to being two different forms of sound, rats exposed to noise during pregnancy had decreased cortical changes unlike rats exposed to music (Kim et al., 2013). In order to discriminate different types of music, complex brain functions should be necessary (Otsuka et al., 2009). Rats could discriminate music and they have higher auditory information processing than humans (Otsuka et al., 2009). For example, classical music therapy might cause lower level of corticosterone on rat pups (Sheikhi & Saboory, 2015). However, a study conducted on chickens claimed that high decibel sounds might increase plasma corticosterone level (Guan et al., 2013). Indeed, prenatal exposure to music resulted in an increased neurogenesis in the rat hippocampus while noise exposure decreased neurogenesis in rats (Kim et al., 2006). However, the molecular mechanisms how music pattern affects the behavior remain elusive. Therefore, we tested the hypothesis that different music pattern exposure during development might result in long-term behavioral and molecular changes in the brain at maturity, and that these effects might vary with the type of music pattern exposure. To achieve this result, after specific behavioral tests that measure anxiety, depression, motor coordination, learning and memory, we performed microarray to determine the overall changes in the hippocampal proteins due to differences in the prenatal music pattern exposure. In addition to the proteins obtained from the microarray data, we determined the alterations in the gene expression of AKT for cell survival, Nuclear factor-kappa B (NF-KB) for neuroinflammation, nerve growth factor (NGF) for

regulation of growth, maintenance, and proliferation of neurons and synaptophysin (SYP) for neuronal functioning and we also determined the protein expression of doublecortin (DCX) for neurogenesis, brain derived neurotrophic factor (BDNF) for neuronal development and neuronal survival, heat shock protein 70 (Hsp70) for neuronal death to correlate the behavioral changes with molecular alterations that might be specific for hippocampus and hippocampus-dependent behavioral outcomes.

2. Materials and Methods

2.1. Subject and Experimental Design

In the current study, 4-month-old male and female Wistar albino rats obtained from Bezmialem Vakif University Research Center were used. Standard conditions were applied to the rats during the experimental procedures (21^0C room temperature and 12-hour light/12-hour dark cycles). The rats were free to obtain food and water. During mating, male rats placed to the cage of individually placed females. After mating, observation of vaginal plug was a sign of fertilization. The gestational day 0 (GD0) was accepted at the time of time of vaginal plug observation (Elibol-Can et al., 2014). In GD0, pregnant female rats were randomly divided into control group (n=4), sufi group (n=4), classical group (n=4), and rock group (n=4). While the control group has no sound exposure, sufi music group had 30 dB (1 kHz) sound (Whirling Derwish/Omar Faruk Tekbilek), classical music group had 60 dB (10^3 kHz) sound (Canon in D Major/Johann Pachelbel), and rock music group had 120 dB (10^9 kHz) sound (In Your Face/Children of Bodom). The sound exposure started at the GD0 and applied one hour per day (the time between 4 pm-6 pm) in soundproof boxes at different rooms. At birth, each litter had 6-8 pups. From birth to the end of the weaning period, mother rats and their pups were caged together to prevent additional stress and music exposure to the dams and their pups continued until weaning age. For behavioral and molecular experiments, a group of male pups was separated at weaning (n=8 for control, n=8 for classical, n=6 for rock, n=5 for Sufi). Animal

experiments were conducted according to guidelines provided by the National Institutes of Health and approved by the Bezmialem Vakif University Local Ethics Committee for Animal Experiments (2015/30).

Behavioral Tests

Anxiety tests, motor-coordination tests, learning and memory tests, and a depression test were applied to a group of animals starting from 60-day old to 75-day old, respectively.

2.1.1. Elevated plus maze:

Elevated plus maze apparatus had two open-armed (50x10 cm) and two closed-armed (50x10x40 cm) platforms. The height of the apparatus was 50 cm. Rats were put to the center of these arms (5x5 cm) and for five minutes in a 350 lux-illuminated room. The measurements performed with a automated video tracking system (Ethovision XT, Noldus Information Technology, The Netherlands). At the end of the 5 min., the duration of rat in open and closed arms were calculated.

2.1.2. Open field test:

The open field apparatus was a square platform (56x56 cm). Their walls were 30 cm in height. Rearing, sniffing, grooming, freezing, the time spent in the center of open field (26x26 cm), and defecations level were measured as parameters for 10 minutes in a 350-lux illuminated room.

2.1.3. Light/dark transition test:

An apparatus with illuminated (1500 lux, 40x30x26 cm) and dark (2 lux, 40x30x26 cm) chambers containing a transition gate (8x4.5 cm) among two chambers was used. Experiment started in illuminated chamber and rats could move freely between two chambers. Total

duration spent in both illuminated and dark chambers, number of transitions between two chambers were recorded for 5 min. (Takao & Miyakawa, 2006).

2.1.4. Motor coordination test

In a day, a battery of motor coordination test was applied to the rats in a 350-lux illuminated room

2.1.4.1. Wire rod test:

This test was used to measure the muscle strength of frontal extremity. A rope highlighted 50 cm above the ground was used and rats held the rope with front grabby part and total duration of hanging on that rope was measured.

2.1.4.2.Beam walking test:

A beam 50 cm long and 50 cm elevated from the floor was used to measure balance and extremity strength of animals. Total duration from start point to the target point was recorded.

2.1.4.3.Rotarod:

Ugo Basile Rota-Rod 47600 was used in the current study. It had a rolling lane (rotation speed of 0~40 rpm) with two-way high walls. The rolling lane began to rotate at a speed of 4 rpm, reaching a maximum rotational speed of 40 rpm in a 120 sec. Total time for staying on accelerating lane was recorded during maximum 300 seconds.

2.1.5. Morris water maze test:

The test apparatus consisted of 210 cm diameter by 51 cm high circular tank. The water in the tank became opaque with nontoxic paint and its temperature was adjusted to the 23 ^{0}C (±1) by an automatic heater. The depth of water was 45 cm. Tracks of the rats in the water during searching for hidden platform using extra-maze cues were recorded by video tracking system.

Four daily trials were applied during place learning for 6 consecutive days. When the rat found the platform or when 60 seconds passed, the trial was considered as completed. After place learning is completed, hidden platform removed from the water and a 60-second probe trial was conducted on the seventh day of Morris water maze testing. A rat's percentage time spent in the platform quadrant was recorded on the probe trial using video tracking system.

2.1.6. Passive avoidance test:

In this test, there were a white-illuminated (1500 lux) compartment and a black-closed (2 lux) compartment. A guillotine door was placed between these compartments for separation of them. There was two sessions in this test. In "the acquisition session", rats were put to the white-illuminated compartment. After 20 sec., the door was opened, and the rats were free to pass to the black-closed compartment. After entering the black-closed compartment of the passive avoidance box, the rats were experienced an electric shock (1 mA) for 2 sec. The "retention session" was applied to the rats after 24h delay. In this session, the rats placed to the white-illuminated compartment and the door was opened after 20 sec. The time of the entrance to the black-closed compartment was recorded with a maximum cut-off time (150 sec). (Isik et al., 2009).

2.1.7. Object recognition test:

In this test, a plexiglass chamber (around 40 cm x 30 cm x 30 cm) and two different shaped and colored objects were used to measure recognition memory in rats. This test was applied for two days. In the training day, two identical objects (yellow cylinders, 7 cm X 9 cm) were placed to the opposite corners of the chamber and rats were allowed to explore them for five minutes. On the second day, the testing day, a new (novel) object (a blue square (6 cm X 6 cm X 6 cm) and an old object (yellow cylinder) were placed to opposite corners of the chamber. Duration of

exploration (sniffing and touching to object, standing closer to the object etc.) for both old object and new object were measured for 5 min. (Leger et al., 2013).

2.1.8. Y-maze test:

Y-shaped maze with three arms (50 cm length, 16 cm width, and 50 cm height for each) developed by Dellu was used (Dellu et al., 1992). Animals were placed on the middle arena of three arms in a 350 lux-illuminated room and order of visited arms were recorded and triple combinations were calculated in a respective manner for 5 min.

2.1.9. Tail suspension test:

On the last day of behavioral experiment, tail suspention test was applied to the rats in a 350 lux-illuminated room. The tails of rats were wrapped to a horizontal bar with adhesive tape without giving harm to the rats. The bar was 50 cm above the ground. Immobility times were recorded during 5 min testing period.

2.2. Molecular Experiments

2.2.1. Enzyme-linked immunosorbent assay (ELISA):

At the 75-day old, rats were decapitated by guillotine under anesthesia when the behavioral experiments were completed. Heart blood was immediately collected after decapitation and serum was separated by centrifugation to store at -80^{0}C. Plasma levels of corticosterone were determined using commercial ELISA kits (Abcam, #ab108821) and the levels of corticosterone were determined at 450 nm at MultiskanTM GO microplate reader (Thermo Fisher Scientific, USA).

2.2.2. Measurement of total antioxidant (TAS) and oxidant (TOS) status:

The levels of TAS and TOS were calculated in the serum of the samples using an automated analyzer (Chromate Manager 4300, Palm City/USA). In the levels of TAS and TOS

determination, a prochromogen solution (Rel Assay Diagnostics, TURKEY) was added to the serum samples and oxidized molecules with hydroxyl radicals were obtained by measuring light intensity at 530 nm and 660 nm for TOS and TAS, respectively. Oxidative stress index (OSI) was determined by estimating TOS and TAS data.

2.2.3. DNA microarray analysis:

Total RNA was extracted from the left hippocampi of rats, which were homogenized with microbeads (Next Advance, #119I2) in the FastPrep®-24 (MP Biomedical, USA), using the kit RNeasy Plus Mini kit (Qiagen #74134) according to the manufacturer's protocol. The RNA concentration was determined using a spectrophotometer (MultiskanTM GO, Thermo Fisher Scientific, USA) and, for each group, all the animals' RNA were pooled nearly 100 ng/µl at a final concentration. Whole transcript expression array was performed using the WT Hibridizasyon Array (#902665 Affymetrix, USA). Briefly, first-strand cDNA and double-strand cDNA synthesis were performed. cRNA was generated using this cDNA as a template by *in vitro* transcription and its amount was measured using a Nanofotometer after purification and fragmentation steps. After second step cDNA synthesis and purification, aliquots of fragmented and labelled ss-cDNA samples were hybridized to WT Hibridizasyon Array. Then, arrays were sequentially washed. GeneChip® 3000 Scanner 7G was used for scanning of arrays. For hybridization defects, quality control procedures of arrays were inspected according to the recommendation of manufacturer. The obtained microarray data were imported. Using The gene expression values' average of the replicated probes were used to determine the expression levels on a transcript level. Fold changes according to control values were calculated for each gene of each group.

2.2.4. Quantitative real-time PCR (RT-PCR) analysis:

Left hippocampus of rats were homogenized. TRİzol was used for total RNA isolation. After incubation with chloroform (at room temperature for 3 min.), samples were centrifuged at 12000 *g* for 15 min at 4C°. Equal amounts of 70% ethanol was added to the transparent part of the supernatant. Using PureLink RNA mini kit columns, the samples were washed and isolated RNAs were separated to eppendorf tubes. The total isolated RNA amount were calculated by a microplate reader (Multiskan™ GO, Thermo Fisher Scientific, The Netherlands). For storage, RNAs were reverse-transcribed to their complementary DNAs (cDNA). On the performing RT-PCR, reaction mixture including Sybr®Green (Bioline, Luckenwalde, Germany) were added to the cDNA mixture and using CFX96 Touch™ RT-PCR (Bio-Rad Laboratories, California) and determining the suitable thermal cycle, reactions were performed. For observation of alterations in the neuronal signalling and function, we studied, *Akt*, *NfkB*, *NGF*, and *synaptophysin* genes with housekeeping *GAPDH* gene by obtaining primer pairs from Sentromer Technology (Istanbul, Turkey). The threshold cycle (CT) relative quantification method were used for determination of expression of genes. The normalization of CT values were performed using the CT value of *GAPDH* gene to calculate ΔCT . The ΔΔCT formula was used to calculate the relative levels of interested gene by subtracting ΔCT (control) valur from ΔCT (TQ group). The $2^{-\Delta\Delta Ct}$ formula was applied to determine the fold induction ratios according to relative quantification.

2.2.5. Western blotting:

Right hippocampal tissue was homogenized using RIPA lysis buffer. After centrifugation, the amount of proteins in supernatants were measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham). The equal amounts of protein were diluted in sample buffer, denaturated by boiling, and loaded onto 4-20% Bis-Tris polyacrylamide gels to perform sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, samples

were transferred from polyacrylamide gels to the PVDF membranes. After transfer, membranes washed with 0.1 M Tris-buffered saline (TBST) and blocking solution (5% milk powder in 0.1% Tween 20/TBST) was added to the membranes. When the blocking step was completed, the mebranes were incubated with primary antibodies (diluted in blocking solution) against to proteins which were obtained from the microarray data and proteins which were chosen from the previous studies related to the behavioral development and disorder. These proteins were Clic6 (Santa Cruz Technology, Cat# sc-365303, RRID:AB_10851345), Kcjn-1 (Abcam Cat#ab80967, RRID:AB_1640783), Klotho (LifeSpan Cat# LS-C8375-50, RRID:AB_835055) and prealbumin (LifeSpan Cat# LS-B775-50, RRID:AB_1812937) from the microarray data and doublecortin (DCX; Cell Signaling Technology, Cat#4604, RRID:AB_561007), brain derived neurotrophic factor (BDNF; Santa Cruz Technology, Cat# sc-546, RRID: AB_630940), heat shock protein 70 (Hsp-70; Cell Signaling Technology, Cat #4872, RRID:AB_2279841) from the literature. The peroxidase-coupled secondary antibody incubation was performent on the following day. The luminol substrate (Advansta, San Francisco, USA) was used for determination of signal intensity under CCD camera (Fusion FX7 system, Vilber Lourmat). Monoclonal mouse antibody against β-actin and β-tubulin (Thermo Fisher Scientific, Paisley, England) were used for controlling the protein loading. ImageJ analysis system (NIH; Washington, USA) was used to quantify immunoreactive protein bands densitometrically.

2.3.Statistical Analysis

Statistical analysis of behavioral and molecular data which were represented as mean ± S.E.M was conducted by SPSS 22.0. One-Way ANOVA test was used for analysis of difference between four music groups. The repeated measure ANOVA was used to analyze Morris water maze training data. For paired comparisons, Fisher's least significant difference (LSD) were performed. Statistical significance for all tests were accepted as the level of $p<0.05$. Partial eta

squared for the effect size (ES) was estimated to determine the strength of relationship between data. The larger the effect size the stronger the relationship between two variables.

3. Results

3.1.Anxiety-Like Behavior Was Lower in Rock Group

As it was seen from Figure 1, in the plus maze closed arms, the time spent were relatively high in the rats of the classical and Sufi group and the rats in the classical group had more entries to that arm (Fig. 1B). The statistical test yielded treatment effect insignificant in time spent ($F_{(3,81)}$=0.007, p=0.999, ES<0.001) and significant main effect in arm entries ($F_{(3,54)}$=5.173, p=0.004, ES=0,911). In addition, a significant effect in the type of arm was noted ($F_{(2,81)}$=412.222, p<0.001, ES=0.184) with arm X group interaction ($F_{(6,81)}$=2.480, p=0.031) for the time spent in the maze and insignificant effect in the type of arm ($F_{(1,54)}$=3.031, p=0.088, ES=0.049) with a significant arm X group interaction ($F_{(3,54)}$=6.233, p=0.001) for the arm entries. It was also observed that each group had the same motor activity which was obtained from the ratio of entries to closed arm entries to total entries in total time (Walf & Frye, 2007) according to the One-way ANOVA ($F_{(3,26)}$=1.288, p=0.302, ES=0.144). In point of detailed statistical analysis, Post Hoc LSD results showed that rock group spent less time in enclosed arm than the rats in the groups of control (p=0.067), classical (p=0.009), and Sufi (p=0.015) (Fig. 1A). According to Post hoc LSD test, the rats in the classical group entered more to the closed arm (p=0.069 for control group; p=0.015 for Sufi group; p=0.005 for rock group) (Fig. 1B). On the other hand, rock group's rats entered to the open arm more times (p=0.001 for control group; p=0.016 for classic group; p<0.001 for Sufi group;) (Fig. 1B). Low number of number of entrances to the open arm was observed in the rats of the Sufi group.

In light/dark transition test, there was a difference for total number of transition ($F_{(3,23)}$=3.878, p=0.02, ES=0.336) among groups (Fig. 1C). LSD results showed that Sufi group had a greater number of transitions between rooms compared to classical and control groups (p=0.007 and

p=0.005, respectively). In addition, we calculated the time spent in the light compartment and the main effect of treatment was insignificant according to the One-way ANOVA ($F_{(3,23)}$=1.691, p=0.197, ES=0.181). Post hoc LSD test showed that the rats in the classical group spent less time in the light compartment compared to rock and control groups (p=0.074 and p=0.070, respectively) (Fig. 1C). The rats in the rock group did not show any change in this test in contrast to the results of the elevated plus maze.

In the present study, we evaluated rearing, sniffing, grooming, freezing, total number of defecations, and total time spent in arena center in the open field arena to test the anxiety levels of animals. There was not statistically significance for grooming, freezing, total number of defecations among groups (p>0.05). Total rearing ($F_{(3,23)}$=3.311, p=0.038, ES=0.302), total sniffing ($F_{(3,23)}$=3.253, p=0.040, ES=0.298), and total time spent in arena center ($F_{(3,23)}$=3.549, p=0.030, ES=0.316) results changed significantly among groups. The rock group had a greater number of rearing behaviors compared to the classical and control groups (p=0.050 and p=0.008, respectively) (Fig. 1D). In the Sufi group, sniffing as an exploration behavior increased compared to the classical and control groups (p=0.008 and p=0.041, respectively) (Fig. 1D). In addition, the rock group spent more time in arena center than control (p=0.014), classical (p=0.021), and Sufi groups (p=0.009) (Fig. 1D).

*3.2.**Both Classical and Rock Groups Had Better Motor Coordination Performance***

On the motor coordination test, a treatment effect was observed according to one-way ANOVA ($F_{(3,23)}$=5.032, p=0.008, ES=0.334). Average duration in rotarod for the classical group was higher compared to the Sufi and control groups (p=0.006 and p=0.025, respectively). In addition, the rats in the rock group showed better performance in the motor coordination compared to the Sufi and control groups (p=0.006 and p=0.023) (Fig. 2).

However, the results of wire rod test ($F_{(3,23)}$=1.255, p=0.313, ES=0.141) and the beam walking test ($F_{(3,23)}$=0.618, p=0.610, ES=0.051) were insignificant among the groups.

3.3.The Types of Music Pattern Differently Affected The Performance of Learning and Memory in Rats

Passive avoidance, object recognition, and Morris water maze were conducted for measuring short/long term memory, fear conditioning memory and recognition memory, respectively. According to training data of Morris water maze, the latency of reaching the platform was decreased in all groups among training days (Fig. 3A). The control and Sufi rats reached the criteria of learning the hidden platform place (~10 sec.) on the fourth day of training whereas the ratss in the classic group reached it on the third day. However, the animals in the rock group did not reach the criteria in the 6 day of training. The day effect was significant according to two-way repeated measure ANOVA ($F_{(5:110)}$=68.410, p≤0.001, ES=0.757). In addition, there was a significance between-subject effect ($F_{(3,22)}$=5.789 p=0.004, ES=0.441). The rats in the rock group showed the worse learning performance on the second day of Morris water maze ($F_{(3,22)}$=9.739, p<0.001, ES=0.561). The worse performance in the rock group continued in the following days (Fig 3A). The rock group also showed worse performance in the probe trial by spending less time in the target quadrant (platform quadrant)as compared to the control group (p=0.046, ES=0.207) (Fig. 3B).

The worst performance and the best performance were obtained respectively by rats in the classical group and in the Sufi group in the passive avoidance retention test while it had no statistically significant difference (p>0.05, ES=0.149). Furthermore, there was a marginal change between the Sufi group and the classical group in the fear memory performance (p=0.066) (Fig. 3C).

In the recognition memory test, an increase in the new object exploration of rats in the control group was observed compared to the old object recognition without any statistically significant change. However, compared to the control group, the Sufi group had more time with new object (p=0.044, ES=0.168), while the time spent in the old object recongnition was insignificant (Fig. 3D). The discrimination index also calculated for the object recognition test and it was found 0.05 for control group, -0.04 for classical group, 0.12 for Sufi group and 0.06 for rock group. In the Y-maze test, we did not obtain any significant change among groups ($F_{(3,23)}$=0.439, p=0.727, ES=0.054).

3.4.Exposure to the Sufi Music Pattern in the Perinatal Period Increases the Depressive-Like Behavior in the Adult Life

Animal immobility time was measured as an indicator of depressive-like behavior in tail suspension test. There was statistically significance between-group effect ($F_{(3,23)}$=3.174, p=0.043, ES=0.293) according to one-way ANOVA. LSD results showed that the Sufi group had more immobility time than the classical and control groups (p=0.008 and p=0.016, respectively) (Fig. 4).

3.5. Rock Music Pattern Increased Corticosterone Level While Classical Music Increased Oxidative Stress Level

A significant between-group difference in the OSI level was noted according to one-way ANOVA ($F_{(3,21)}$=3.264, p=0.046, ES=0.352). The level of TAS decreased in the classical group than the rock group (p=0.029, ES=0.226) (Fig. 5A). The change in the TOS level among groups was insignificant, while a relative increase was observed in the classical group (Fig. 5B). In the calculation of oxidative stress, it was observed that the classical group had higher OSI level than rock and control groups (p=0.014 and p=0.023, respectively) (Fig. 5C).

The level of corticosterone was measured to evaluate the level of physiological stress and we found that listening to the rock music pattern in the perinatal period increases the corticostereone level (Fig. 5D). However, this increase was significant when we compared the rock group to the Sufi group (p=0.05, ES=0.171).

3.6.Exposure to Different Patterns of Music in the Perinatal Period Affects the Levels of Some Genes

The present study showed some alterations in the gene expression due to different patterns of music exposure in the perinatal period acoording to microarray dat. According to fold change against to the control group, we observed different number of gene which were upregulated and downregulated in response to different music patterns (Fig 6A). As you can see from the figure, the number of common altered gene expression in both Sufi and classical music pattern was higher than classical and rock or Sufi or rock. Two common genes (one of them is mir296 and the other one is undefined) were altered in all groups. The other known common genes between groups and their fold changes presented in the figure 6B. According to this, mir296 expression was affected from all types of music pattern. The upregulation of some genes such as klotho, transthyretin, kcnj13, clic6, slc4a5, fibromodulin, and Igf2 were high in the classical group (Fig. 6B). The expression of klotho, transthyretin, and kcnj13 were also upregulated in the Sufi group. The eIF5 gene expression downregulated in both classical and Sufi groups. The common upregulated genes between the rock and Sufi groups were carboxyesterase-like gene in addition to olfactory receptor genes (Fig. 6B).

In the pathway analysis, we observed that Sufi music pattern exposure had an effect mostly in the regulation of biological processes and the response to stimulus. In addition, classical music pattern exposure had effects on the nervous system development and some transport mechanisms and metabolic function that include sulfur. On the other hand, rock music pattern exposure had the most gene ontology terms in biological processes which associated with the

regulation of biological processes and the response to stimulus as Sufi music pattern exposure (Fig. 6C).

In addition, we performed RT-PCR for further analysis of some specific genes that are responsible for cell signalling in the neuronal development and maintenance (Fig. 7). In the current study, we did not observe any change in the AKT expression ($p>0.05$, ES=0.128) (Fig. 7). On the other hand, rock music exposure during perinatal life increased the expression of NFkB (4.24-fold) in comparison with the values in the control group ($p=0.018$, ES=0.272). A small change was observed in the expression of NGF in the rock group as a 3.45-fold increase when compared both control group ($p=0.067$, ES=0.209) and classical group ($p=0.060$). Interestingly, rock music pattern exposure increased SYP expression as compared to other music groups ($p=0.028$ for classical and $p=0.045$ for Sufi, ES=0.258) (Fig. 7).

3.7.The Concentration of Proteins were also Affected from Perinatal Music Exposure

For microarray validation, western blot analysis was performed using antibodies against to proteins (Klotho, Transthyretin (Prealbumin), Kcnj13, and Clic-6) whose expressions were altered in the microarray data.

The between-group change in the protein expression of these genes was significant (*klotho*: $F_{(3,11)}=8.253$, $p=0.008$, ES=0.759; *transthyretin:* $F_{(3,11)}=23.209$, $p\leq0.001$, ES=0.890; *Kcnj13:* $F_{(3,11)}=37.759$, $p\leq0.001$, ES=0.933; and *Clic-6:* $F_{(3,11)}=17.212$, $p=0.001$, ES=0.867). The expression level of klotho was presented in Figure 8A. The similar pattern seen in microarray data (20.97 and 2.74-fold change, respectively) was validated in the protein expression of Klotho as an increase in the classical ($p=0.001$) and Sufi ($p=0.037$) groups in comparison to the control group. In addition, the rock group also had higher klotho level than the control group ($p=0.015$). Furthermore, the rise of klotho expression was higher in the classical group than that of the Sufi group ($p=0.042$). In parallel to microarray data, the protein expression of

transthyretin (prealbumin) was affected from different music pattern types (Fig. 8B). The expression level of prealbumin protein was higher in the classical group ($p=0.033$) and lower in the Sufi music group ($p=0.001$) than that of the control group. In the Sufi group, the decrease in the prealbumin level was also different than the rock ($p=0.006$) and classical ($p\leq0.001$) groups. Lastly, there was a significant expressional level of prealbumin between the rock and classical groups ($p=0.002$). The other protein which affected from perinatal music exposure according to the microarray data was potassium voltage-gated channel subfamily J member 13 (KCNJ-13) (Fig. 8C). In contrast to microarray data, a dramatic decrease in the Kcnj-13 protein level was observed in the Sufi group as compared to all other groups ($p\leq0.001$). In parallel to the microarray data (17-fold increase), we observed an increase in the expression of Kcnj-13 in the classical group in comparison to the control group, however, this rise was not significant. On the other hand, this increase produced a difference in the level of Kcnj-13 between the classical and rock groups ($p=0.036$). The last protein which was validated after the observation of nearly 10-fold increase in the classical group was the Chloride Intracellular Channel 6 (CLIC-6) protein (Fig. 8D). The perinatal music exposure effect in the Clic-6 expression was significant in all groups ($p=0.001$ in the classical group; $p\leq0.001$ in the rock group; $p=0.002$ in the Sufi group). In addition, the rise in the Clic-6 expression was notable in the rock group than that of the Sufi group ($p=0.053$).

In the current study, we also investigated selected additional proteins related neurogenesis (DCX), neuron development (BDNF) and stress protection (Hsp-70). According to Post Hoc LSD analysis after one-way ANOVA, compared to the control group, there was a decrease in the DCX expression in the Sufi ($p\leq0.001$) and rock ($p=0.007$) groups whereas this difference was absent in the classical group ($ES=0.876$) (Fig. 9A). In addition, the lowest level of DCX was present in the hippocampus of rats in the Sufi group in comparison with the classical group ($p=0.001$) and rock group ($p=0.015$). The expression level of DCX protein was also different

between the rock group and classical group (p=0.041). Parallel to DCX expression, in the Sufi group, the maturation of BDNF was also lowest (p=0.046 for control; p≤0.001 for classical; and p=0.004 for rock, ES=0.834) (Fig. 9B). On the other hand, classical music pattern exposure during the perinatal period increased the ratio of mature BDNF to pro-BDNF in comparison with the ratiof of mature BDNF to pro-BDNF in the control group (p=0.006). The expression of heat shock protein-70 (Hsp-70) which helps to protect cells from stress was affected only in the Sufi group as a decrease in this group when compared to other groups (p=0.023 in the control and the rock groups, and p=0.026 in the classical group, ES=0.603) (Fig. 9C).

4. Discussion

During prenatal development, the fetus is exposed to many environmental factors. Music is one of these factors known to have an impact on human development. Studies have shown that when pregnant women are exposed to music particularly in the period of brain development, the babies gain positive effects such as enhancing learning capacity and memory consolidation. In a previous study, Celma-Miralles & Toro (2020) were observed that rat could sense the rhythmicity of a music rather than other features like decibels, frequency or pitch when they exposed to rats with a part of the Happy Birthday song. In addition, rodents can discriminate the Beatles and Mozart (Okaichi & Okaichi, 2001) or Stravinsky and Bach (Otsuka et al., 2009) due to differences in the tune. Therefore, in the present study, we investigated the effects of different sound patterns (at different unit of loudness and rhythmicity) exposed during perinatal period on behavioral characteristics such as anxiety, motor coordination, learning, memory and depression, and we aimed to determine the underlying molecular mechanisms that might cause these effects.

In this study, the rats exposed to rock music pattern manifested significantly lower anxiety-like behavior as compared to the rats in other groups. Exposure to noise during prenatal or postnatal period has been found to increase anxiety-like behavior in animals (Naqvi et al., 2012). In

keeping with our findings, the levels of anxiety and depression among metal music listeners are similar to and lower than those among the general population. (Recours et al., 2009). In addition, Tornek et al. (2003) recorded a more positive EEG signals with a decrease in the anxiety level and cortisol concentration of mothers who exposed to rock music. In our study, we used the number of arm entries and the time spent in the closed/open arms of the elevated plus maze, the time spent in the center of the open arena, the stereotypic behaviors of the rats as an anxiety level. The lesser time in the closed arm and the highest number of entries to the open arm, the more time in the open arena center and the increase in the rearing behavior supported the low anxiety level in the rock group. An increase in the time spent in the middle area of the open field and a decrease in the closed arm of the elevated plus maze also showed as an indicator for the decrease in the anxiety and increase in the exploratory activity in animals, as noted in our rock group animals (Küçük & Gölgeli, 2005). The rearing behavior which is mostly performed by the unstressed animal is an exploratory behavior for the environment (Küçük & Gölgeli, 2005). When the anxiety state is increased in rats, this behavior decreases (Kenntner-Mabiala et al., 2007). For example, in a previous study (Poltyrey et al., 1996), prenatal stress produced by noise exposure during pregnancy resulted in a reduction in the number of rearing suggesting a suppression of exploratory activity. Herein, the smallest number of rearings were observed in the rats of the classical group. On the contrary to our results, Papadakakis et al. showed that 12-hour/day exposure to Mozart's K. 448 sonata (65-70 dB) during postnatal days 2 and 14 preserved maternal separation induced anxiety and depression (Papadakakis et al., 2019). However, we did not observe another anxiety sign in the classical group except the rearing and sniffing behavior alteration.

In other respects, the light/dark transition and the sniffing behavior in the Sufi group were higher compared to behavior in the control and classical groups suggesting an increase in the exploratory behavior. The light-dark transitions have been reported to be an index of activity-

exploration because of habituation over time and an increase in transitions is considered to reflect anxiolytic activity (Borin & Hascöet, 2003; Zhang et al., 2014; Morgan & Pfaff, 2002). However, due to the lack of supporting data for the anxiolytic effect which was obtained from the elevated plus maze and open arena, the higher transition between light and dark compartments did not completely suggest the anxiolytic effect of Sufi music on the rats as seen in the data of previous studies obtained by classical music exposure in rats (Chikahisa et al., 2007). On the other hand, a high level of sniffing behavior may be considered as a stress sign (Boukouvalas et al., 2008). However, as we investigated the plasma corticosterone level as a molecular sign of stress, we did not observe any effect of the perinatal Sufi and classical music sound pattern exposure on the corticosterone level. Interestingly, we obtained higher plasma corticosterone level in the rock group that that of the Sufi group suggesting that Sufi music pattern causes a reduction in the physiological stress as compared to rock music pattern.

In contrast to the relation between stress and depression, we noted an increase in the depressive-like behavior in the Sufi group than that of the classical and the control groups. Previous studies showed that corticosterone subserves important adaptive functions during stress exposure by limiting its own secretion as a negative feedback regulation of HPA axis. While initially adaptive, chronic stress response may be involved in the development of disease states such as depression (Herman & Cullinan, 1997; McGonagle & Kessler, 1990). In addition, according to the microarray data, the molecules for the response to stress pathway were activated only in the rats of the Sufi group than the rats in the control group whereas there was a slight decrement in the corticosterone level of the Sufi group rats in comparison with control group rats. At the molecular level, in the Sufi group, we observed a decrease in some proteins related with neurogenesis (DCX), neuroprotection (Transthyretin/Prealbumin), synaptic plasticity (Kcnj-13/Kir7.1), and neuronal survival (BDNF and HSP-70) which are mostly affected from physiological and cellular stress conditions (Gobinath et al., 2017; Sousa et al., 2004; Pulga et

al., 2016; Boersma et al., 2014; Sharp et al., 2013). Sufi music is the devotional music of the Sufis which is played with the Ney, an end-blown flute. In our study, as seen from the experimental results, the rhythmicity of this mystic music pattern produces an unsettling external factor for the dams and their pups due to being stranger to these types of sounds. Exposing these external stimuli during perinatal development produces a negative effect on the DCX expression in the hippocampus of rats as seen in a prenatal stress-related previous study (Lucassen et al., 2009). In a long-term stress condition, a displayed depressive-like behavior is correlated with decreased neurotrophic factor for normal neuronal development such as neurotrophic factor-α1 and fibroblast growth factor 2 (FGF2) following a reduced neurogenesis obtained by a decrease in the DCX expression (Cheng et al., 2015). Herein, we also observed a parallel reduction in neurogenesis and neurotrophic factor, BDNF, in the Sufi group. The decrease in the BDNF level is also correlated with development of mood disorders due to its critical role in the modulation of neuronal signaling (Boersma et al., 2014). On the other hand, in a previous study, perinatal classical music exposure enhanced learning performance and reduced the BDNF levels in the cortex of the rats in their adult life (Chikahisa et al., 2007). Another molecule that play a role in the survival of neuron like BDNF is Hsp70 whose level also reduced with Sufi music pattern exposure during prenatal period. Hsp70 can also inhibit apoptosis by binding different types of proteins involved in apoptotic pathway (Joly et al., 2010). In addition, an increase in the Hsp70 protein acts as a neuroprotectant for the damaged neurons (Sharp et al., 2013). It may be concluded that the depressive effect of Sufi music pattern can change the balance between cell survival and apoptosis by reducing the level of Hsp70. The neuroprotectant molecule, prealbumin (Nunes et al., 2009) also reduced by Sufi music pattern exposure in the adult hippocampus. Previous studies showed that maternal separation stress resulted with low level of the prealbumin expression in the adult rats' hippocampi suggesting its role in the sensitivity of stress (Kohda et al., 2006) and leading to depression (Sullivan et al.,

2006). Lastly, the decrease in the Kir 7.1 expression in hippocampus (Partiseti et al., 1998) supported the reduction of synaptic plasticity because inwardly rectifying K+ channels interact with the cytoskeleton to mediate aspects of synaptic plasticity (Cohen et al., 1996) due to Sufi music pattern exposure-related depression-like behavior.

In addition, motor coordination abilities were better in the classical and rock groups. In one of the previous studies, it was found that both classical music and variety music can decrease the motor reaction time due to greater activation of the right cerebral hemisphere from emotional structures (Zakharova & Ivashchenko, 1984). The enhancement effect of music on the motor function can be proved by the previous studies showing the ameliorative effects of music therapy including drum and piano on the neurological movement disorders such as stroke (Altenmuller et al., 2009). On the other hand, it was showed that prenatal exposure to comportable music like classical music promoted neurogenesis in the rat pups' motor cortex resulting an amelioration in the motor functions (Chen et al., 1994; Kim et al., 2013). This increase in the motor coordination can be related with the higher level of synaptophysin expression because there is a correlation between synaptophysin level and motor coordination impairments (Valencia et al., 2019). At the same time, it was supported that prenatal sound stimulation using patterned sounds enhances the expression of synaptophysin (Alladi et al., 2002).

Previous studies also showed that prenatal exposure to different types of music significantly affects the learning and memory abilities of the pups in their adult life. In the current study, a positive effect on the object recognition memory was noted in the rats prenatally exposed to Sufi music pattern. As parallel, the level of Klotho protein was increased. It was previously showed that overexpression of Klotho in the CA1 region of hippocampus improved object recognition task due to the function of Klotho on the enhancement of the activity-dependent synaptic plasticity (Li et al., 2019). The low level of Klotho expression in the brain is related to

the memory loss because the proteolytic shedding of Klotho performed by a disintegrin and metalloproteinase domain-containing proteins (ADAMs) which is responsible for the cleavage of the amyloid precursor protein (APP) and result in Alzheimer's Disease (Saftig & Lichtenthaler, 2015; Peron et al., 2018).Therefore, increase in the level of Klotho is related to the learning and memory improvements in rats of the Sufi group as well as in rats of the classical group. The rats in the classical group reached the criteria faster than other groups in the spatial learning paradigm suggesting the best learning performance among the other groups (Vorhees & Williams, 2006). There are many studies related to the ameliorative effect of classical music on learning and memory (Xing et al., 2016b; Xiong et al., 2018; Amagdei et al., 2010). For example, Rauscher observed that exposure to Mozart during in utero and 2 months after birth resulted more rapid in the completition of task in the maze with few errors (Rauscher et al., 1998). It was suggested that the enhacement in the spatial memory is mostly related to the increased neurogenesis in developing rats due to prenatal 65 dB comfortable music exposure (Kim et al., 2006). Herein, parallel to the increase in the learning performance, classical music pattern exposure increased BDNF level supporting to the notion related with the modulation of the hippocampal activity by new-age type music and classical music exposure through the level of BDNF (Chikahisa et al., 2006; Angelucci et al., 2007; Xing et al., 2016a). However, in different type of music such as retrograde Mozart music reversed this effect (Xing et al., 2016b). In addition to the Klotho, the microarray data showed that all other proteins related to the nervous system development and functioning such as Transthyretin, KCNJ and Clic6 were upregulated in the classical group. In support of microarray data, the protein expression of Klotho, prealbumin and Clic6 were higher in the classical music rats. These results suggested that classical music pattern exposure could ameliorate the synaptic plasticity which also observed in a previous study showing the relation between classical music exposure and hippocampal spine density (Papadakakis et al., 2019). As mentioned before, there is a positive

relation between the level of Klotho and learning performance (Saftig & Lichtenthaler, 2015; Peron et al., 2018). Also, prealbumin is a protein which preventing Aβ aggregation and maintaining of learning capacities during aging (Bastianetto et al., 2007).

Interestingly, the increase in the Klotho level did not point an enhancement of learning and memory abilities in the rock group. The rats in this group showed learning disabilities such as, in the Morris water maze, they did not reach the criteria of the time spent for learning the place of the hidden platform and their memory capacity were the lowest compared to the other groups. It is also supported that prenatal noise exposure delays brain development and impairs cognitive functions (Williams et al., 1998). According to the previous literature of emphasizing the relation between neurogenesis and memory enhancement, a decrease in the DCX levels was noted in the hippocampal tissue of the rock group. In addition, the increase in the NF-kB expression pointing to the neuroinflammation may be a cause of rock music pattern-related memory impairment (Degaspari et al., 2015). In contrast to other music patterns, listening to rock music pattern can increase the noradrenaline in plasma and compromises the spatial learning and memory through neuroinflammation due to its high intensity (Sanyal et al., 2013). To sum up, we observed that listening to regularly the Sufi music pattern during pregnancy might make people more prone to stress effect in their adult life by decreasing neurogenesis and neuronal survival. On the other hand, listening to the rock music pattern during pregnancy might decrease the anxiety and improve the motor coordination whereas a remarkable decrease in the learning and memory performance due to decreas in neurogenesis and increas in neuroinflammation. Supporting to the previous literature, listening to the classical music pattern might enhance the learning abilities and motor functions by an increase in the molecular markers of neurogenesis and synaptic plasticity. Therefore, it was concluded that perinatal music exposure may be an important epigenetic factor for functioning of higher brain areas in the adult life.

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Figure Legends

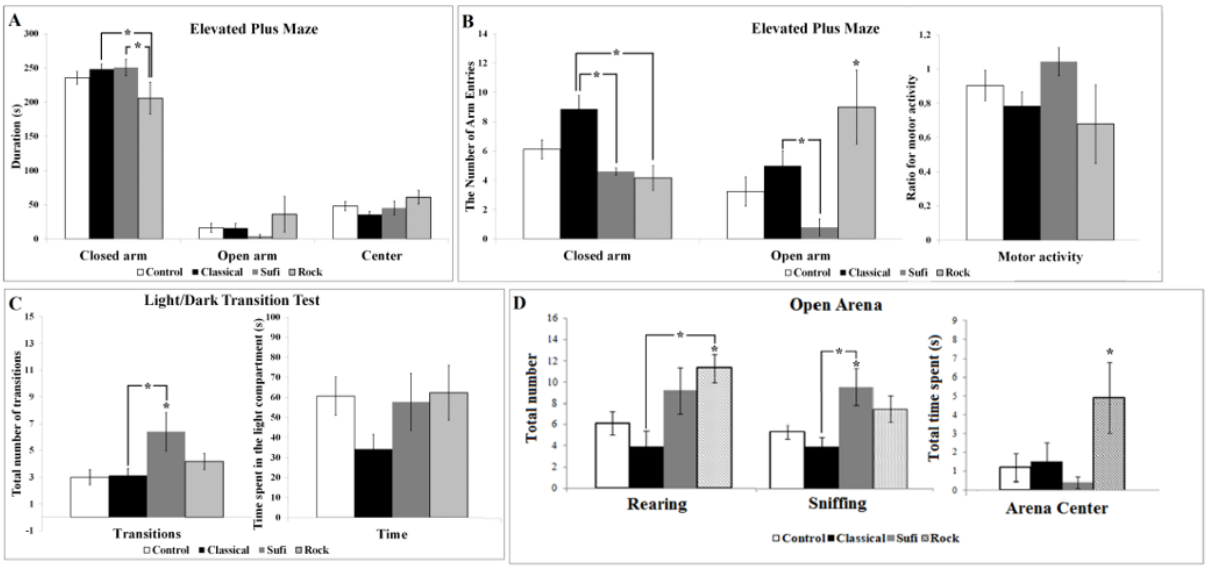


Figure 1: Comparison of the anxiety levels of pups by **A)** The time in the closed arm, open arm and center of the elevated plus maze **B)** The total entry numbers to the closed and open arms and the motor activity in the elevated plus maze **C)** The number of transitions and the time spen in the light compartment of the light/dark transition test **D)** The number of rearing and sniffing behavior and the time in the open arena center. Error bars denote standard error of mean (SEM). * indicates significant difference at $p<0.05$ according to control group. The lines represent the difference at two groups.

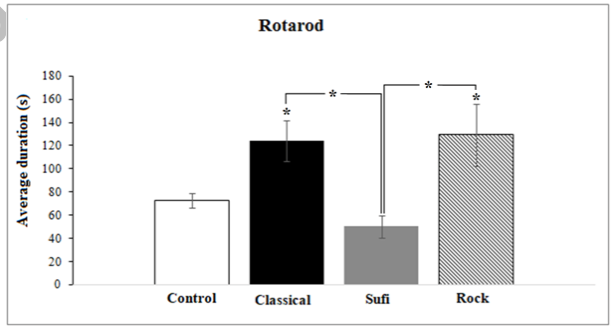


Figure 2: Comparison of sensory motor parameters of pups by Rotarod. Error bars denote standard error of mean (SEM). * indicates significant difference at $p<0.05$ according to control group. The lines represent the difference at two groups.

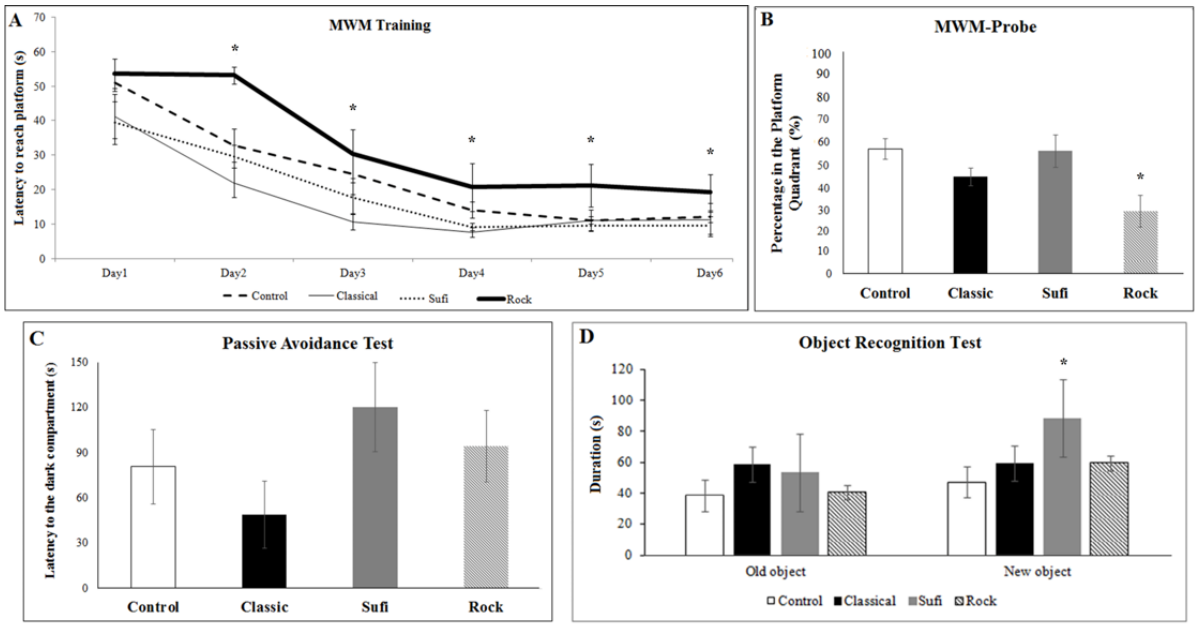


Figure 3: Comparison of the learning and memory **A)** Mean swim latency to reach hidden platform during Morris water maze training sessions **B)** The percentage of time in the platform quadrant on the Morris water maze test (probe) trial **C)** The time to enter the black-closed compartment of the passive avoidance box for fear memory retention **D)** Mean time spent with the novel and old object for the retention of object recognition. Error bars denote standard error of mean (SEM). * indicates significant difference at $p<0.05$ according to control group.

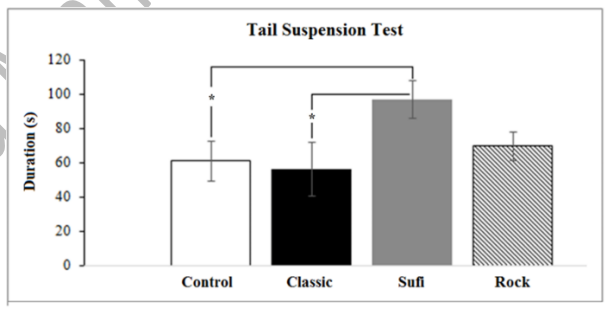


Figure 4: Comparison of the depressive-like behavior of the pups by tail suspension test. Error bars denote standard error of mean (SEM). * indicates significant difference at $p<0.05$ according to control group. The lines represent the difference at two groups.

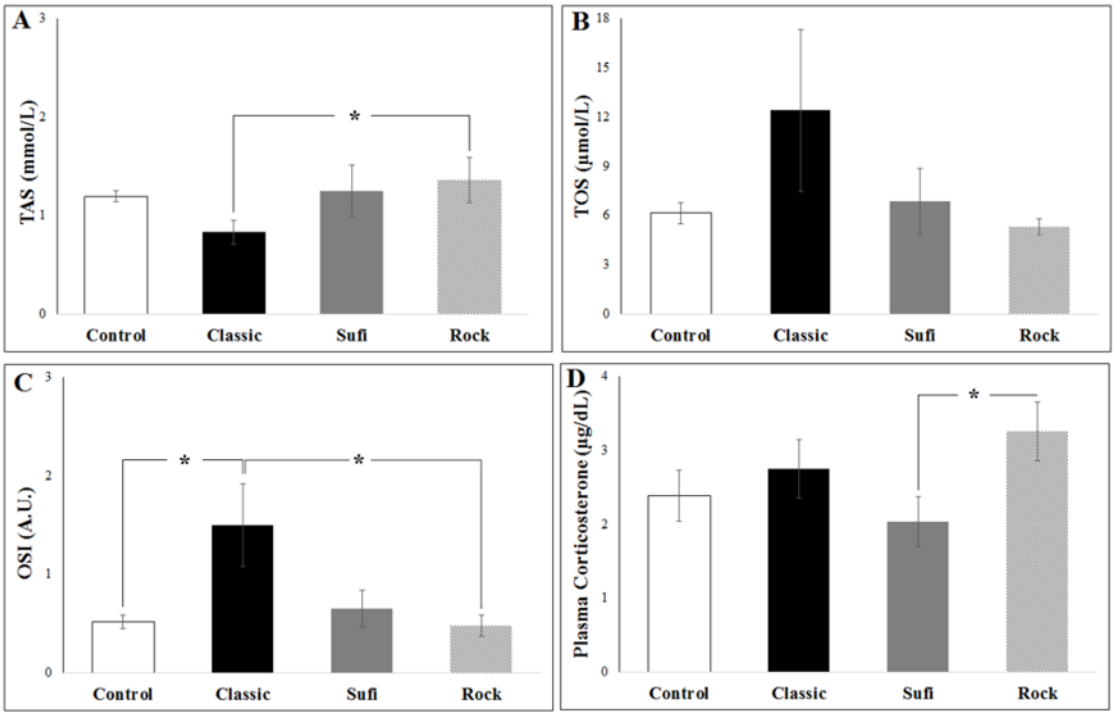
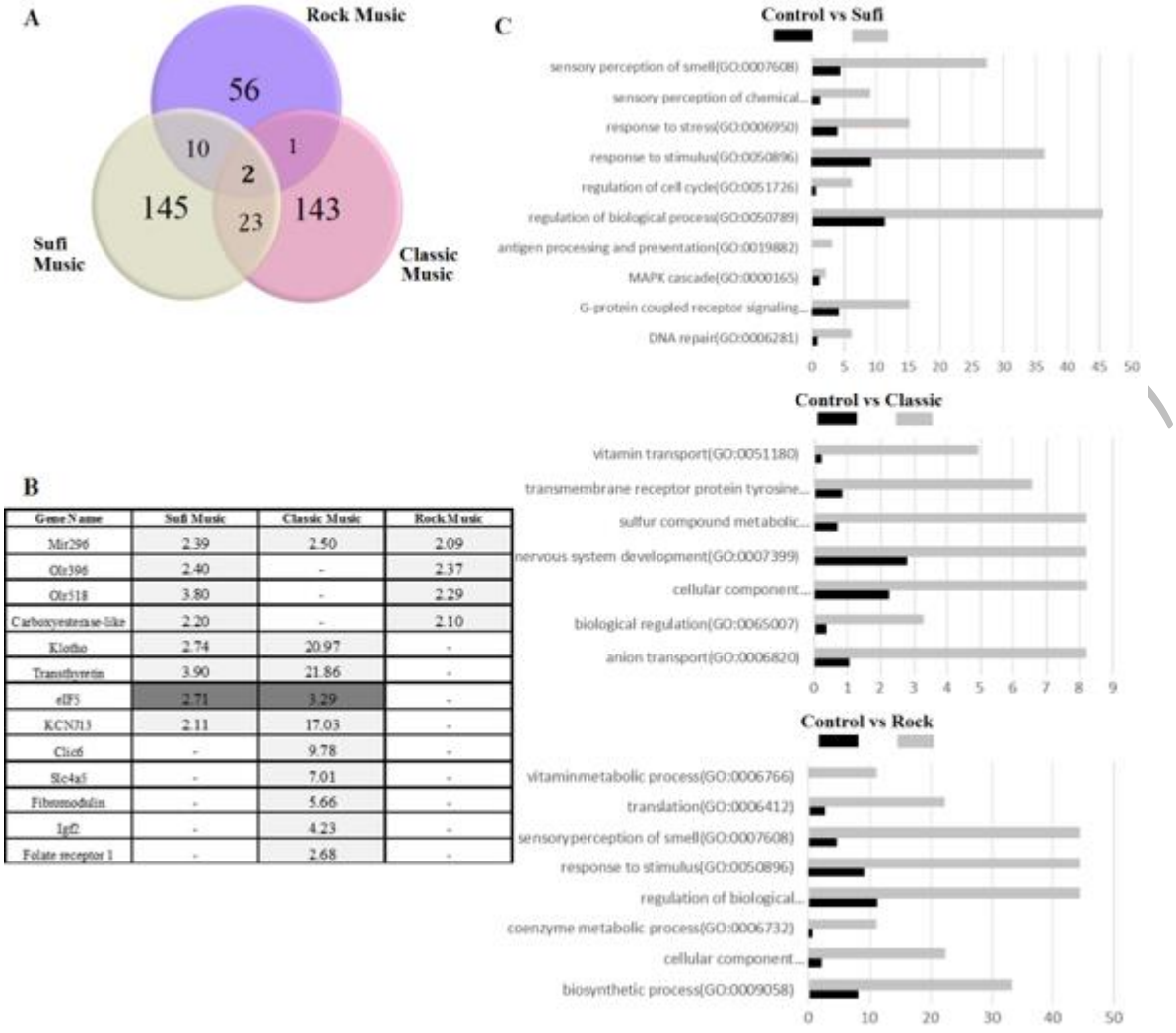


Figure 5: Comparison of the levels of **A)** TAS (mmol/L), **B)** TOS (µmol/L), **C)** OSI, and **D)** Plasma corticosterone levels at µg/dL in control, classical, Sufi and rock groups. Error bars denote standard error of mean (SEM). * indicates significant difference at $p<0.05$ according to control group. The lines represent the difference at two groups.



Gene Name	Sufi Music	Classic Music	Rock Music
Mir296	2.39	2.50	2.09
Olr396	2.40	-	2.37
Olr518	3.80	-	2.29
Carboxylesterase-like	2.20	-	2.10
Klotho	2.74	20.97	-
Transthyretin	3.90	21.86	-
eIF5	2.71	3.29	-
KCNJ13	2.11	17.03	-
Clic6	-	9.78	-
Slc4a5	-	7.01	-
Fibromodulin	-	5.66	-
Igf2	-	4.23	-
Folate receptor 1	-	2.68	-

Figure 6: Comparison of the microarray analysis results of the pups' hippocampal tissue**A)** The Venn diagram for the number of altered common gene expression for Sufi classical, and rock groups, **B)** The fold changes against to control group for some specific genes. White boxes for increase and gray boxes for decreases, **C)** The top biological pathways estimated by gene counts compared to background genes are shown by the horizontal bar length

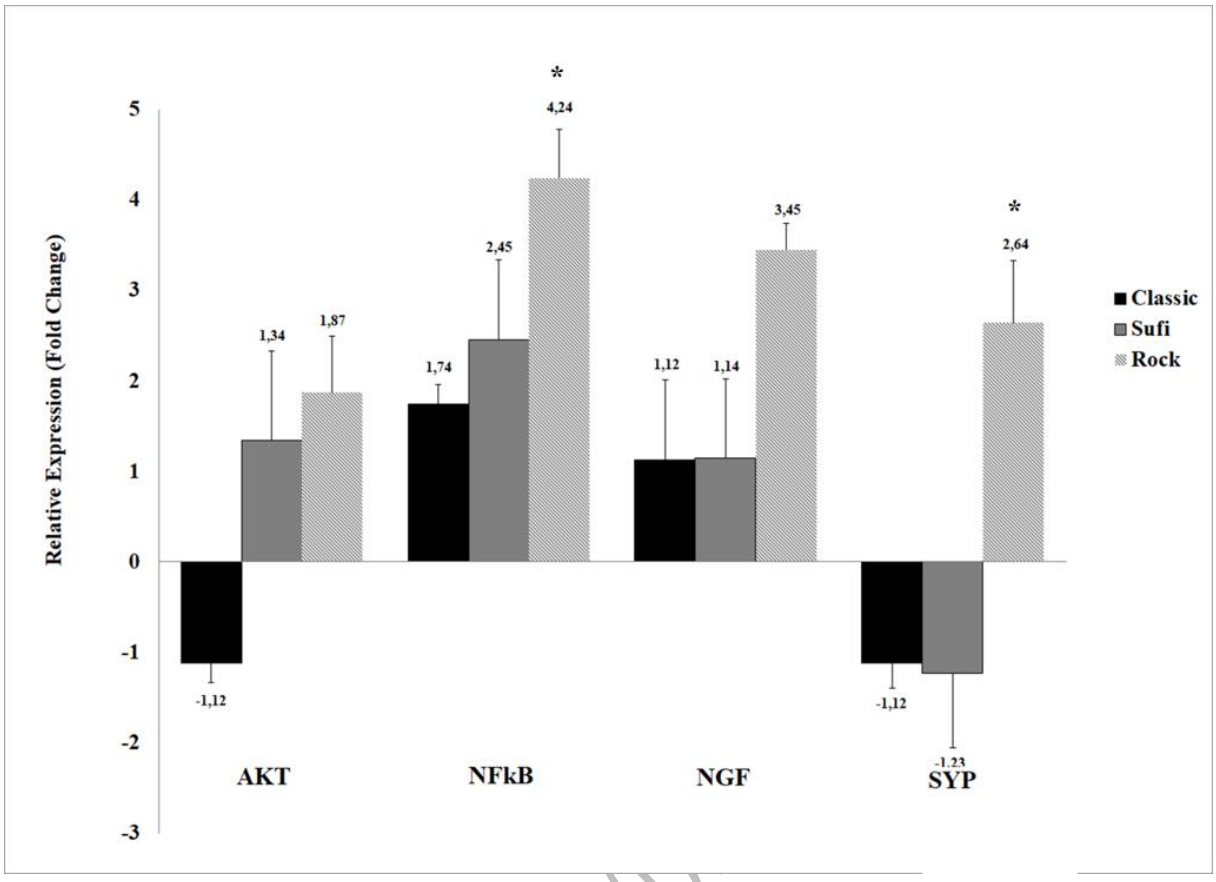


Figure 7: RT-PCR analysis for *AKT, NFκB, NGF*, and *Synaptophysin (SYP)* genes. After calibration of ΔCt data of each music pattern exposure groups according to the control group, relative fold change values were estimated. Error bars denote standard error of mean (SEM). * indicates significant difference at $p<0.05$ according to control group. The lines represent the difference at two groups.

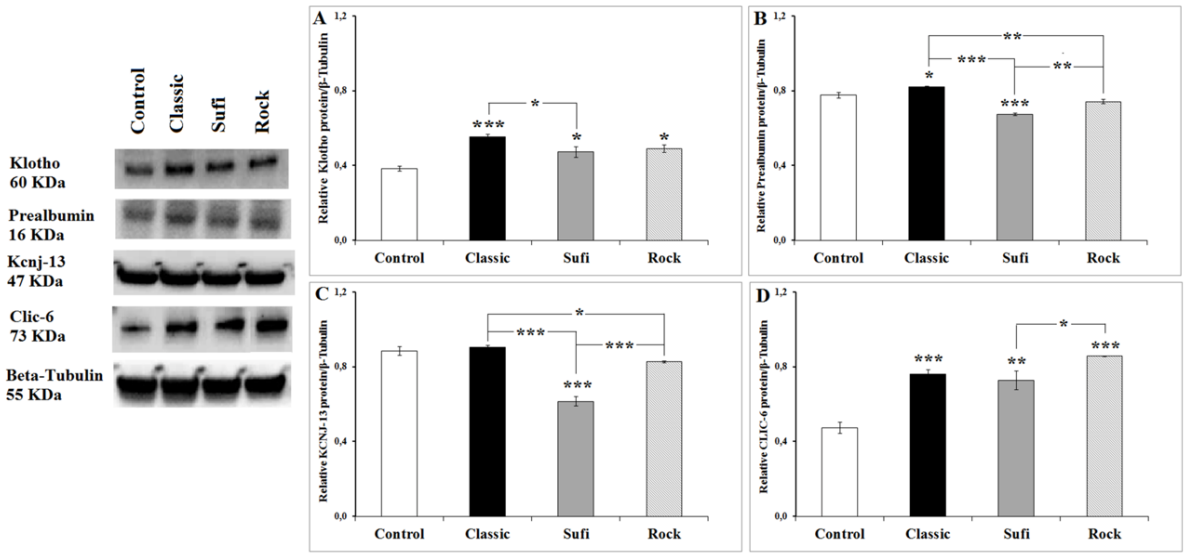


Figure 8: The relative amounts of **A)** Klotho, **B)** Prealbumin, **C)** KCNJ-13 and **D)** Clic-6 proteins to beta tubulin protein that were estimated by western blotting for control, classical, Sufi, and rock groups. Error bars denote standard error of mean (SEM). *for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ indicate significant difference. The lines represent the difference at two groups.

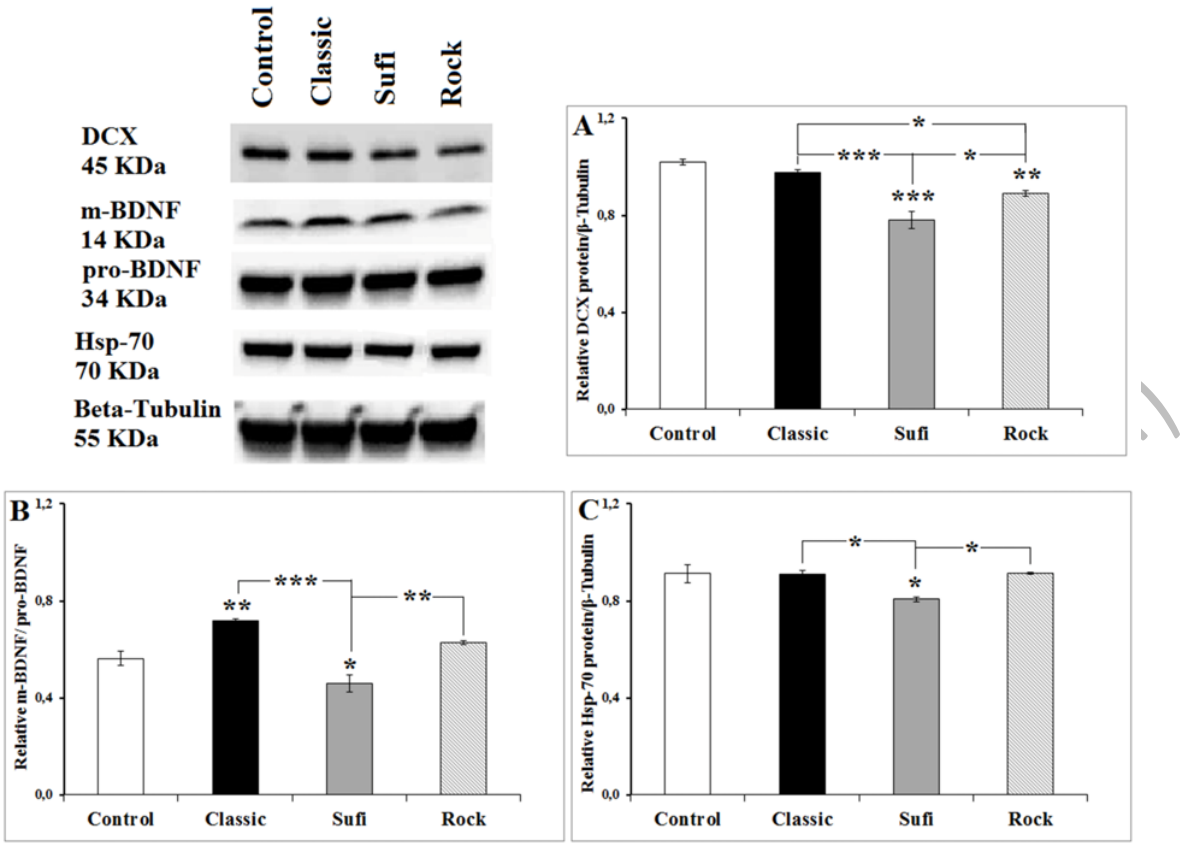


Figure 9: The relative amounts of **A)** DCX to beta tubulin, **B)** mature BDNF to pro BDNF, and **C)** Hsp-70 to beta tubulin protein that were estimated by western blotting for control, classical, Sufi, and rock groups. Error bars denote standard error of mean (SEM). *for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ indicate significant difference. The lines represent the difference at two groups.