

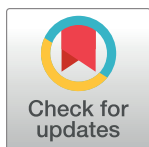
RESEARCH ARTICLE

Melatonin influence on miRNA expression in sperm, hypothalamus, pre-frontal cortex and cerebellum of Wistar rats

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Abstract

Melatonin is a pineal hormone synthesized exclusively at night, in several organisms. Its action on sperm is of particular interest, since they transfer genetic and epigenetic information to the offspring, including microRNAs, configuring a mechanism of paternal epigenetic inheritance. MicroRNAs are known to participate in a wide variety of mechanisms in basically all cells and tissues, including the brain and the sperm cells, which are known, respectively, to present 70% of all identified microRNAs and to transfer these molecules to the embryo. MicroRNAs from sperm have been associated with modulation of embryonic development and inheritance of psychiatric symptoms, including autism. Given that microRNAs and melatonin are ubiquitous molecules with important roles in the organism, the aim of this study was to investigate the expression of specific microRNAs in sperm, brain and cerebellum of pinealectomized rats. For this study, Wistar rats had their pineal gland removed at 60 *post-partum*. Part of these rats received exogenous melatonin until the day of the euthanasia. The control group did not receive any treatment or manipulation. The sperm, hypothalamus, prefrontal cortex and cerebellum were collected for analysis of microRNA expression by RT-qPCR. The results suggest that melatonin absence caused by pinealectomy increases the expression of the target microRNAs in the sperm. Although the data suggest an alteration (increase or decrease depending on the region and microRNA) of expression levels of some microRNAs in the brain and cerebellum of pinealectomized rats, the differences were not statistically significant. This seems to be a consequence of the intragroup variation. Melatonin administration restored the levels of the target microRNAs in the sperm. Additional studies are needed to understand the impact of the alterations of microRNA expression to the pinealectomized rats as well as to their descendants.

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Introduction

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone produced in several organisms and synthesized exclusively at night. In mammals, melatonin is primarily produced by the pinealocytes, in the pineal gland, through the uptake of the amino acid tryptophan in the absence of light [1]. The pineal gland initiates melatonin synthesis under the command of a vast neural network that receives photoperiodic information from the environment. Due to the amphiphilic nature of melatonin, it is not stored and is destined directly for central and peripheral tissues. Melatonin concentration in plasma indicates the night period and allows physiological adaptations during the seasons [2]. Due to this function, it is referred as a chronobiotic substance [3].

Although melatonin is commonly referred to as the “sleep hormone”, it acts in a wide variety of physiological phenomena, working as an antioxidant and anti-inflammatory substance [4]. The neuroprotective role of melatonin, for example, has been reported in different psychiatric conditions, such as autism, ADHD, depression and anxiety [5–8], as well as in neuroplasticity [9]. The role of melatonin as an antidepressant has also been suggested, making it a possible adjuvant treatment to depression symptoms [10]. The molecular mechanisms involved in these melatonin actions in the brain include free radical scavenging as well as inhibition of pro-apoptotic factors and of pro-inflammatory substances such as interleukins. Melatonin action on neural stem cell proliferation and differentiation has also been documented [11, 12].

Some cellular actions of melatonin depend on specific cellular receptors. In humans, the MT1 (MTNR1A) and MT2 (MTNR1B) receptors are well known. Studies show that nuclear receptors for melatonin allow its action in the regulation of gene expression and genetic modulation [13]. Melatonin also influences epigenetic mechanisms in different cells and tissues by acting on microRNA (miRNA) expression, histone acetylation/phosphorylation and DNA methylation (Reviewed by [14]). Melatonin has been shown to act on the reduction of DNA-methyl transferases (DNMTs) expression, leading to a decrease of DNA methylation [15].

A recent study indicates that the pattern of melatonin is associated with the differential methylation of clock genes related to the circadian rhythm [16]. The interaction of melatonin with small non-coding RNAs (sncRNA) has also been reported in the regulation of its receptors [17], in its synthesis [18, 19], in spermatogenesis *in vitro* [20] and in various pathological conditions [21]. However, most data refer to exogenous melatonin. Little information is available about the action of endogenous melatonin on different cells and tissues that are responsive to exogenous melatonin.

MicroRNAs (miRNAs) are a type of sncRNA that control gene expression through pre and post-transcriptional mechanisms [22]. It has been shown that the brain expresses 70% of all identified miRNAs, illustrating their importance for this organ. Indeed, miRNAs control synapsis and neuron morphology, neurogenesis and neuron migration and metabolism. In addition, alterations in miRNA levels were detected in psychiatric and degenerative disorders. In the sperm cells, miRNA exact role has not been elucidated yet, although their importance to spermatogenesis and relationship with male infertility have been documented [23, 24]. It has also been shown that miRNA biogenesis occurs in the sperm and that these miRNAs are important to initial embryo development [25].

The influence of the administration of exogenous melatonin on the profile of miRNAs during spermatogenesis *in vitro* has been reported [20]. The action and influence of melatonin on spermatogenesis is of particular interest because the sperm transfer not only genetic but also epigenetic information to the embryo. Studies show that miRNAs are transferred from sperm to the embryo and can configure a mechanism of paternal epigenetic inheritance [26, 27]. In

addition, some miRNAs whose expression is common to the brain and sperm, such as miR-34a and miR-132, have been related to the intergenerational transmission of neural and stress characteristics [28, 29]. Consequently, it is possible to hypothesize that sperm may also mediate the epigenetic inheritance of psychiatric disorders.

Considering that lifestyle has changed significantly in the last decades, leading to increased exposure to light, and that melatonin and miRNA act widely in the organism, we hypothesize that alterations in melatonin levels lead to alteration of miRNA levels in the brain and in the sperm. Thus, in view of the importance of miRNAs and melatonin for the central nervous system and the sperm, as well as the lack of information about the functions of endogenous melatonin, the objective of the present study was to evaluate the influence of melatonin absence on miRNA profile of sperm, hypothalamus, prefrontal cortex and cerebellum from pinealectomized rats.

Materials and methods

Animals and pinealectomy

For this study, 18 adults male Wistar rats (*Rattus norvegicus albinus*) were randomly distributed into three groups (n = 6): control, PINX and PINX-MEL. The PINX group consisted of pinealectomized rats without melatonin replacement. The PINX-MEL group was composed of pinealectomized rats that received melatonin replacement therapy. The animals in the control group were not pinealectomized and did not receive melatonin replacement. Surgery to remove the pineal gland was performed at 60 days of postnatal life (60dpn), the age at which the spermatogenesis is complete, and sperm have reached epididymis cauda. The sham group was not included in this study because it has been previously shown that the sham rats work like a control group [30–32]. All procedures were approved by the research ethics committee of the Federal University of São Paulo (CEUA N° 8074220415) and followed the recommendations of the International Standards for the Care and Use of Laboratory Animals.

The surgical technique of pineal removal described below is intended for experimentation with rats. The equipment and procedures carried out are part of the protocol intended for this species. The pinealectomy was performed at ZT 2 (abbreviation for Zeitgeber Time—time signal of the circadian system), that is, 2 hours after the lights were turned on in the animal facility. ZT 0 represents the beginning of the light phase, when the lights are turned on, while ZT 12 represents the beginning of the dark phase, when the lights go off. At the time of pineal removal, the rats were sedated and anesthetized using 10 mg/kg of xylazine and 40 mg/kg of ketamine. The animal was positioned in a stereotaxic apparatus in prone position and excess hair from the upper region of the head was removed. Using a scalpel blade, a longitudinal incision of approximately 2–3 cm was made in the skull and a circular trephine was used to expose the pineal gland, which was removed using forceps in a single movement. The blood was cleaned, and the removed cranial fragment was repositioned in the skull. Finally, the animals were placed back in the cages and remained under observation until their reflexes were recovered.

Melatonin replacement therapy

PINX-MEL rats received melatonin (0.5mg/kg) that was added to water bottles during the dark phase of the light-dark cycle for 70 days from the same day of surgery to the euthanasia. This interval between pineal removal and euthanasia comprised at least one complete period of spermatogenesis, which lasts approximately 53 to 54 days in rats [16]. The dose of melatonin used in this study is higher than physiological concentrations (0.1mg/kg) to ensure that a basal concentration was consumed by each rat. Melatonin solution (Sigma-Aldrich, Cat. M5250)

was prepared daily and administered at the beginning of the dark period (ZT 13), that is, 1 hour after the lights went off. Full access to the water bottle was allowed during the dark phase period. The rats were weighed weekly and the melatonin concentration for the PINX-MEL group was adjusted according to the average weight of the animals in each box and according to the daily water intake. The bottles with melatonin were removed one hour before the beginning of the light phase, at ZT 23 and replaced with bottles containing only water. As the half-life of melatonin is 20 minutes, the removal of the bottles with melatonin occurred at ZT 23 to ensure that the rats overcome the dark-light transition with ideal concentrations of melatonin. This process occurred every day until the day of euthanasia. Throughout the experiment, the animals were kept under controlled conditions of temperature (21 to 23°C), humidity and light (12h light/12h dark cycles).

Euthanasia and sample collection

Euthanasia was carried out by decapitation at 130dpn and occurred at ZT 1. For sperm collection, the epididymis was isolated through abdominal incision. A small incision was made in the epididymis cauda to allow the contents to leak. The cauda was placed in a 1.5mL tube containing pre-heated Phosphate-Buffered Saline solution (PBS, pH 7.4) and incubated at 37°C for 10 minutes for sperm dispersion. The fragments of the epididymis cauda were removed and the solution containing the sperm was centrifuged. The pellet was resuspended in Somatic Cell Lysis Buffer Buffer (SCLB: 0.1% SDS and 0.5% Triton in ddH₂O) on ice for 20 minutes, centrifuged for 15 minutes at 4°C and the supernatant was removed. This process was repeated, and the pellet was washed in 3mL of PBS to remove SCLB residues.

To collect the hypothalamus, prefrontal cortex and cerebellum, the encephalon was removed and placed in a Petri dish on ice; the tissues were directly allocated into 1.5mL tubes containing RNAlater Stabilization Solution® (ThermoFisher®) according to manufacturer specifications and stored at -70°C until the moment of RNA extraction.

miRNA extraction and qPCR

The miRNA from sperm and brain regions was extracted using the mirVana® miRNA Isolation kit (Thermo Fischer Scientific, Cat. AM1560), according to the manufacturer instructions, except for the addition of a heating step (65°C for 10 minutes with low agitation) during incubation in Lysis Binding Buffer in the case of sperm samples. The synthesis of miRNA cDNA was performed using the TaqMan Advanced miRNA cDNA Synthesis® kit (ThermoFisher Scientific, Cat. A28007), according to the manufacturer instructions.

MiRNA expression was analyzed by RT-qPCR using the Roche LightCycler® 96 System platform. The target miRNAs analyzed were: miR-18a-5p, miR-34a-5p, miR-132-5p, miR-195-5p, miR-219a-5p, miR-182-5p and Let-7g-5p (Table 1). These target miRNAs were chosen

Table 1. Target miRNA information.

Gene	Assay Name	Assay ID	miRNA Sequence
Let-7a	rno-let-7a-1-3p	rno481486_mir	CUAUACAAUCUACUGUCUUUCC
Let-7g-5p	rno-let-7g-5p	478580_mir	UGAGGUAGUAGUUUGUACAGUU
miR-18a-5p	rno-miR-18a-5p	rno480968_mir	UAAGGUGCAUCUAGUGCAGAUAG
miR-34a-5p	rno-miR-34a-5p	rno481304_mir	UGGCAGUGUCUUAGCUGGUUGU
miR-132-5p	rno-miR-132-5p	rno481320_mir	ACCGUGGCUUUCGAUUGUUACU
miR-182-5p	rno-miR-182-5p	477935_mir	UUUGGCAAUGGUGAAGACUCACACU
miR-195-5p	rno-miR-195-5p	rno480882_mir	UAGCAGCACAGAAUAUUGGC
miR-219a-5p	rno-miR-219a-5p	rno481348_mir	UGAUUGUCCAAACGCAAUUCU

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based on data from the literature, which show that these miRNAs might be related to neuro-physiology and/or sperm physiology. MiRNA Let-7a and Let-7g were used as reference for the data normalization from the hypothalamus, prefrontal cortex and cerebellum. MiRNA expression was analyzed using TaqMan assays (ThermoFisher Scientific, [Table 1](#)). PCR conditions were used as follows: preincubation at 55°C for 120s (1 cycle), enzyme activation at 95°C for 20s (1 cycle), denaturation at 95°C for 3s, annealing/extension at 60°C for 30s (40 cycles). To perform the ΔCq calculations, the average of the Cq values of Let-7a and Let-7g was obtained. For data from sperm, the average of the Cq of all target miRNA combined was used. The expression level was obtained using the $2^{-\Delta Cq}$ method [33, 34]. All reactions were performed as duplicates and each reaction was repeated once. The data distribution was analyzed using the Shapiro-Wilk test. For those that showed normal distribution, the one-way ANOVA test was applied, followed by Tukey test when the differences were significant. For data that did not present a normal distribution, the Kruskal-Wallis multiple comparison test was applied, followed by Dunn test when significant differences occurred. The analyses were performed using the RStudio (version 4.3.1) program and differences were considered statistically significant when $p \leq 0.05$.

Biological pathways of the target miRNA

To obtain information about the possible biological meaning of the data obtained, the biological pathways of the miRNA were analyzed. First, the target genes of the miRNA were identified using the TargetScan (https://www.targetscan.org/vert_80/) and miRDB (<https://mirdb.org/>) databases. Then, the gene list resulting from TargetScan and miRDB was uploaded to Metascape database (<https://metascape.org/>) and heat maps indicating the enriched pathways for the target genes of the miRNAs of interest were generated.

Results

miRNA expression in sperm

A previous study carried out by our group (data not published) indicated that the Let-7g-5p miRNA is a good endogenous control for analyzing miRNA expression in brain and sperm samples. Furthermore, other studies [35–38] and data from the SpermBase platform (<http://spermbase.org/>) also indicate that this miRNA is a good reference option. However, in the present study it was observed that the absence of the pineal led to changes in the Cq of Let-7g in the sperm in relation to the control group, impeding this miRNA from being used as a reference for calculating ΔCq . Likewise, the Cq of all other target miRNAs in this study were also altered in the sperm, making their use as a reference miRNA unfeasible. Two other miRNAs, Let-7a and Let-7c, were also investigated as possible reference miRNAs for sperm analysis, but no consistent amplification was detected in the samples in this study. Thus, for relative expression analyses, the global average of Cq values obtained from all target miRNAs, including Let-7g, was used as the reference value to obtain ΔCq for each target miRNA [39].

The expression level of all miRNAs (miR-18a, miR-34a-5p, miR-132-5p, miR-195-5p, miR-219a-5p, miR-182-5p and Let-7g-5p) was altered in the sperm of the PINX group ([Fig 1](#)). The miRNA that showed the most pronounced change was miR-182, followed by Let-7g and miR-195, while miR-132 showed the lowest degree of change. The administration of exogenous melatonin led to a reduction of the expression of all miRNAs analyzed in the sperm, leading the values to become close to the expression in the control group ([Fig 1](#)).

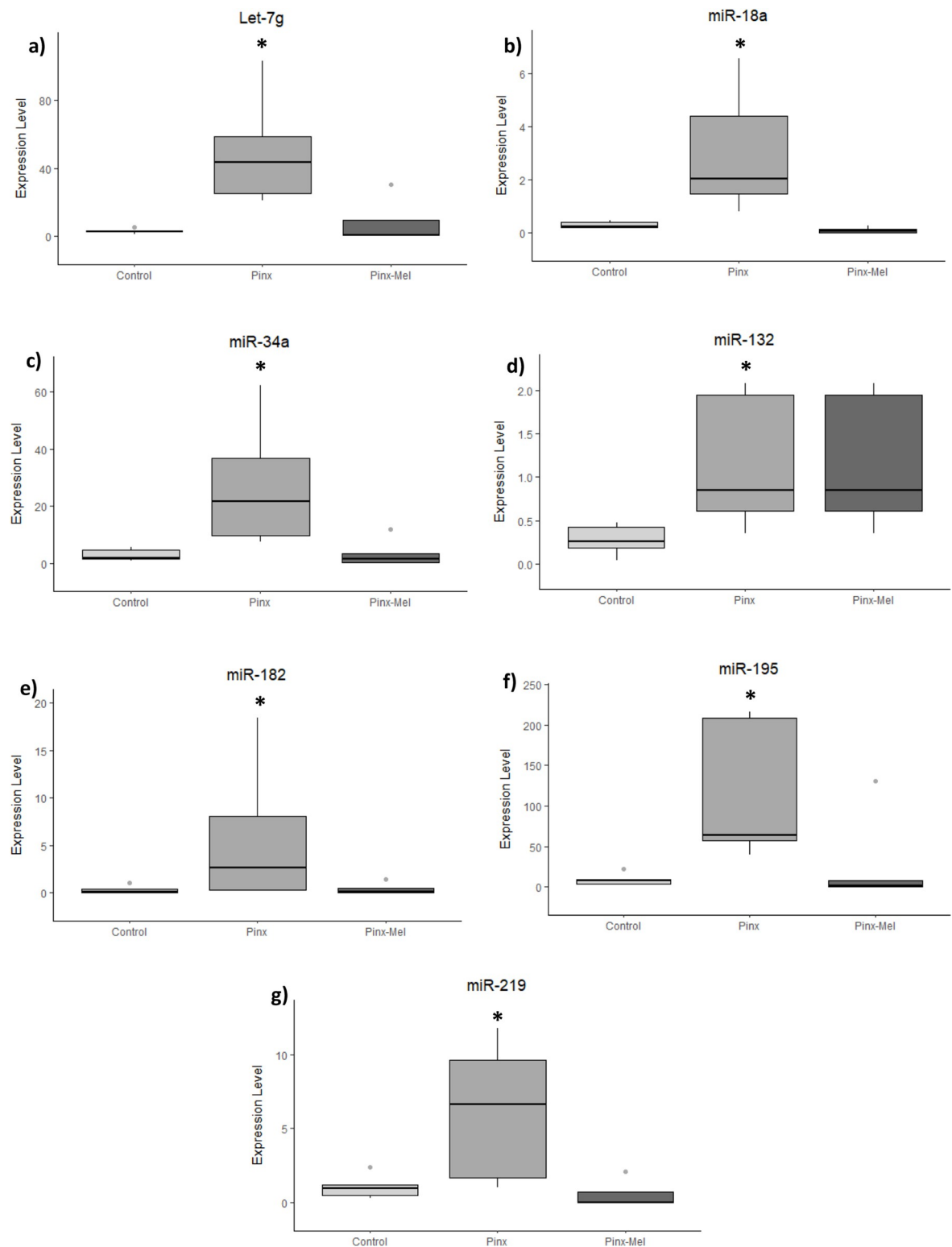


Fig 1. Expression level (2^{-ΔCq}) of microRNA in the sperm of control, pinealectomized (PINX) and melatonin-treated (PINX-MEL) rats. In the sperm, all miRNA analyzed showed increased expression in the PINX group when compared with the control group. Melatonin administration led to a reduction in the expression of these miRNAs towards the values observed in control rats.

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miRNA expression in hypothalamus, prefrontal cortex and cerebellum

The removal of the pineal gland caused no significant alteration in miRNA expression in the hypothalamus (Fig 2), prefrontal cortex (Fig 3) and cerebellum (Fig 4), although a decreasing tendency was observed for miR-34a, miR-132, miR-195 and miR-219 in the hypothalamus (Fig 2), whereas an increasing tendency was observed as well as for miR-34a and miR-132 in the prefrontal cortex of the PINX group (Fig 3). The administration of exogenous melatonin led to a pronounced and statistically significant increase of the expression of miR-132 in the hypothalamus of PINX-MEL group when compared to the PINX group (Fig 2). In the prefrontal cortex the expression of miR-132 showed a tendency of decrease after melatonin administration to pinealectomized rats (Fig 3). In the cerebellum no alteration of miRNA expression was detected after melatonin administration (Fig 4). MiR-18a and miR-182 were not detected in the prefrontal cortex and in the cerebellum.

Enriched biological pathways

To obtain information about the biological meaning of changes in miRNA expression, bioinformatics analyzes were carried out using consolidated databases such as miRDB, TargetScan and Metascape. This investigation generated heat maps of enriched pathways for genes targeted by the miRNA studied here. The 20 most enriched pathways (top 20 clusters) generated by Metascape were considered (Fig 5A–5G). The total number of clusters generated for the genes targeted by each miRNA is presented as supplementary material (S1–S7 Figs).

Among the top 20 clusters, several biological pathways such as rhythmic processes, phenomena related to the central nervous system and its development, as well as behavior and reproductive processes (Fig 5A–5G) are indicated as a result of the Metascape analysis. In this analysis, miR-132 stands out, showing greater relevance for related processes (Fig 5C). Table 2 presents the miRNAs and the pathways in which they are potentially involved.

Discussion

MiRNA are subject of numerous studies that aim to elucidate epigenetic mechanisms involved in biological phenomena, including those related to the nervous system and gametes [40, 41] given the importance of these molecules for neurodevelopment and gametogenesis. Like miRNAs, melatonin has been identified as a molecule that performs important functions in the brain, providing protection against oxidative stress [42], neuronal apoptosis and neuroinflammation [43]. In this study, miRNAs that target genes related to neurogenesis, neurodevelopment, synaptogenesis, behavior modulation, circadian rhythm and other processes involved with the nervous system were analyzed [44, 45]. Data obtained from RT-qPCR suggest that melatonin absence caused by pineal removal led to changes in the expression of all analyzed miRNAs in sperm and of specific miRNAs in the hypothalamus, prefrontal cortex and cerebellum. Previous studies have shown that pinealectomized rats that did not receive melatonin replacement present desynchronization of the biological clock rhythm in Leydig cells due to rhythmic disorganization [46, 47], which agrees with the alteration in the expression of the miRNAs miR-34a, miR-132 and miR-195 in the brain, which are involved in the regulation of the circadian rhythm [48, 49].

The role of miRNAs in melatonin synthesis [19] as well as melatonin influence on miRNA expression has been previously reported [50–52]; however, the direct relationship of this hormone or its deficiency with the levels of miRNA in different tissues has been little explored so far, and the data that are available are still indirect and/or controversial [53]. Thus, the action of melatonin on miRNA expression has been inferred based on data about the role of these miRNAs in each tissue and cell, that is, from the understanding of the independent function of

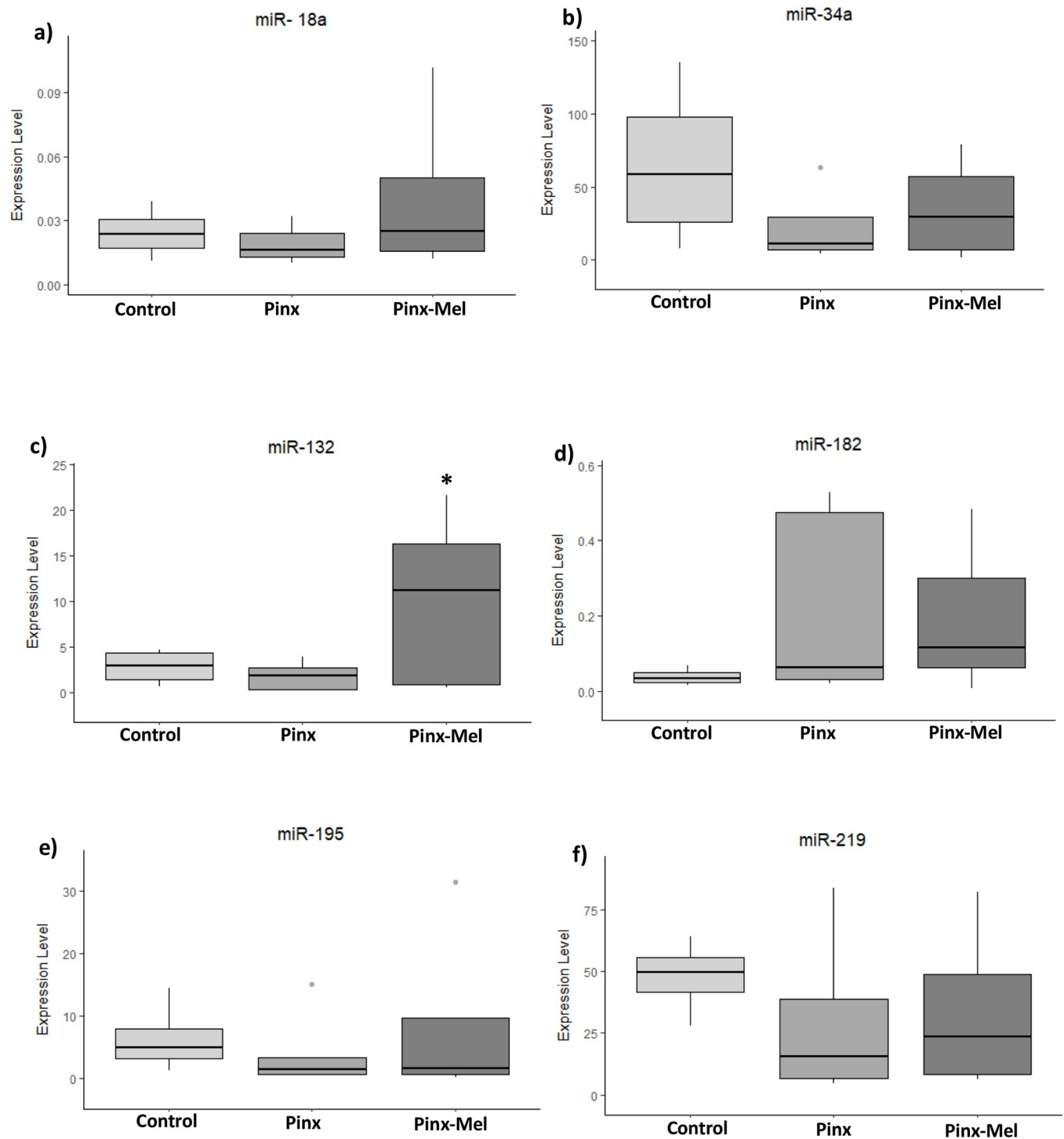


Fig 2. Expression level (2^{-ΔCq}) of microRNA in the hypothalamus of control, pinealectomized (PINX) and melatonin-treated (PINX-MEL) rats. Although the differences were not significant, a tendency of increase in the expression of miR-34a, miR-132, miR-195 and miR-219 can be noticed in the hypothalamus of the PINX group. A significant increase of miR-132 expression was observed in the PINX-MEL group (Fig 2C, asterisk).

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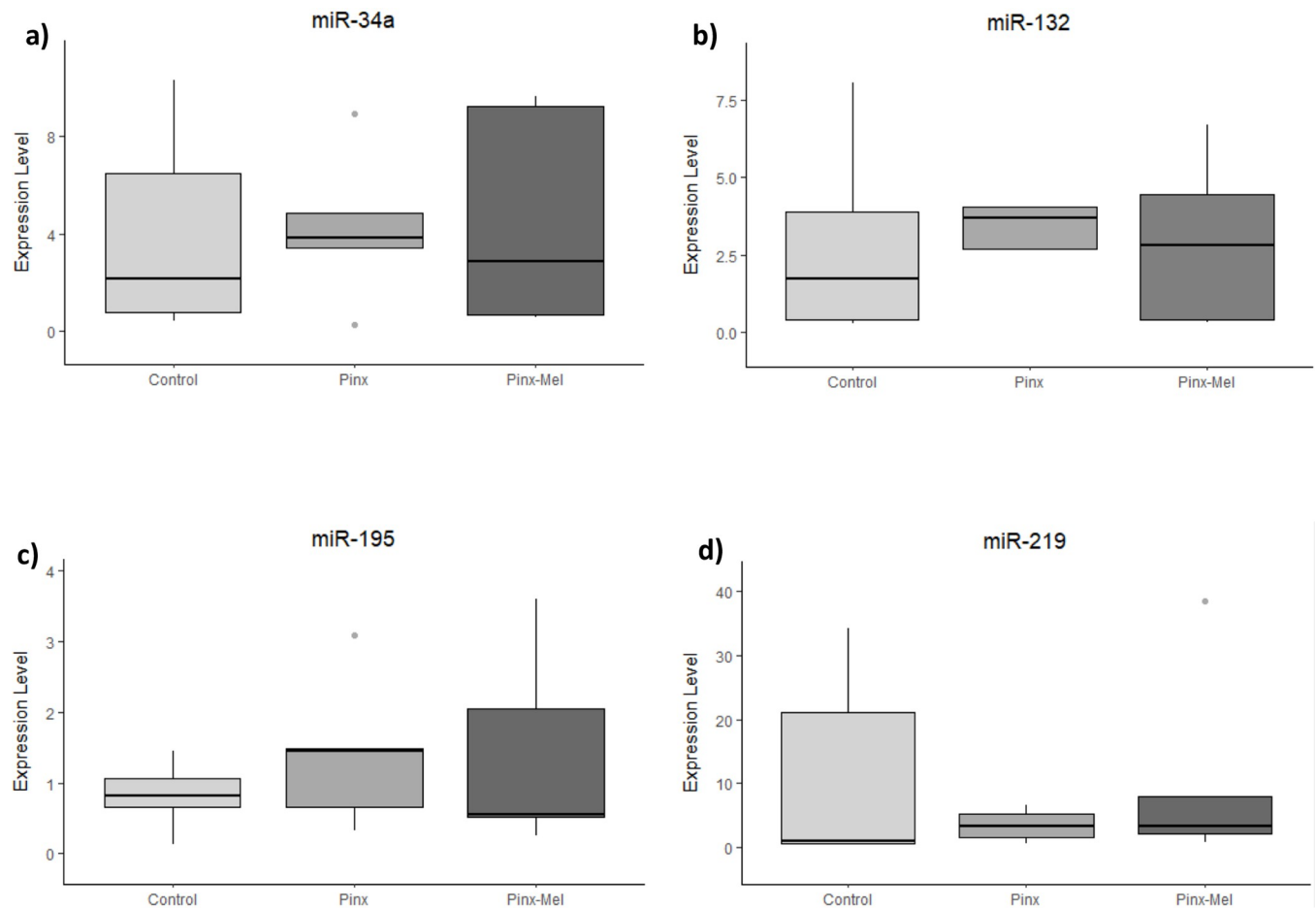


Fig 3. Expression level (2^{-ΔCq}) of microRNA in the prefrontal cortex of control, pinealectomized (PINX) and melatonin-treated (PINX-MEL) rats. As observed for the hypothalamus, no significant difference was observed among the groups, although a tendency of increase in the expression of miR-34a, miR-132, miR-195 and miR-219 can be noticed in the PINX group.

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miRNAs and melatonin in these tissues and cells. The role of exogenous melatonin administration has also been explored due to increasing data about its functions and to the increasing use of this hormone as a dietary supplement. Studies show that miRNAs participate in different mechanisms by which exogenous melatonin acts on cellular stress [54], cognitive [55] and general inflammatory processes [56], in Alzheimer's [52] and in memory [57]. Thus, the existence of a relationship between melatonin and miRNAs in neurophysiological processes seems clear. On the other hand, the meaning and the implications of the changes in miRNA expression observed here still need to be clarified.

RT-qPCR data showed that removal of the pineal gland led to a more marked alteration in the expression of miRNAs in sperm than in the brain and cerebellum. This data suggest that melatonin has remarkable influence on the physiology of these gametes. The effects of exogenous melatonin on general male reproductive parameters have been explored under different conditions and in different species. Its administration can improve spermatogenesis and fertility after exposure to toxic substances [58–64], but the molecular mechanisms by which melatonin acts on sperm are still unknown. *In vitro* studies suggest that melatonin acts on spermatogonia lineages by regulating the expression of miRNAs [20, 65]. However, no studies were found in the literature that investigated the influence of melatonin on sperm miRNA

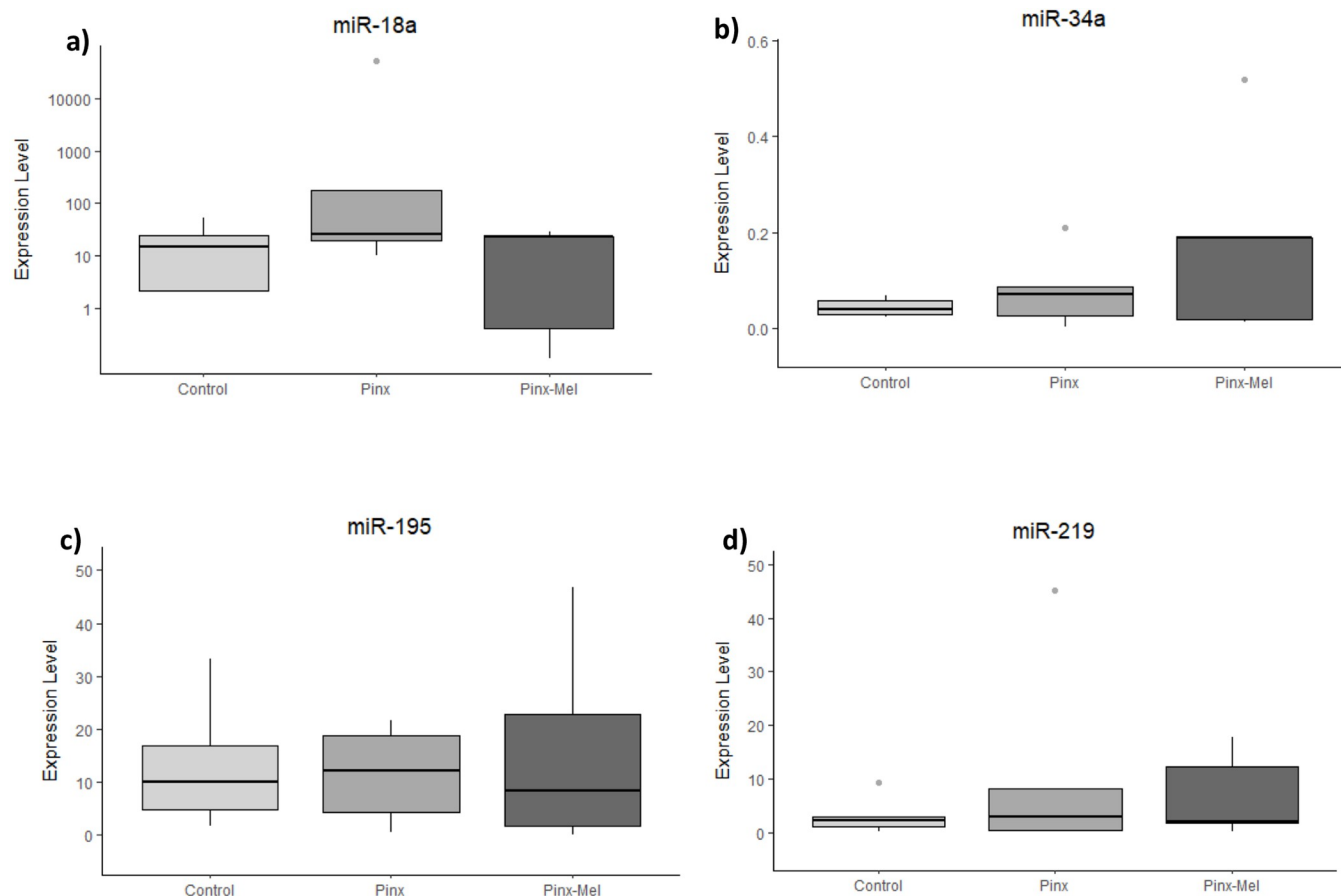


Fig 4. Expression level (2^{-ΔCq}) of microRNA in the cerebellum of control, pinealectomized (PINX) and melatonin-treated (PINX-MEL) rats. As observed for the hypothalamus and prefrontal cortex, no significant difference was observed among the groups.

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expression or during spermatogenesis *in vivo*; so, to the best of our knowledge, this is the first study to explore this relationship. In addition to the implications for their own physiology, changes in the expression of sperm miRNAs raise the question about their possible implications for embryonic development and about the possibility of transmitting these changes to descendants through epigenetic inheritance. Studies show that changes in the sperm miRNA profile can be transmitted to future generations [66–68]; so, it is reasonable to consider that the extensive change observed in the present study can potentially influence the next generation.

It is important to highlight that the administration of exogenous melatonin led to a relevant recovery in the expression of miRNAs in sperm, but not in all the nervous tissues analyzed. This data is consistent with those from the pinealectomized rats, reinforcing that sperm are very sensitive to melatonin, responding intensely to both the lack of it and its replacement. This is in line with the various studies that show that melatonin acts positively on different physiological aspects of sperm, leading, for example, to an improvement of their quality in *in vitro* fertilization protocols in different species, including humans [69–72].

Although the biological response to the changes in miRNA levels needs investigation, the high susceptibility of sperm to melatonin draws attention and suggests that this topic requires consideration, given the function of these cells to transmit genetic and epigenetic characteristics. To evaluate the possible biological meanings of the changes in the expression of miRNAs

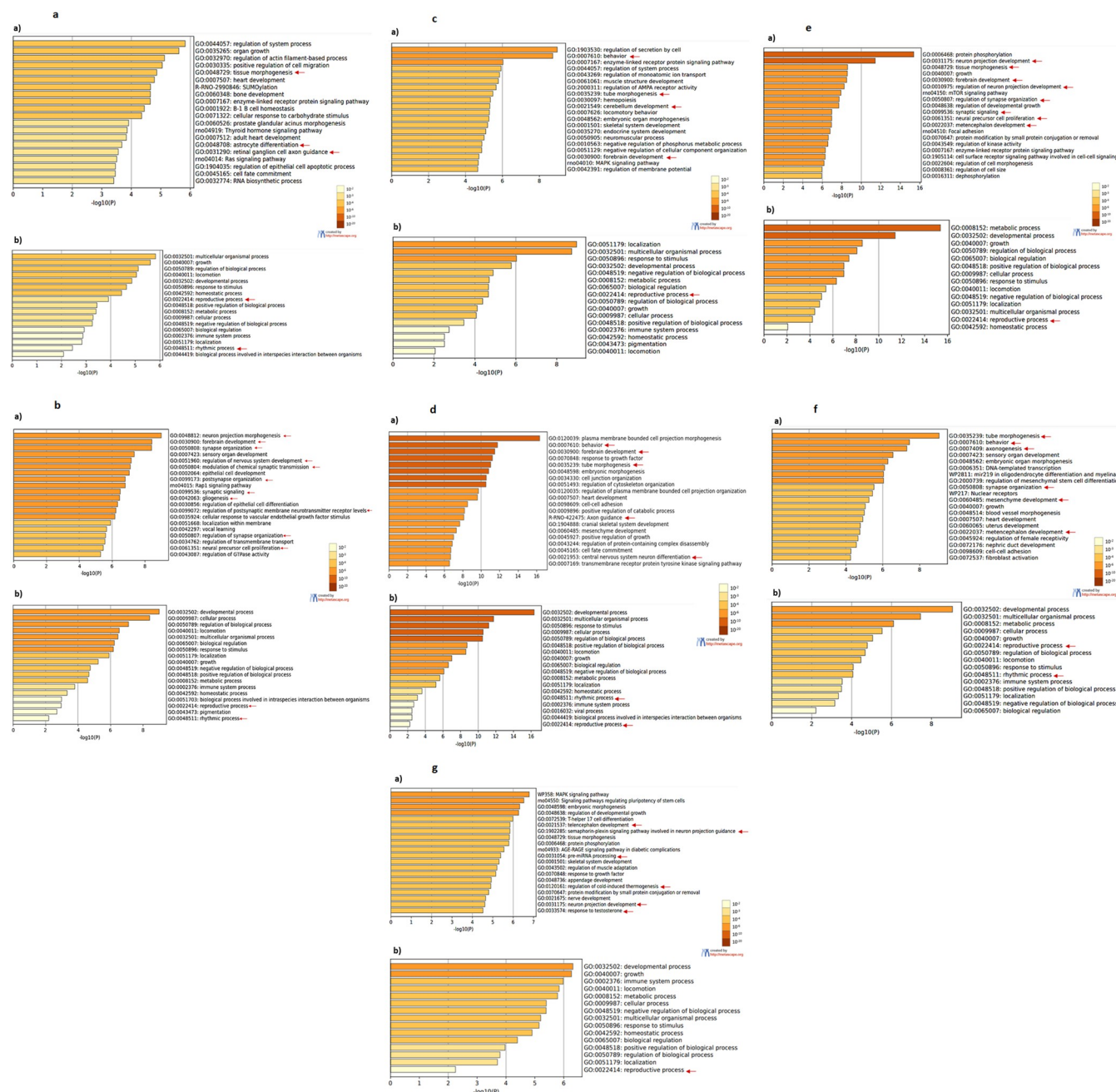


Fig 5. a: Heat map showing the enriched pathways (a) and ontology analysis (b) for miR-18a target genes. Although the enriched pathways do not show biological processes of interest (a), the gene ontology analysis (b) indicate the involvement of miR-18a in reproductive and rhythmic processes (arrows) among the 20 most enriched processes. b: Heat map showing the enriched pathways (a) and ontology analysis (b) for miR-34a target genes. The analysis returned processes related to neurodevelopment and neurophysiology (arrows) among the top 20 most enriched pathways (a). The gene ontology analysis (b) indicates the participation of genes targeted by miR-34a in rhythmic and reproductive processes (arrows). c: Heat map showing the enriched pathways (a) and ontology analysis (b) for miR-132 target genes. The top 20 enriched pathways (a) returned for miR-132 include neurodevelopment processes as well as behavior (arrows). The gene ontology analysis (b) show that the genes targeted by miR-132 are related to reproductive processes (arrow). d: Heat map showing the enriched pathways (a) and ontology analysis (b) for miR-182 target genes. It is indicated that the genes targeted by miR-182 participate on processes related to neurodevelopment and behavior (a), whereas the gene ontology indicate their involvement with rhythmic and reproductive processes (b). e: Heat map showing the enriched pathways (a) and ontology analysis (b) for miR-195 target genes. The analysis returned processes related to neurodevelopment (arrows) among the top 20 most enriched pathways (a). In the gene ontology analysis (b), the involvement of miR-195 targeted genes in reproductive processes (arrow). f: Heat map showing the enriched pathways (a) and ontology analysis (b) for miR-219a target genes. The top 20 enriched pathways (a) returned for miR-219a include neurodevelopment and neurophysiology processes (arrows), whereas the gene ontology (b) indicate the involvement of genes targeted by miR-219a with rhythmic and reproductive processes (arrows). g: Heat map showing the enriched pathways (a) and ontology analysis (b) for Let-7g target genes. It is indicated

that the genes targeted by Let-7g are involved in neurodevelopment (arrows), whereas the gene ontology indicate the participation of the Let-7g targeted genes in reproductive processes (arrow).

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observed in this study, bioinformatics tools based on consolidated databases were used. The genes targeted by the miRNAs studied here were involved in biological processes related to the physiology and/or development of the central nervous system, such as synaptic organization, differentiation of neurons and glial cells, development of the brain and cerebellum and behavior. These results were expected, since the miRNAs analyzed in this study were chosen based on their relationship with neurodevelopment and neurophysiology. In addition to the relationship with neurodevelopment and neurophysiology, bioinformatics revealed that the genes targeted by miR-34a, miR-18a, miR-182 and miR-219, such as *Per1*, *Clock* and *Cry2*, are also involved in rhythmic processes, suggesting that these miRNAs play a role in these processes. Although the bioinformatic analysis has suggested the involvement of the miRNAs studied here in reproductive processes, the relevance with which these processes appear is low. This may be due to the lack of data about the action of miRNAs on sperm in these databases. Therefore, it is important to assess the implications of the changes observed here for these gametes and for the embryos generated by them.

The variety of processes, tissues and cells highlighted by bioinformatics analysis reinforces the ubiquitous characteristic of melatonin and miRNAs in the body, which explains the broad alteration in the expression of miRNAs observed in this study, especially in sperm, but also in the brain and cerebellum. This ubiquitous characteristic makes data interpretation quite complex and challenging. In this context, it was surprising that melatonin absence caused more pronounced changes in the expression of miRNAs in sperm than in the brain and cerebellum, since the functions of melatonin in neurophysiology are much better documented than in sperm. On the other hand, the central nervous system is considered the richest in diversity of miRNAs in comparison to other systems of the organism [73], what, combined with the multiplicity of genes targeted by these miRNAs, might suggest that the functions of these miRNAs coincide with other miRNA, generating mechanisms of compensation which prevent an imbalance in the synthesis of these molecules.

Table 2. Pathways with potential involvement of the target miRNAs.

Target miRNA	Potentially involved pathways
miR-18a	Retinal ganglion cell axon guidance and astrocyte differentiation.
miR-34a	Neuronal projection morphogenesis, forebrain development, regulation of nervous system development, gliogenesis, neural precursor cell proliferation, synaptic organization and regulation, modulation of chemical synaptic transmission, postsynapse organization, synaptic signaling, and regulation of neurotransmitter receptor levels of the postsynaptic membrane.
miR-132	Tubular morphogenesis, development of the cerebellum and forebrain, and involvement with target genes associated with behavior.
miR-182	Tubular morphogenesis, forebrain development, axon guidance and differentiation of neurons in the central nervous system.
miR-195	Development of neuronal projection and forebrain, proliferation of neural precursor cells, development of the hindbrain, regulation of synaptic organization and synaptic signaling.
miR-219a	Tube morphogenesis, axonogenesis, oligodendrocyte differentiation and myelination, hindbrain development, synapse organization, and target genes associated with behavior.
Let-7g	Telencephalon development, semaphorin-plexin signaling pathway involved in neuronal projection guidance, nerve development, neuronal projection development, response to testosterone, regulation of cold-induced thermogenesis and pre-miRNA processing.

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Considering that epigenetic inheritance is a relatively new topic and that very little is known about this subject, further studies are of great importance, especially given the high and indiscriminate consumption of melatonin throughout the world. Added to this is the fact that melatonin is being used to improve some undesirable symptoms that are more common in psychiatric disorders, such as difficulty sleeping, for example [74]. Much progress has been made in knowledge about psychiatric disorders and their heredity has been the subject of many studies. Thus, the relationship between melatonin and the brain in different conditions and the epigenetic characteristics of sperm emerges as an important aspect to be considered in the approach to psychiatric disorders and their inheritance.

In conclusion, data of the present study suggest that melatonin deficiency influence the expression of miRNAs related to neurodevelopment and neurophysiology in sperm and, to a lesser extent, in the brain and cerebellum. Additional studies are needed to better explore these results and to clarify the biological meanings of these changes for pinealectomized animals and whether they can have an impact on their offspring.

Supporting information

S1 Fig. Expanded heat maps showing the enriched pathways. The pathways for the genes targeted by miR-18a are presented.
(TIF)

S2 Fig. Expanded heat maps showing the enriched pathways. The pathways for the genes targeted by miR-34a are presented.
(TIF)

S3 Fig. Expanded heat maps showing the enriched pathways. The pathways for the genes targeted by miR-132 are presented.
(TIF)

S4 Fig. Expanded heat maps showing the enriched pathways. The pathways for the genes targeted by miR-182 are presented.
(TIF)

S5 Fig. Expanded heat maps showing the enriched pathways. The pathways for the genes targeted by miR-195 are presented.
(TIF)

S6 Fig. Expanded heat maps showing the enriched pathways. The pathways for the genes targeted by miR-219a are presented.
(TIF)

S7 Fig. Expanded heat maps showing the enriched pathways. The pathways for the genes targeted by Let-7g are presented.
(TIF)

S1 File. Raw data. Details of qPCR results and statistical analysis.
(XLSX)

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References

1. Amaral FGD, Cipolla-Neto J. A brief review about melatonin, a pineal hormone. *Arch Endocrinol Metab.* 2018; 62(4):472–479. <https://doi.org/10.20945/2359-3997000000066> PMID: 30304113
2. Castro Neto. Melatonina, ritmos biológicos e sono—uma revisão da literatura. *Revista Brasileira de Neurologia.* 2008; 44(1): 5–11.
3. Amaral FGD, Andrade-Silva J, Kuwabara WMT, Cipolla-Neto J. New insights into the function of melatonin and its role in metabolic disturbances. *Expert Rev Endocrinol Metab.* 2019; 14(4):293–300. <https://doi.org/10.1080/17446651.2019.1631158> PMID: 31192707
4. Guo Y, Sun J, Li T, Zhang Q, Bu S, Wang Q, et al. Melatonin ameliorates restraint stress-induced oxidative stress and apoptosis in testicular cells via NF- κ B/iNOS and Nrf2/ HO-1 signaling pathway. *Sci Rep.* 2017; 7(1):9599. <https://doi.org/10.1038/s41598-017-09943-2> PMID: 28851995
5. Chini Mattia, and Hanganu-Opatz Ileana L. “Prefrontal Cortex Development in Health and Disease: Lessons from Rodents and Humans.” *Trends in neurosc.* 2021; 44(3): 227–240. <https://doi.org/10.1016/j.tins.2020.10.017> PMID: 33246578
6. Bernstein Hans-Gert et al. “The hypothalamus and neuropsychiatric disorders: psychiatry meets microscopy.” *Cell and tissue res.* 2019; 375(1): 243–258. <https://doi.org/10.1007/s00441-018-2849-3> PMID: 29767278
7. Ohtsuki G, Kim T, Sun P, Chen Y, D’Angelo EU. “Editorial: Cerebellum-related learning and psychiatric diseases.” *Frontiers in cellular neurosc.* 2023; 13:17:1132286. <https://doi.org/10.3389/fncel.2023.1132286> PMID: 36860375
8. Argyropoulos GPD, Lupo M, Olivito G. Editorial: The cerebellar role in psychiatric disorders: Emerging evidence and future perspectives. *Front Behav Neurosci.* 2022; 16:1075679. <https://doi.org/10.3389/fnbeh.2022.1075679> PMID: 36386782
9. Lee JG, Woo YS, Park SW, Seog DH, Seo MK, Bahk WM. The Neuroprotective Effects of Melatonin: Possible Role in the Pathophysiology of Neuropsychiatric Disease [published correction appears in *Brain Sci.* 2019;9(12):]. *Brain Sci.* 2019 oct. 2019; 9(10):285. <https://doi.org/10.3390/brainsci9100285> PMID: 31640239
10. Wang YQ, Jiang YJ, Zou MS, Liu J, Zhao HQ, Wang YH. Antidepressant actions of melatonin and melatonin receptor agonist: Focus on pathophysiology and treatment. *Behav Brain Res.* 2022; 420:113724. <https://doi.org/10.1016/j.bbr.2021.113724> PMID: 34929236
11. Chu Jiaqi et al. “Effects of melatonin and its analogues on neural stem cells.” *Mol and cel endocrinol.* 2016; 420:169–79. <https://doi.org/10.1016/j.mce.2015.10.012> PMID: 26499395
12. Mendivil-Perez Miguel et al. “Melatonin enhances neural stem cell differentiation and engraftment by increasing mitochondrial function.” *J pineal res.* 2017; 63(2): 10.1111/jpi.12415. <https://doi.org/10.1111/jpi.12415> PMID: 28423196
13. Ferreira CS, Maganhin CC, Simões RS, Girão MJBC, Baracat EC, Soares JM Jr. Melatonina: modulador de morte celular. *Rev. Assoc. Med. Bras.* 2010; 56(6). <https://doi.org/10.1590/S0104-42302010000600024>.
14. Monayo Seth Mikaye, and Liu Xin. “The Prospective Application of Melatonin in Treating Epigenetic Dysfunctional Diseases.” *Frontiers pharmacol.* 2022; 13:867500. <https://doi.org/10.3389/fphar.2022.867500> PMID: 35668933
15. Korkmaz Ahmet, and Russel J Reiter. “Epigenetic regulation: a new research area for melatonin?.” *J pineal res.* 2008; 44(1): 41–4. <https://doi.org/10.1111/j.1600-079X.2007.00509.x> PMID: 18078446
16. Ritonja JA, Aronson KJ, Leung M, Flaten L, Topouza DG, Duan QL, et al. Investigating the relationship between melatonin patterns and methylation in circadian genes among day shift and night shift workers. *Occup Environ Med.* 2022; 79:673–680. <https://doi.org/10.1136/oemed-2021-108111> PMID: 35501127

17. Esposito T, Magliocca S, Formicola D, Gianfrancesco F. piR_015520 belongs to Piwi-associated RNAs regulates expression of the human melatonin receptor 1A gene. *PLoS One*. 2011; 6(7):e22727. <https://doi.org/10.1371/journal.pone.0022727> PMID: 21818375
18. Su SC, Reiter RJ, Hsiao HY, Chung WH, Yang SF. Functional Interaction between Melatonin Signaling and Noncoding RNAs. *Trends Endocrinol Metab*. 2018; 29(6):435–445. <https://doi.org/10.1016/j.tem.2018.03.008> PMID: 29631868
19. Clokie SJ, Lau P, Kim HH, Coon SL, Klein DC. MicroRNAs in the pineal gland: miR-483 regulates melatonin synthesis by targeting arylalkylamine N-acetyltransferase. *J Biol Chem*. 2012; 287(30):25312–25324. <https://doi.org/10.1074/jbc.M112.356733> PMID: 22908386
20. Li C, Chen S, Li H, Chen L, Zhao Y, Jiang Y, et al. MicroRNA-16 Modulates Melatonin-Induced Cell Growth in the Mouse-Derived Spermatogonia Cell Line GC-1 spg Cells by Targeting Ccnd1. *Biol Reprod*. 2016; 95(3):57. <https://doi.org/10.1095/biolreprod.115.138313> PMID: 27465135
21. Mehrzadi S, Pourhanifeh MH, Mirzaei A, Moradian F, Hosseinzadeh A. An updated review of mechanistic potentials of melatonin against cancer: pivotal roles in angiogenesis, apoptosis, autophagy, endoplasmic reticulum stress and oxidative stress. *Cancer Cell Int*. 2021; 21(1):188. <https://doi.org/10.1186/s12935-021-01892-1> PMID: 33789681
22. Catalanotto C, Cogoni C, Zardo G. MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *Int J Mol Sci*. 2016; 17(10):1712. <https://doi.org/10.3390/ijms17101712> PMID: 27754357
23. Barbu MG, Thompson DC, Suciu N, Voinea SC, Cretoiu D, Predescu DV. The Roles of MicroRNAs in Male Infertility. *Int J Mol Sci*. 2021; 22(6):2910. <https://doi.org/10.3390/ijms22062910> PMID: 33805594
24. Sinaei R., Jamebozorgi K., Mirshekarpour H. Poormasoumi H, Mahdizadeh A, Akbari Z, et al. The role of miRNAs in the diagnosis and treatment of male infertility: a review study. *Egypt J Med Hum Genet* 2023; 40 <https://doi.org/10.1186/s43042-023-00419-9>
25. Liu Wei-Min et al. "Sperm-borne microRNA-34c is required for the first cleavage division in mouse." *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109(2): 490–4. <https://doi.org/10.1073/pnas.1110368109> PMID: 22203953
26. Zhang Y, Shi J, Rassoulzadegan M, Tuorto F, Chen Q. Sperm RNA code programmes the metabolic health of offspring. *Nat Rev Endocrinol*. 2019; 15(8):489–498. <https://doi.org/10.1038/s41574-019-0226-2> PMID: 31235802
27. Xavier MJ, Roman SD, Aitken RJ, Nixon B. Transgenerational inheritance: how impacts to the epigenetic and genetic information of parents affect offspring health. *Hum Reprod Update*. 2019; 25(5):518–540. <https://doi.org/10.1093/humupd/dmz017> PMID: 31374565
28. Benito E, Kerimoglu C, Ramachandran B, Pena-Centeno T, Jain G, Stilling RM, et al. RNA-Dependent Intergenerational Inheritance of Enhanced Synaptic Plasticity after Environmental Enrichment. *Cell Rep*. 2018; 23(2):546–554. <https://doi.org/10.1016/j.celrep.2018.03.059> PMID: 29642011
29. Dickson DA, Paulus JK, Mensah V, Lem J, Saavedra-Rodriguez L, Gentry A, et al. "Reduced levels of miRNAs 449 and 34 in sperm of mice and men exposed to early life stress." *Translational psychiatry*. 2018; 8(1):101. <https://doi.org/10.1038/s41398-018-0146-2> PMID: 29795112
30. Ferreira SG, Peliciari-Garcia RA, Takahashi-Hyodo SA, Rodrigues AC, Amaral FG, Berra CM, et al. Effects of melatonin on DNA damage induced by cyclophosphamide in rats. *Braz J Med Biol Res*. 2013; 46(3):278–86. <https://doi.org/10.1590/1414-431x20122230> PMID: 23471360
31. Coelho, et al. Pineal melatonin deprivation alters the mRNA expression of melatonin and steroidogenic-related receptor genes in rat oviduct and uterus during the estrus stage of estrous cycle. *Melatonin Res*. 2022; 5(1):68–83. <https://doi.org/10.32794/mr112500121>
32. Kim Mikyung et al. "Pinelectomy increases thermogenesis and decreases lipogenesis." *Molecular Med. Rep*. 2020; 22(5): 4289–4297. <https://doi.org/10.3892/mmr.2020.11534> PMID: 33000192
33. Livak K.J, and Schmittgen T D. "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." *Methods*. 2001; 25(4): 402–8. <https://doi.org/10.1006/meth.2001.1262> PMID: 11846609
34. Schmittgen, Thomas D, and Livak Kenneth J. "Analyzing real-time PCR data by the comparative C(T) method." *Nature protocols*. 2008; 3(6): 1101–8. <https://doi.org/10.1038/nprot.2008.73> PMID: 18546601
35. Kim AH, Reimers M, Maher B, Williamson V, McMichael O, McClay JL, et al. MicroRNA expression profiling in the prefrontal cortex of individuals affected with schizophrenia and bipolar disorders. *Schizophr Res*. 2010 Dec. 2010; 124(1–3):183–191. <https://doi.org/10.1016/j.schres.2010.07.002> PMID: 20675101
36. Smalheiser NR, Lugli G, Rizavi HS, Zhang H, Torvik VI, Pandey GN, et al. MicroRNA expression in rat brain exposed to repeated inescapable shock: differential alterations in learned helplessness vs. non-

- learned helplessness. *Int J Neuropsychopharmacol*. 2011; 14(10):1315–1325. <https://doi.org/10.1017/S1461145710001628> PMID: 21275079
37. Geaghan M, Cairns MJ. MicroRNA and Posttranscriptional Dysregulation in Psychiatry. *Biol Psychiatry*. 2015; 78(4):231–239. <https://doi.org/10.1016/j.biopsych.2014.12.009> PMID: 25636176
 38. Lai CY, Lee SY, Scarr E, Yu YH, Lin YT, Liu CM, et al. Aberrant expression of microRNAs as biomarker for schizophrenia: from acute state to partial remission, and from peripheral blood to cortical tissue. *Transl Psychiatry*. 2016; 6(1):e717. <https://doi.org/10.1038/tp.2015.213> PMID: 26784971
 39. Becker C, Hammerle-Fickinger A, Riedmaier I, Pfaffl MW. mRNA and microRNA quality control for RT-qPCR analysis. *Methods*. 2010; 50(4):237–43. <https://doi.org/10.1016/j.ymeth.2010.01.010> PMID: 20079844
 40. Forero DA, van der Ven K, Callaerts P, Del-Favero J. miRNA genes and the brain: implications for psychiatric disorders. *Hum Mutat*. 2010; 31(11):1195–1204. <https://doi.org/10.1002/humu.21344> PMID: 20725930
 41. Ran M, Chen B, Yin J, Yang A, Jiang M. Advances in miRNA research related to testis development and spermatogenesis. *Yi Chuan*. 2014; 36(7):646–654. <https://doi.org/10.3724/SP.J.1005.2014.0646> PMID: 25076028
 42. Xu D, Liu L, Zhao Y, Yang L, Cheng J, Hua R, et al. Melatonin protects mouse testes from palmitic acid-induced lipotoxicity by attenuating oxidative stress and DNA damage in a SIRT1-dependent manner. *J Pineal Res*. 2020; 69(4):e12690. <https://doi.org/10.1111/jpi.12690> PMID: 32761924
 43. Rehman SU, Ikram M, Ullah N, Alam SI, Park HY, Badshah H, et al. Neurological Enhancement Effects of Melatonin against Brain Injury-Induced Oxidative Stress, Neuroinflammation, and Neurodegeneration via AMPK/CREB Signaling. *Cells*. 21;8(7):760. <https://doi.org/10.3390/cells8070760>
 44. Miranda-Riestra A, Estrada-Reyes R, Torres-Sanchez ED, Carreño-García S, Ortiz GG, Benítez-King G. Melatonin: A Neurotrophic Factor?. *Molecules*. 2022; 27(22):7742. <https://doi.org/10.3390/molecules27227742> PMID: 36431847
 45. Moriya T, Horie N, Mitome M, Shinohara K. Melatonin influences the proliferative and differentiative activity of neural stem cells. *J Pineal Res*. 2007; 42(4):411–418. <https://doi.org/10.1111/j.1600-079X.2007.00435.x> PMID: 17439558
 46. Yang M, Guan S, Tao J, Zhu K, Lv D, Wang J, et al. Melatonin promotes male reproductive performance and increases testosterone synthesis in mammalian Leydig cells. *Biol Reprod*. 2021; 104(6):1322–1336. <https://doi.org/10.1093/biolre/iaab046> PMID: 33709108
 47. Baburski AZ, Sokanovic SJ, Janjic MM, Stojkov-Mimic NJ, Bjelic MM, Andric SA, et al. Melatonin replacement restores the circadian behavior in adult rat Leydig cells after pinealectomy. *Mol Cell Endocrinol*. 2015; 413:26–35. <https://doi.org/10.1016/j.mce.2015.05.039> PMID: 26116827
 48. Aten S, Hansen KF, Price KH, Wheaton K, Kalidindi A, Garcia A, et al. miR-132 couples the circadian clock to daily rhythms of neuronal plasticity and cognition. *Learn Mem*. 2018; 25(5):214–229. <https://doi.org/10.1101/lm.047191.117> PMID: 29661834
 49. Cheng HY, Papp JW, Varlamova O, Dziema H, Russell B, Curfman JP, et al. microRNA modulation of circadian-clock period and entrainment. *Neuron*. 7;54(5):813–29. <https://doi.org/10.1016/j.neuron.2007.05.017>
 50. Gu J, Lu Z, Ji C, Chen Y, Liu Y, Lei Z, et al. Melatonin inhibits proliferation and invasion via repression of miRNA-155 in glioma cells. *Biomed Pharmacother*. 2017; 93:969–975. <https://doi.org/10.1016/j.biopha.2017.07.010> PMID: 28724215
 51. Khan HL, Bhatti S, Abbas S, Kaloglu C, Qurat-Ul-Ain Zahra S, Khan YL, et al. Melatonin levels and microRNA (miRNA) relative expression profile in the follicular ambient microenvironment in patients undergoing in vitro fertilization process. *J Assist Reprod Genet*. 2021; 38(2):443–459. <https://doi.org/10.1007/s10815-020-02010-2> PMID: 33226531
 52. Chen D, Lan G, Li R, Mei Y, Shui X, Gu X, et al. “Melatonin ameliorates tau-related pathology via the miR-504-3p and CDK5 axis in Alzheimer’s disease.” *Transl Neurodegener*. 2022; 11(1):27. <https://doi.org/10.1186/s40035-022-00302-4> PMID: 35527277
 53. Lu J, Luo Y, Mei S, Fang Y, Zhang J, Chen S. The Effect of Melatonin Modulation of Non-coding RNAs on Central Nervous System Disorders: An Updated Review. *Curr Neuropharmacol*. 2021; 19(1):3–23. <https://doi.org/10.2174/1570159X18666200503024700> PMID: 32359338
 54. Wu C, Du M, Yu R, Cheng Y, Wu B, Fu J, et al. A novel mechanism linking ferroptosis and endoplasmic reticulum stress via the circPtpn14/miR-351-5p/5-LOX signaling in melatonin-mediated treatment of traumatic brain injury. *Free Radic Biol Med*. 2022; 178:271–294. <https://doi.org/10.1016/j.freeradbiomed.2021.12.007> PMID: 34883251
 55. Carloni S, Favrais G, Saliba E, Albertini MC, Chalon S, Longini M, et al. Melatonin modulates neonatal brain inflammation through endoplasmic reticulum stress, autophagy, and miR-34a/silent information

- regulator 1 pathway. *J Pineal Res.* 2016; 61(3):370–380. <https://doi.org/10.1111/jpi.12354> PMID: 27441728
56. Cho JH, Bhutani S, Kim CH, Irwin MR. Anti-inflammatory effects of melatonin: A systematic review and meta-analysis of clinical trials. *Brain Behav Immun.* 2021; 93:245–253. <https://doi.org/10.1016/j.bbi.2021.01.034> PMID: 33581247
 57. Wang X, Wang ZH, Wu YY, Tang H, Tan L, Wang X, et al. Melatonin attenuates scopolamine-induced memory/synaptic disorder by rescuing EPACs/miR-124/Egr1 pathway. *Mol Neurobiol.* 2013; 47(1):373–381. <https://doi.org/10.1007/s12035-012-8355-9> PMID: 23054680
 58. Ateşşahin A, Sahna E, Türk G, Ceribaşı AO, Yılmaz S, Yüce A, et al. Chemoprotective effect of melatonin against cisplatin-induced testicular toxicity in rats. *J Pineal Res.* 2006; 41(1):21–27. <https://doi.org/10.1111/j.1600-079X.2006.00327.x> PMID: 16842537
 59. Bashandy SAE, Ebaid H, Al-Tamimi J, Ahmed-Farid OA, Omara EA, Alhazza IM. Melatonin Alleviated Potassium Dichromate-Induced Oxidative Stress and Reprotoxicity in Male Rats. *Biomed Res Int.* 2021; 2021:3565360. <https://doi.org/10.1155/2021/3565360> PMID: 34222468
 60. Moradi M, Goodarzi N, Faramarzi A, Cheraghi H, Hashemian AH, Jalili C. Melatonin protects rats testes against bleomycin, etoposide, and cisplatin-induced toxicity via mitigating nitro-oxidative stress and apoptosis. *Biomed Pharmacother.* 2021; 138:111481. <https://doi.org/10.1016/j.biopha.2021.111481> PMID: 33752059
 61. Wang Z, Teng Z, Wang Z, Song Z, Zhu P, Li N, et al. Melatonin ameliorates paclitaxel-induced mice spermatogenesis and fertility defects. *J Cell Mol Med.* 2022; 26(4):1219–1228. <https://doi.org/10.1111/jcmm.17177> PMID: 35001532
 62. Olayaki L, Adeyemi W, Alabi Q, et al. Melatonin ameliorates some biochemical alterations following ketoconazole administration in rats. *J Basic Clin Physiol Pharmacol.* 2020; 31(4):/j/jbcp.2020.31.issue-4/jbcp-2019-0155/jbcp-2019-0155.xml. <https://doi.org/10.1515/jbcp-2019-0155> PMID: 31926082
 63. Peng Z, Xin WZ, Sheng ZY, Zi T, Nan LY, Lin WZ, et al. Melatonin alleviates cisplatin-induced mice spermatogenesis defects. *Reprod Toxicol.* 2023; 119:108391. <https://doi.org/10.1016/j.reprotox.2023.108391> PMID: 37149204
 64. Venditti M, Ben Rhouma M, Romano MZ, Messaoudi I, Reiter RJ, Minucci S. Evidence of melatonin ameliorative effects on the blood-testis barrier and sperm quality alterations induced by cadmium in the rat testis. *Ecotoxicol Environ Saf.* 2021; 226:112878. <https://doi.org/10.1016/j.ecoenv.2021.112878> PMID: 34634736
 65. Zhu X, Chen S, Jiang Y, Xu Y, Zhao Y, Chen L, et al. Analysis of miRNA expression profiles in melatonin-exposed GC-1 spg cell line. *Gene.* 2018; 642:513–521. <https://doi.org/10.1016/j.gene.2017.11.068> PMID: 29197587
 66. Liang G, Zhu X, Zhang Z, Chen L, Feng H, Zhan S, et al. microRNAs in aged sperm confer psychiatric symptoms to offspring through causing the dysfunction of estradiol signaling in early embryos. *Cell Discov.* 2022; 8(1):63. <https://doi.org/10.1038/s41421-022-00414-1> PMID: 35790721
 67. Rodgers AB, Morgan CP, Leu NA, Bale TL. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proc Natl Acad Sci U S A.* 2015; 112(44):13699–13704. <https://doi.org/10.1073/pnas.1508347112> PMID: 26483456
 68. Sharma U. Paternal Contributions to Offspring Health: Role of Sperm Small RNAs in Intergenerational Transmission of Epigenetic Information. *Front Cell Dev Biol.* 2019; 7:215. <https://doi.org/10.3389/fcell.2019.00215> PMID: 31681757
 69. Asadi E, Najafi A, Benson JD. Exogenous Melatonin Ameliorates the Negative Effect of Osmotic Stress in Human and Bovine Ovarian Stromal Cells. *Antioxidants (Basel).* 2022; 11(6):1054. <https://doi.org/10.3390/antiox11061054> PMID: 35739950
 70. Ortiz A, Espino J, Bejarano I, et al. High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. *J Pineal Res.* 2011; 50(2):132–139. <https://doi.org/10.1111/j.1600-079X.2010.00822.x> PMID: 20964711
 71. Succu S, Berlinguer F, Pasciu V, Satta V, Leoni GG, Naitana S. Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. *J Pineal Res.* 2011; 50(3):310–318. <https://doi.org/10.1111/j.1600-079X.2010.00843.x> PMID: 21214627
 72. Alevra Alexia & Achillefs Makris & Kanaki AP& Exadactylos, Athanasios MenteEleni Mylonas, Constantinos, et al. The effects of melatonin in preserving gilthead seabream (*Sparus aurata*) spermatozoa: preliminary results. Conference: Aquaculture Europe 2022, European Aquaculture Society (EAS)At: Rimini, Italy: pp. 34–35.
 73. Fineberg SK, Kosik KS, Davidson BL. MicroRNAs potentiate neural development. *Neuron.* 2009; 64(3):303–309. <https://doi.org/10.1016/j.neuron.2009.10.020> PMID: 19914179

74. Moon E, Kim K, Partonen T, Linnaranta O. Role of Melatonin in the Management of Sleep and Circadian Disorders in the Context of Psychiatric Illness. *Curr Psychiatry Rep.* 2022; 24(11):623–634. <https://doi.org/10.1007/s11920-022-01369-6> PMID: 36227449