Research Article

Monosodium glutamate administration early in life alters pineal melatonin nocturnal profile in adulthood

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Running title: MSG alters melatonin nocturnal profile in the pineal glands

ABSTRACT

The pineal gland synthesizes melatonin exclusively at night, which gives melatonin the characteristic of a temporal synchronizer of the physiological systems. Melatonin is a regulator of insulin activities centrally and also peripherally and its synthesis is reduced in diabetes. Since monosodium glutamate (MSG) is often used to induce the type 2 diabetic and metabolic syndrome in animal models, the purpose of this work is to evaluate the potential effects of MSG given to neonates on the pineal melatonin synthesis in different aged male and female rats. Wistar rats were subcutaneously injected with MSG (4mg/g/day) or saline solution (0.9%) from the second to eighth post-natal day. The circadian profiles both melatonin levels and AANAT activity were monitored at different ages. Body weight, naso-anal length, adipose tissues weight, GTT, ITT and serum insulin levels were also evaluated. Typical obesity with the neonatal MSG treatment was observed, indicated by a great increase in adipose depots without a concurrent increase in body weight. MSG treatment did not cause hyperglycemia or glucose intolerance, but induced insulin resistance. An increase of melatonin synthesis at ZT 15 with phase advance was observed in in some animals. The AANAT activity was positively parallel to the melatonin circadian profile. It seems that MSG causes hypothalamic obesity which may increase AANAT activity and melatonin production in pineal gland. These effects were not temporally correlated with insulin resistance and hyperinsulinemia indicating the hypothalamic lesions, particularly in arcuate nucleus induced by MSG in early age, as the principal cause of the increase in melatonin production.

Key words: Monosodium glutamate, melatonin, pineal gland, hypothalamic obesity, insulin resistance.

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1. INTRODUCTION

Serum melatonin is majorly synthesized by the pineal gland in vertebrates; however, melatonin is also produced in other cells and tissues functioning as the autocrine and paracrine. Thus, plasma melatonin reflects melatonin produced in the pineal gland under the physiological condition and its synthesis is restricted to the night, signaling the environmental light/dark cycle to the internal organs of organisms. This signaling allows organisms to adjust their physiology and behavior to adapt the environmental changes related to the natural geophysical daily cycles (1, 2). The circadian melatonin synthesis is controlled by the biological clock i.e., the hypothalamic suprachiasmatic nuclei (SCN), that project to the hypothalamic paraventricular nuclei (PVN), that send direct or indirect projections to the intermediolateral column of the spinal cord. From the superior cervical ganglia, postsynaptic axons of the sympathetic nerves reach the pineal gland releasing norepinephrine (NE) in its interstices during dark and triggering melatonin synthesis. NE interacts with $\alpha1$ and $\beta1$-adrenoceptors to activate the arylalkylamine N-acetyltransferase (AANAT), the rate-limiting enzyme in the melatonin synthetic pathway, catalyzing the transformation of serotonin to N-acetylserotonin which is converted to melatonin by acetylserotonin methyltransferase (ASMT) (1, 2).

Pineal melatonin regulates several physiological processes including cardiovascular, immunological and metabolic functions, the sleep-wake cycle, among others, adapting them to the day/night environmental variations (3). For carbohydrate metabolism, melatonin exhibits a synergistic effect with insulin, inducing tyrosine phosphorylation and activation of the tyrosine kinase β-subunit of the insulin receptor prior to insulin signaling mediated by MT1 receptor activation (4). In addition, melatonin regulates GLUT4 synthesis in adipose and muscle tissues. Thus, melatonin sensitizes central and peripheral insulin action. The absence or reduction of pineal melatonin production, whether by the effect of light, aging or pinealectomy, could lead to insulin resistance and this resistance could be reversed by melatonin replacement (5, 6). On the other hand, type 1 diabetes, either in humans or experimentally in animals can cause an accentuated reduction in melatonin synthesis (7). A similar phenomenon has been observed in type 2 diabetes patients. The occurrence of type 2 diabetes is reported to associate with mutated variants of the melatonin receptor 2 (MT2) (8-11). Obesity is an important etiology of insulin resistance and type 2 diabetes. This is characterized by a chronic systemic low-grade inflammation which leads to a desensitization of insulin signaling (12-15). Obesity and diabetes are public health threats with high prevalence and there is a growing interest in their prevention.

Monosodium glutamate (MSG) is often used to generate a specific obese animal model which is referred to as hypothalamic obesity. MSG treatment can also cause type 2 diabetes and metabolic syndrome in animals (16-18). MSG treated newborns (rats or mice) will develop a metabolic syndrome in the young adulthood characterized by obesity, growth impairment, hyperinsulinemia and hyperleptinemia, without increasing food intake (17). In older animals, the insulin resistance and hyperglycemia are also present (16, 19). MSG induced pathophysiology is a consequence of glutamate excitotoxicity which is associated with the immaturity of the blood brain barrier in newborns. The circumventricular structures including hypothalamic arcuate nucleus, ventromedial nucleus and median eminence are most affected (17, 20). In humans, ingestion of MSG in the diet has been reported to cause obesity (21-24).

The aim of this study is to find a potential association between MSG administration in newborns and pineal melatonin production amongst different ages of male and female rats. For these purposes, the circadian profiles of pineal melatonin content and AANAT activity were measured. At the same time, the developments of hyperglycemia and insulin resistance, body weight, adipose tissue weight, naso-anal length, Lee Index and insulinemia were also monitored. These parameters were used to uncover the potential associations of pineal melatonin production and the metabolic disorder induced by MSG.

2. MATERIALS AND METHODS

2.1. Animals.

Male and female Wistar rats were kept under a 12:12 h light-dark cycle in a temperature-controlled room (21±2°C) with water and food *ad libitum*. All experiments were performed in accordance to the guidelines of the Brazilian College for Animal Experimentation (COBEA) and approved by the Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences (CEUA – Permit number: 199/11), University of São Paulo (São Paulo, Brazil). Animals were allowed to mate at three months of age, two females and one male were housed per cage for one week. Pregnant females were individually housed. Pups were injected subcutaneously with MSG (4mg/g of body weight/ day) (MSG group) (Sigma-Aldrich Chemicals, St. Louis, MO, USA) or with 0.9% saline (control group) from the second to the eighth postnatal day. After weaning, male and female rats were separated and housed in cages (5/cage) with water and food *ad libitum*. Care has always been taken to reduce animal pain.

2.2. Experimental protocol.

MSG treated and control rats (male and female, n=5/group), were euthanized at 2, 3, 4, 5 and 6 months of age in the dark period under red light. Pineal glands and retroperitoneal and periepididimal adipose tissues were collected at ZTs (zeitgeber time) 12, 15, 18, 21 and 24. ZT12 represents the transition from light to dark and ZT24 represents the transition from dark to light. Pineal glands were placed in microcentrifuge tubes and kept in freezer -80°C until assayed. Retroperitoneal and periepididimal adipose tissues were weighed.

2.3. Evaluation of body weight, naso-anal length and Lee index

Body weight (n=20/group) and the naso-anal length (n=5/group) were evaluated the day before euthanasia. Lee index that measures adiposity index was calculated by dividing the cube root of the body weight (g) by the naso-anal length (cm).

2.4. Melatonin measurement – high-pressure liquid chromatography.

Melatonin was determined by high-pressure liquid chromatography in a HPLC apparatus (Ultimate 3000; Dionex, Sunnyvale, CA, USA) with electrochemical detection (ESA Coulochem III, with guard cell 5020 and amperometric analytical cell 5041 with a glassy carbon ceramic target eletrode). The system was controlled by a Chromeleon software (Dionex 3000, ThermoFisher, USA) and isocratically operated with the following mobile phase: 0.1 M sodium acetate, 0.1 M citric acid, 0.15 mM EDTA, 30% methanol, pH 3.7, at a flow rate of 0.135 mL/min. The electrochemical detector potential was adjusted 700 mV vs PD reference electrode (5041 analytical cell). A guard cell was used and the potential was set at + 750 mV. The elution time for melatonin was about 10 min.

Each pineal gland was sonicated (Microson XL 2005, Heat System Inc., Farmingdale, USA) in a solution of 0.1 M perchloric acid (120 µL), containing 0.02% EDTA and 0.02% sodium bisulfate. After centrifugation (20 min, 14,000 g, Eppendorf 5415C centrifuge, Brinkman Instruments Inc., Westbury, USA), 40 µL of the supernatant was injected into the chromatographic system (WPS-3000TSL autosampler with sample thermostating). The melatonin peak in the samples was determined by a comparison of the retention time to the one obtained for the standards. Melatonin concentration in the samples was determined against a

standard curve (peak area X concentration) freshly prepared, with seven different and known concentrations (0.145–9.28 ng/40 L).

2.5. AANAT activity determination.

AANAT activity was measured by a radiometric assay (25, 26). Briefly, 100 µL of 0.1M sodium phosphate buffer, pH 6.8, containing 40mM tryptamine and [^{3}H]-acetyl coenzyme A (2mM, final specific activity = 4 mCi/mmol) (Amersham Biosciences, Piscataway, NJ, USA) were added to a microcentrifuge tube containing one gland kept at 4°C. The glands were sonicated and then incubated at 37°C for 20 min. The reaction product N-^{3}[H]-acetyltryptamine was extracted with chloroform (1 mL). Samples of 500 µL were evaporated until dryness in a scintillation vial (with 3mL of BCS-NA non-aqueous biodegradable counting scintillant, Amersham Bioscience, Piscataway, NJ, USA) and radioactivity was determined with a Beckman LS6500 β counter (Beckman Coulter Inc., Fullerton, CA, USA).

2.6. Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT).

MSG treated and control rats (male and female) aged 3 and 5 months were fasted for twelve hours. For the GTT, a 20% glucose solution (2g/kg of body weight) was injected intraperitonially and the tail blood was collected 0, 10, 15, 30, 60 and 120 minutes after the injection (n=6/group). Blood glucose was evaluated and the area under the curve (AUC) was estimated in relation to basal glycaemia (27). For the ITT, the animals were intraperitonially injected with insulin (2UI/kg of body weight) and the tail blood was collected 0, 10, 15, 25 and 30 minutes after the injection (n=6/group) for glycaemia assessment. Glycaemia values were converted to natural logarithm (Ln), the slope was calculated by linear regression (time x Ln glucose) and multiplied by one hundred to obtain the decay per minute of the glucose rate during the insulin tolerance test (kITT) (28).
The prior described parameters and insulinemia quantification were evaluated in 3- and 5-month-old male and female rats, in order to analyze putative alterations in melatonin production at an early (3 months) and a more advanced (5 months) age.

2.7. Insulin quantification.

Three and 5-month-old male and female MSG-treated and control rats (n=7/group) were euthanized and trunk blood was collected. Plasma insulin was quantified by immunoassay using the Rat Insulin Elisa kit (Cat. number E-EL-R2466, Elabscience, Houston, Texas, USA) according to the manufacturer's instructions.

2.8. Statistical analysis.

Statistical analysis was conducted using the computational program Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). All parameters were expressed as mean ± SEM and analyzed by two-way ANOVA and Bonferroni posthoc test or Student t-test. The diurnal profiles of melatonin and AANAT activity were analyzed by Cosinor (29) that consists of fitting a cosine curve to the data by the least square method and determining the presence of a circadian rhythm by testing if the amplitude of the fitted curve was different from zero. Three parameters are obtained from the fitted curve: mesor (oscillation medium level), amplitude (distance between mesor and maximal value) and acrophase (time when the amplitude is maximal). These parameters were compared between groups by Student t-test.

3. RESULTS

Compared to control rats, MSG-treated male and female rats at all ages showed increased adipose mass as evaluated by retroperitoneal (male and female) and periepididimal (male) white adipose tissues (Figure 1). The increment in adipose tissue mass was always greater in females than that in males (2-month-old: F=183%, M=101%; 3-month-old: F=234%, M=223%; 4-month-old: F=173%, M=115%; 5-month-old: F=138%, M=89%; 6-month-old: F=269%, M=211%).

Body weight of MSG neonatally-treated rats showed minor changes and were mostly expressed by a reduction as observed in males aged 2, 3 and 5 months. The other groups presented no alterations in body weight except females aged 6 months in which body weight was greater in MSG-treated than in the control group. On the other hand, naso-anal length was consistently reduced in all male and female MSG-treated animals, at all ages. Lee Index, that correlates body weight with naso-anal length and represents an adipose index that validates the occurrence of obesity, was increased in MSG-treated animals at all ages, even though body weight was almost unchanged (Figure 1).
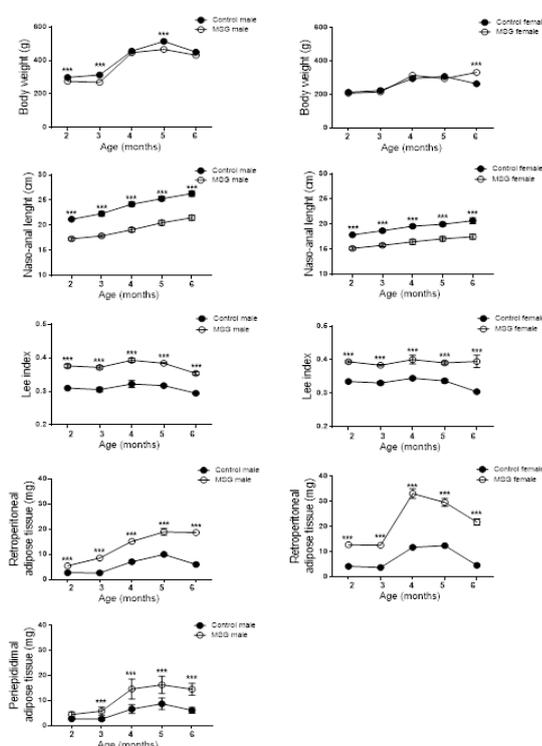

Fig. 1. Body weight, naso-anal length, lee index, retroperitoneal and periepididimal adipose tissues weight.

Parameters obtained from male and female rats aged 2, 3, 4, 5 and 6 months, with or without MSG (4mg/g/day from 2nd to 8th postnatal day). Body weight, Retroperitoneal and Periepididimal tissues: n=20/group; Naso-anal length and Lee Index: n=5/group. ***p<0.001 vs control.

Glucose tolerance was assessed by GTT in 3- and 5-months-old male and female rats. GTT curves are presented in Figure 2A. Area under the curve (AUC) was calculated from GTT curves and showed no change by MSG treatment, confirming the absence of glucose intolerance (3 months: Male control = 4026 ± 614, Male MSG = 3258 ± 269, Female control = 4111 ± 448, Female MSG = 2533 ± 789; 5 months: Male control = 7395 ± 1183, Male MSG = 9468 ± 2894, Female control = 7049 ± 689, Female MSG = 6376 ± 367) (Figure 2B).
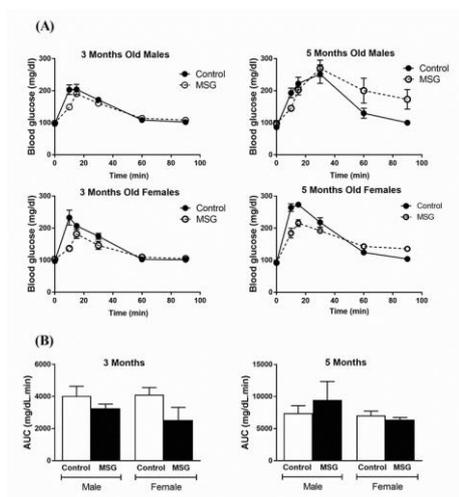
Fig. 2. GTT (glucose tolerance test) and Area under the curve.

GTT was performed by intraperitonial glucose administration (20% glucose solution - 2g/kg of body weight) and tail blood collection 0, 10, 15, 30, 60 and 120 minutes after injection. The test was performed in 3- and 5-months-old male and female rats with or without MSG in the neonatal period (4mg/g/day from 2nd to 8th postnatal day). (B). Area under the curve obtained from the curves of GTT. T test: there was no difference between groups. N=6/group

Insulin resistance was assessed by ITT in 3 and 5-months-old male and female rats. There was a significant difference between control and MSG-treated animals (except for 3-month-old males) showing the presence of insulin resistance as confirmed by kITT values (3-month-old: Male control = 3.66 ± 0.27, Male MSG = 3.87 ± 0.31, Female control = 5.33 ± 0.35, Female MSG = 2.85 ± 0.52; 5-month-old: Male control = 3.65 ± 0.41, Male MSG = 2.46 ± 0.19, Female control = 3.40 ± 0.39, Female MSG = 1.52 ± 0.48) (Figures 3A and 3B).
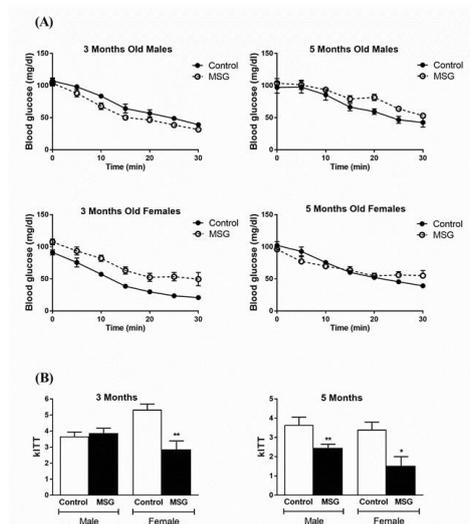

Fig. 3. ITT (insulin tolerance test) and kITT (decay of the glucose rate during the insulin tolerance test per minute).

(A) ITT was performed by intraperitonial insulin injection (2UI/kg of body weight) and tail blood collection at the time points 0, 10, 15, 25 and 30 minutes. It was performed in 3- and 5-months-old male and female rats with or without MSG in the neonatal period (4mg/g/day from 2nd to 8th postnatal day). (B): kITT was obtained from ITT data. There was a significant difference between MSG and control groups for 5-month-old male and female and 3-month-old female rats. T test: *p<0.05; **p<0.01.vs control, N=6/group.

Insulinemia was also evaluated in 3- and 5-months-old male and female rats. There was an increase in plasma insulin in MSG-treated 5-month-old animals, males and females, but not for 3-month-old animals (3-month-old: Male control = 4.70 ± 0.59, Male MSG = 3.79 ± 0.48, Female control = 4.08 ± 0.30, Female MSG = 4.33 ± 0.41; 5-month-old: Male control = 3.70 ± 0.24, Male MSG = 4.96 ± 0.27, Female control = 3.39 ± 0.32, Female MSG = 4.83 ± 0.55) (Figure 4). The normal ranges of glycemia observed for MSG-treated 5-month-old animals seems to be maintained at the expense of increased insulin secretion in order to compensate the insulin resistance.
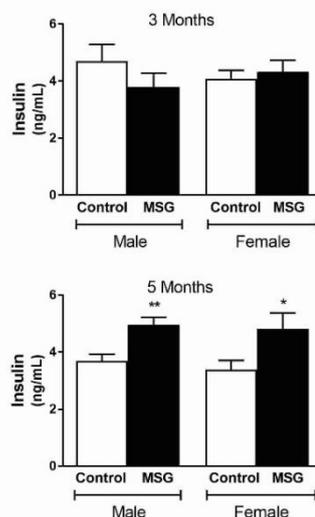

Fig. 4. Plasma insulin.
　Trunk blood was collected from 3- and 5-months-old male and female rats with or without MSG in the neonatal period (4mg/g/day from 2nd to 8th postnatal day). T test: *p<0.05; **p<0.01 vs the respective control group. N=7/group.

　Melatonin content was evaluated in the pineal glands of male and female rats at several ages (2- to 6-month-old) at 3h intervals during the night, characterizing the nocturnal profile of melatonin synthesis. The most remarkable effect observed was an increase in melatonin synthesis by the pineal gland at ZT=15 induced by MSG treatment for males and females at several ages. The exceptions were 2-month-old males and females and 3-month-old males (Figure 5).
　AANAT activity followed the same pattern as observed in melatonin's profile. There was a difference in ZT15, with MSG-treated groups exhibiting increased activity at this time point. The exceptions were 2-month-old males and females. Besides, alterations were also verified for other ages at ZTs 18 and 21 (Figure 6).
　Cosinor analysis, which fits a cosine curve to the raw data, enabled the analysis of several parameters such as the presence of 24h rhythm (circadian rhythm), mesor, amplitude and acrophase, revealing some changes in rhythmicity. For the investigated melatonin and AANAT activity profiles, MSG treatment did not alter the presence of circadian rhythm, i.e., the 24h-period oscillation (Figures 7 and 8). Considering pineal melatonin content, minor changes were observed for acrophase, representing phase advances (Figure 9). AANAT activity presented significant changes in acrophase, mesor and amplitude. The phase advances occurred in 3-, 4-, 5-month-old males and 3-, 5-, 6-month-old females. Increased mesor was observed in 4- and 5-month-old males and in 2-, 3-, 4-, 6-month-old females. Increased amplitude was found in 4- and 5-month-old males and in 2- and 4-month-old females (Figure 10). Table 1 shows a summary of all results.
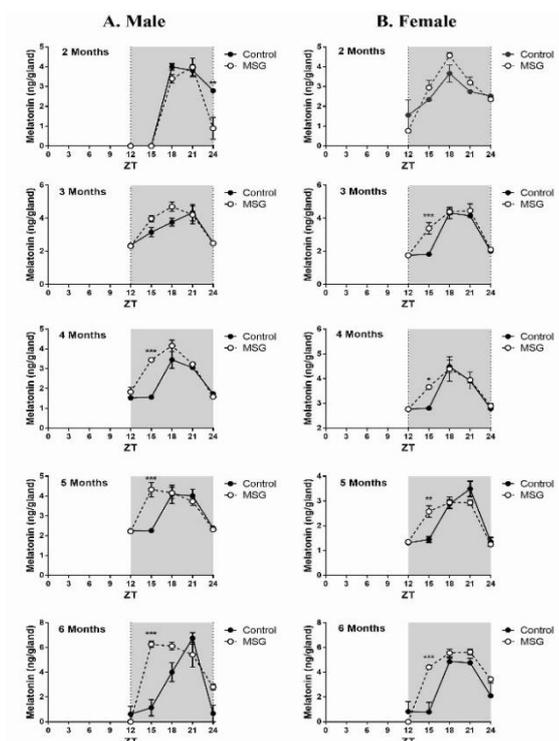
Fig. 5. Nocturnal melatonin profile.

The pineal glands collected from male and female rats with or without MSG in the neonates (4mg/g/day from 2nd to 8th postnatal day) aged 2, 3, 4, 5 and 6 months. Bonferroni post hoc test: *p<0.05; **p<0.01; ***p<0.001 vs the respective control groups. N=5/group/time point.
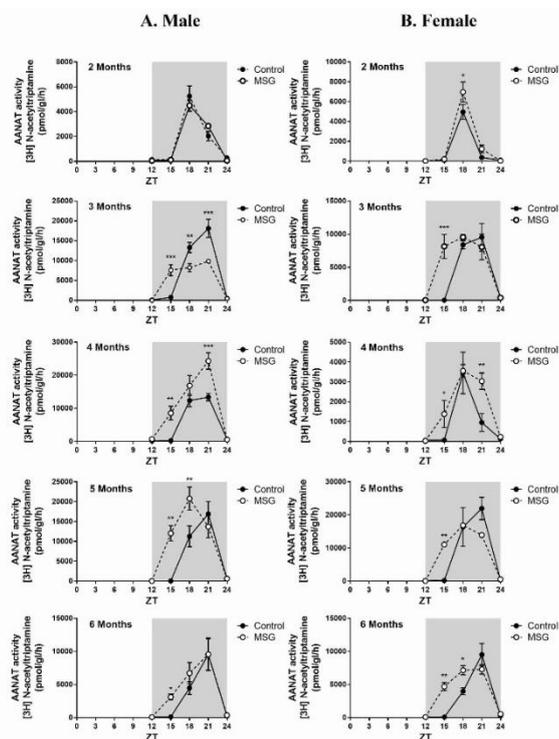

Fig. 6. Nocturnal AANAT activity profile.

The pineal glands collected from male and female rats with or without MSG in the neonates (4mg/g/day from 2nd to 8th postnatal day) aged 2, 3, 4, 5 and 6 months. Bonferroni post hoc test: *p<0.05; **p<0.01; ***p<0.001 vs the respective control groups. N=5/group/time point.
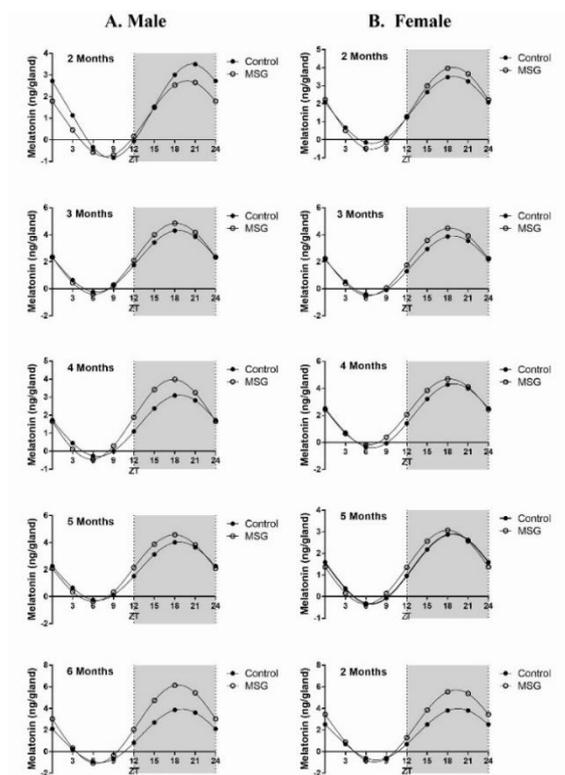
Fig. 7. **Melatonin cosine curve**.

It was adjusted to the melatonin nocturnal profile data as showed in Figure 9. 24-oscillation period was maintained in the MSG treated rats as well as it was observed for control groups.
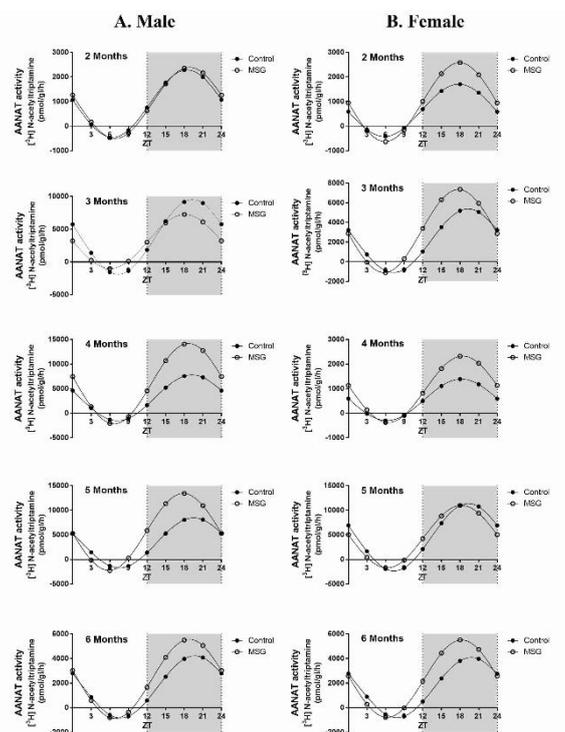

Fig. 8. AANAT cosine curve.

It was adjusted to the AANAT activity nocturnal profile data as showed in Figure 10. 24-oscillation period was maintained in the MSG treated rats as well as was observed for control group.
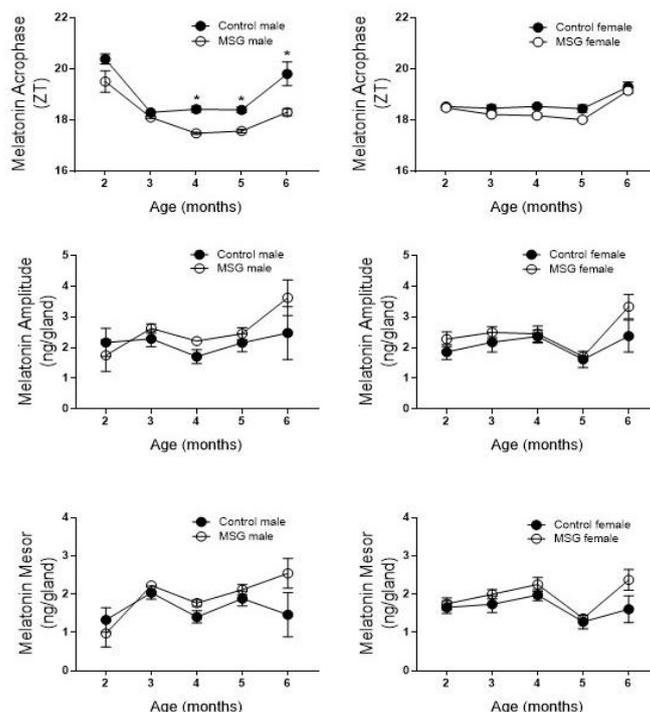
Fig. 9. **Melatonin Acrophase, Amplitude and Mesor**.

*Parameters obtained from the cosine curves adjusted to melatonin nocturnal profile for 2-, 3-, 4-, 5- and 6-month-old male and female rats with or without MSG in the neonatal period (4mg/g/day from 2nd to 8th postnatal day). Mesor represents the medium oscillation level, amplitude is the distance between the mesor and the maximal value and acrophase is the time when the maximal amplitude occurs. T test: *p<0.05 vs the respective control.*
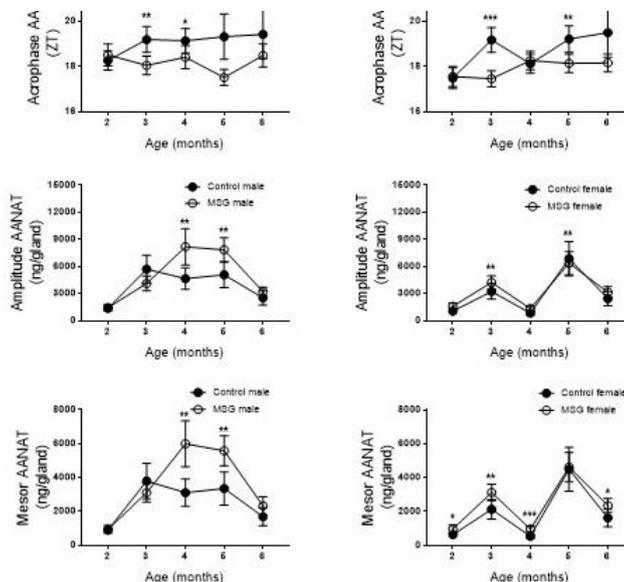

Figure 10. **AANAT Acrophase, Amplitude and Mesor**.

*Parameters obtained from the cosine curves adjusted to AANAT activity nocturnal profile for 2-, 3-, 4-, 5- and 6-month-old male and female rats with or without MSG in the neonatal period (4mg/g/day from 2nd to 8th postnatal day). Mesor represents the medium oscillation level, amplitude is the distance between the mesor and the maximal value and acrophase is the time when the maximal amplitude occurs. T test: *p<0.05; **p<0.01; ***p<0.001 vs the respective control.*

Table 1. Results summary table.

Gender	Age (Month)	Body weight	N-A Length	Lee index	RAT weight	PAT weight	GTT (AUC)	kITT	Ins	Mel	AANAT activity
Male	2	<	<	>	>	>	-----	-----	-----	n	n
	3	<	<	>	>	>	n	n	n	n	>
	4	n	<	>	>	>	-----	-----	-----	>	>
	5	<	<	>	>	>	n	<	>	>	>
	6	n	<	>	>	>	-----	-----	-----	>	>
Female	2	n	<	>	>	-----	-----	-----	-----	n	n
	3	n	<	>	>	-----	n	<	n	>	>
	4	n	<	>	>	-----	-----	-----	-----	>	>
	5	n	<	>	>	-----	n	<	>	>	>
	6	>	<	>	>	-----	-----	-----	-----	>	>

Comparisons between control and MSG groups from 2-, 3-, 4-, 5- and 6-month-old male and female rats are showed for body weight, naso-anal length (N-A lengh), Lee Index, retroperitoneal (RAT) and periepididimal adipose tissues weight (PAT), glucose tolerance test (GTT), kITT (decay constant of the insulin tolerance test), insulinemia (Ins), melatonin (Mel) and AANAT activity (at ZT=15). Alterations are expressed as: (>) if it was increased by the MSG-treatment, (<) if it was decreased by the MSG-treatment, (n) if there was no alteration by the MSG-treatment, (-----) if the parameter was not measure.

4. DISCUSSION

It is well-known that administered MSG to neonates causes animal obesity in adulthood due to an injury to the hypothalamic nuclei which are involved in energy balance control (17, 20). This MSG animal model mainly affects periventricular structures with the hypothalamic arcuate nuclei being the most affected one (16, 17, 20). Arcuate neurons are crucial for the regulation of metabolic homeostasis, including insulin secretion and action.

In this study, we have confirmed the MSG induced obesity in adult male and female rats as reported by others (19, 30, 31). A systematic study addressing the temporal development of obesity, its associated pathologies and melatonin synthesis and AANAT activity has been performed in this study and this has not been reported previously.

Obesity was observed in male and female rats consistently from 2- to 6-month-old after MSG treatment. The same pattern was observed for body length and Lee Index in the MSG group at all ages in both genders. Body weight was almost unchanged with slight variations in which predominated weight reduction except for 6-month-old female rats that showed weight gain. In fact, the white visceral adipose tissue weight in females was always higher than in males, which could explain the weight gain at 6 months of age (19). Lee Index represents an index of adiposity correlating body weight with naso-anal length, showing in this model an increase in adiposity.

The body length reduction imposed by neonatal MSG-treatment is due to a decrease in the growth hormone-releasing hormone as a consequence of arcuate nuclei lesion (32). The arcuate lesion is also responsible for an imbalance in the regulation of energy metabolism that results in obesity (16, 17). The disturbance in metabolic functions observed since 2-month-old seems to be a consequence of NPY reduction in the PVN (33) since a great number of NPYergic fibers that projects to the PVN come from the arcuate nuclei. MSG treatment, by damaging these

nuclei, suppress a tonic inhibitory modulation imposed to the PVN (34). In this model, leptin signaling is also impaired (16).

Although adiposity was increased in MSG-treated rats as early as 2-month-old, other parameters took longer to change. This was the case for insulin resistance and hyperinsulinemia that occurred in 5-month-old animals, although insulin resistance was also observed earlier in female rats (at 3-month-old).

The insulin resistance and hyperinsulinemia induced by MSG administration could be attributed to a reduction of GLUT4 transporter and insulin receptor expression in insulin sensitive tissues leading to an impaired insulin signaling effectiveness (31).

The work also uncovered the alterations in melatonin synthesis and AANAT activity in MSG-treated animals regardless of the gender and age. Melatonin level was increased in the early hours of the night, at ZT 15, did as the AANAT activity. Thus, the elevated melatonin level could be explained by the increased AANAT activity since AANAT is the rate-limiting enzyme in melatonin synthesis pathway (1, 2). These results differ from what is observed in rats with type 2 diabetes or even type 1 diabetes in which melatonin synthesis is reduced (7, 35, 36) and this reduction is attributed to the presence of hyperglycemia (7). Actually, MSG-treated rats show a clinical characteristic of pre-diabetes, presenting hyperinsulinemia and insulin resistance without showing hyperglycemia. According to the review from Bahadoran et al. (16) higher doses than 4mg/g/day of MSG are required to develop a model of type 2 diabetes, although other authors demonstrated the occurrence of hyperglycemia in older animals with lower doses (19).

The modifications in melatonin and AANAT activity are accompanied by changes in Lee Index and adipose tissue weight, i.e., melatonin and AANAT activity alterations followed the same pattern of obesity parameters. However, the time frame for the appearance of hyperinsulinemia and insulin resistance is longer than for melatonin and AANAT activity increase, pointing to an independence of melatonin alterations in relation to insulin signaling desensitization. Thus, the most probable cause of changes in melatonin and AANAT activity is related to the well-known central lesion. In fact, NPYergic fibers that reach PVN negatively modulating these nuclei could be responsible for an inhibitory influence on melatonin synthesis (33, 34, 37). The withdrawal of an inhibitory NPYergic afference to the PVN may possibly explain increased AANAT activity and melatonin production at ZT 15. However, the influence of insulin in melatonin production at 5-month-old male and female rats cannot be excluded, as an insulin increment was also present at this stage of development and it was reported that insulin was a potentiating factor on melatonin synthesis (38, 39).

When cosinor model was adjusted to the data it did not always reveal the alterations observed by parametric analysis. The elevated melatonin synthesis and AANAT activity that were systematically observed at ZT 15 was not always associated with an alteration in the adjusted cosine curve parameters. The most relevant effect revealed by cosinor analysis was the melatonin and AANAT activity phases advance which occurred almost at all age groups of both male and female rats (Figures 9 and 10). The increased mesor and amplitude were also demonstrated at ZT15 for AANAT activity.

Cosinor analysis also evidenced the presence of melatonin and AANAT circadian rhythms. Although some rhythm parameters were altered by MSG treatment, the 24h-oscillation seems to be preserved. The same was observed by other authors (40-43) that reported the maintenance of the 24h-rhythm and entrainment to the light-dark-cycle of activity and body temperature cycles. These data evidenced the integrity of the suprachiasmatic nuclei as the central biological clock and of the retinohypothalamic tract (RHT), responsible for the pacemaker entrainment to the light-dark cycle. It is known that neonatal MSG treatment induces a lesion in the inner retina (43, 44), but the photopigment responsible for the circadian system synchronization, melanopsin, located in the inner retina, seems to be preserved, as well as the

RHT (43). It could be speculated that the RHT is spared in the neonatal MSG treated animals because of the administration time window in which this pathway is still in development and thus it could be plastic enough to preserve its neural connections. The same could be in relation to the neural pathway that connects the suprachiasmatic nuclei to the pineal gland that is only completely mature at 2-week-old pups (45).

In conclusion, the parenteral MSG model of hypothalamic obesity increased melatonin and AANAT activity in the early hours of the night in male and female rats at the age of 3 to 6 months what is, in some cases, manifested as a temporally advanced phase. Neonatal MSG treatment constitutes a model of pre-diabetes up to 6 months of age in rats, with characteristics of hyperinsulinemia and insulin resistance without the occurrence of hyperglycemia.

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AUTHORSHIP

Janaína Barduco Garcia: acquisition of the data, data analysis/interpretation; Fernanda Gaspar do Amaral: acquisition of data, critical revision of the manuscript; Daniela do Carmo Buonfiglio: acquisition of data; Rafaela Fadoni Alponti Vendrame: acquisition of data; Patrícia Lucio Alves: acquisition of data; Maria Eliza Ferreira do Val de Paulo: acquisition of data; Julieta Helena Scialfa: acquisition of data; Paulo Flávio da Silveira: concept/design; José Cipolla Neto: funding acquisition, critical revision of the manuscript, concept/design; Solange Castro Afeche: concept/design, data analysis, interpretation, drafting of the manuscript, project administration.

CONFLICT INTEREST

The authors declare no conflict of interest.

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