RESEARCH ARTICLES

J. Sci. Soc. Thailand, 10 (1984) 89 - 99

DIFFERENCE BETWEEN PATTERNS OF MELATONIN UPTAKE IN RAT BRAIN AFTER THE MORNING AND THE AFTERNOON MELATONIN ADMINISTRATION

B. WITHYACHUMNARNKUL and W.K. TRAKULRUNGSI

Department of Anatomy, Faculty of Science Mahidol University, Rama 6 Rd., Bangkok 10400, Thailand.

(Received 23 March 1984)

Abstract

^{3}H-Melatonin uptake was determined in the brain of rats infused with ^{3}H-melatonin into the right common carotid artery. When the infusion was performed in the morning (4 h after onset of light), ^{3}H-melatonin uptake in the hypothalamus was higher than that in the cerebrum, the cerebellum, and the midbrain. In contrast, when the infusion was performed in the afternoon (3 h before onset of darkness), the uptake in the hypothalamus was equal to that found in the morning but the uptake in all other regions increased significantly comparable to that in the hypothalamus. The uptake in the anterior pituitary and the pineal gland was lower than that in the brain. Total radioactivity in serum of the morning and the afternoon rats were comparable, whereas melatonin radioactivity in serum of the afternoon rats was significantly higher than that of the morning rats. These data suggest that melatonin may act primarily in the hypothalamus but the action has to be facilitated by melatonin interaction to other brain regions which occurs only during the late light phase. The increased melatonin binding in the brain may retard the rate of melatonin degradation in the liver.

Introduction

Melatonin, 5-methoxy-N- acetyltryptamine, is generally considered as a pineal hormone. Since its discovery by Lerner et al in 1958[1], numerous studies have appeared in leterature regarding its function in mammals. At present, it is reasonable to state that melatonin is antigonadotropic in hamsters, rats, and mice[2-6] and probably, in dogs and

humans. The effects caused by exogenous melatonin included delayed sexual maturity, gonadal atrophy, decreased spermatogenesis and oogenesis, decreased LH and FSH, etc. However, to achieve these effects, it requires that melatonin must be given only at a certain period of the day. In hamsters and white-footed mice, melatonin given a few hours before the onset of darkness was antigonadotropic; whereas melatonin given during the early light phase showed either no effect or counter antigonadotropic effect.[3-6, 10-15] In male rats, long term melatonin injection at the late light phase retarded body growth, decreased pituitary and accessory sex organ weights, and decreased serum PRL.[16] The site of melatonin action is, by most lines of evidence, in the hypothalamus although the pituitary gland and gonads can not be entirely excluded[2, 8].

The high sensitivity to exogenous melatonin at the specific, late light phase is interesting and its underline mechanism needs further study. A hypothesis was, thus, set forth that the difference in sensitivity might be due to difference in melatonin binding to the hypothalamus or other brain regions in rats treated with melatonin at different times of a day. This experiment was carried out to test the hypothesis.

Materials and Methods

Animals. Adult male Sprague-Dawley rats, 8-10 wks of age, were kept in a room with ambient temperature about 25-28 C and a light : dark cycle being 12:12 h. Light was on between 06:00-18.00 h. Food and water were given *ad lib.* To allow complete accommodation to the lighting condition, the animals were kept for 2 wks before operation.

Chemicals. ^{3}H-Melatonin or acetyl-5-methoxytryptamine, N-2-aminoethyl-2-^{3}H (specific activity=32 Ci/mM) and NCS tissue solubilizer were purchased from New England Nuclear Corporation , U.S.A. Indoleamines used as markers in thin-layer chromatography are melatonin, serotonin, tryptophan, 5 hydroxy-tryptophol, 5 methoxy-tryptophol, 5 hydroxy indoleacetic acid, and 5 methoxy-indole acetic acid; all were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Chemicals for liquid scintillation cocktail, ie (1, 4-bis 2-5-phenyloxazolyl) benzene; 2, 2-p-phenylenebis (5-phenyloxazole) (POPOP), 2, 5-diphenyloxazole (PPO), Triton X-100, and toluene were also from Sigma Chemical Company.

^{3}H-Melatonin Infusion. The animals were anaesthetized by intraperitoneal injection of sodium pentobarbital (5.75 mg/100 g B.W.) and placed in supine position. The right common carotid artery was exposed by standard surgical technique. The artery was then cannulated and the brain infused with 100 ng of ^{3}H-melatonin (approximately 2.5 x 10^{5} dpm) in 1.0 ml of 0.01 M phosphate buffered saline (PBS), at the rate of 100 µl/min. It would take, therefore, 20 min to complete the infusion, after which the rat was decapitated, trunk blood collected; and the brain, the pituitary gland, and the pineal gland isolated. The blood was centrifuged at 500 xg, 4C, for 20 min and the serum isolated.

One group of rats (AM group) were operated during 1000-1100 h, approximately 4 h after onset of light ; another group (PM group) were operated during 1500-1600 h, approximately 3 h before onset of darkness.

Determination of Melatonin Radioactivity. The brain tissue was divided and four regions were sampled for ^{3}H-melatonin determination :

(1) The hypothalamus; A tissue block was cut from the base of the brain by making two coronal, one sagittal, and one horizontal sections. The two coronal sections were at 3 mm rostral to the optic chiasm and at the posterior border of the mamillary body. The sagittal cut was made along the optic tract and the horizontal one was at the level of the anterior commissure. The hypothalamic block preparation as such would include preoptic nucleus, suprachiasmatic nucleus, paraventricular nucleus, supraoptic nucleus, arcuate nucleus, dorsomedial nucleus, ventromedial nucleus, mammillary area, and the whole median eminence[17].

(2) The cerebrum ; A piece of tissue weighed around 50 mg was sampled from the frontal lobe.

(3) The cerebellum; A similar piece of tissue was sampled from the dorsal surface of the cerebellum.

(4) The midbrain ; A piece of tissue including the midbrain colliculi and tegmentum was sampled.

The pituitary gland was carefully dissected out of its posterior neural part, only the anterior pituitary was left for analysis. All tissue samples from the brain were exactly weighed and homogenized in ice-cold PBS to make 50 mg tissue/ml homogenate. The anterior pituitary and the pineal were homogenized in ice-cold 1 ml PBS. The whole brain was also homogenized to make 5 mg/ml and 1 ml/ml homogenates which could be compared to the anterior pituitary and the pineal, respectively.

Aliquots of 1 ml homogenate and 200 µl serum were then extracted with 10x volume chloroform, N_2-dried, and redissolved in 150 µl absolute ethanol. Duplicates of 50 µl ethanol extract were further analyzed for ^{3}H-melatonin content by two-dimensional thin layer chromatography as described by Klein and Notides[18]. Melatonin spot on sillica gel was isolated and dissolved in 0.5 ml absolute ethanol, mixed with 10 ml liquid scintillation cocktail (containing POPOP 0.25 g, PPO 8.25 g. Triton X-100 500 ml, in toluene 1,000 ml) and counted in a liquid scintillation counter.

Extraction yield was determined by adding 5 ng (1.25×10^4 dpm) of ^{3}H-melatonin into 1 ml brain homogenate (50 mg/ml) from non-operated animal. The homogenate was then processed as described. ^{3}H-Melatonin recovered was then calculated as percentage of the originally added ^{3}H-melatonin. By this procedure, the extraction yield was 76.5%; all values obtained were, therefore, corrected to 100% by this number.

In serum, in addition to ^{3}H-melatonin determination, total radioactivity was also determined by adding 10 ml of the cocktail into 50 ul serum and counted as such.

Data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. The Student's t test was also used when only two groups of data were compared.

Results

The rats receiving ^{3}H-melatonin in the morning (AM group) showed higher levels of melatonin radioactivity in the hypothalamus, compared to that in the cerebrum, the cerebellum, and the midbrain (Figure 1). Statistical analysis revealed significant difference between the hypothalamic uptake and the cerebral uptake, and between the hypothalamic uptake and the midbrain uptake. In contrast to the AM uptake, the rats receiving ^{3}H-melatonin in the afternoon (PM group) showed, statistically, no difference in ^{3}H-melatonin uptake among the various brain regions (Figure 2). The values In the cerebrum, the cerebellum, and the midbrain of the PM group were all significantly higher than those in the respective tissue of the AM group (Table 1). However, there is no significant difference in the hypothalamic ^{3}H-melatonin content between the AM and the PM groups

Because of their small size, the uptake in the anterior pituitary and pineal was calculated as dpm/5 mg and dpm/1 mg, and compared to the uptake in 5 mg and 1 mg brain tissue, respectively. This would reduce possible gross error which actually occurs when data of the small-sized tissue were compared to those of the much larger-sized tissue by using a multiplying factor. On this comparison basis, the anterior pituitary and the pineal uptake is always non-significantly lower than that of the 5 mg and 1 mg brain tissue, respectively. This lower uptake was found in both the AM and PM groups (Figure 3). Comparing the uptake between the AM-PM pairs of the anterior pituitary and the pineal, the PM values are always non-significantly higher than the AM values.

When total radioactivity in sera between the AM and PM groups were compared, no significant difference was observed. But when the serum ^{3}H-melatonin was compared, the PM value was significantly higher than the AM value (Table 2). A large fraction of serum radioactivity in the AM group was, therefore, not ^{3}H-melatonin.

Discussion

The higher ^{3}H-melatonin uptake in the hypothalamic tissue of the AM rats, compared to that in other brain regions, is in agreement with others' findings that melatonin action is in the hypothalamus.[2, 5, 7, 19, 20] However, the uptake pattern of the PM rats showing no preferential binding to the hypothalamus was quite unexpected. In fact, the hypothalamic uptake remained unchanged, whereas the uptake in other areas rose to the same levels as in the hypothalamus. Considering the high sensitivity of exogenous melatonin during late light phase, these data offer at least two possible mechanisms for this peculiar phenomenon. Firstly, melatonin might produce its

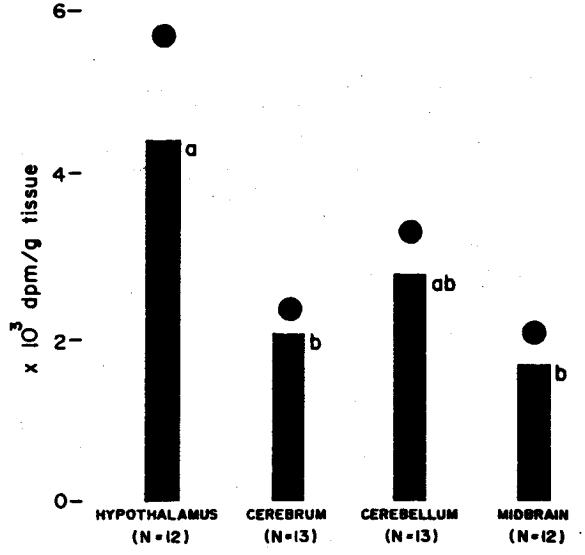

Figure 1. ^{3}H-Melatonin uptake in various brain regions after ^{3}H-melatonin infusion during the AM period. Each bar represents $\bar{X} \pm SE$. Different superscripts at each bar indicate statistical significance.

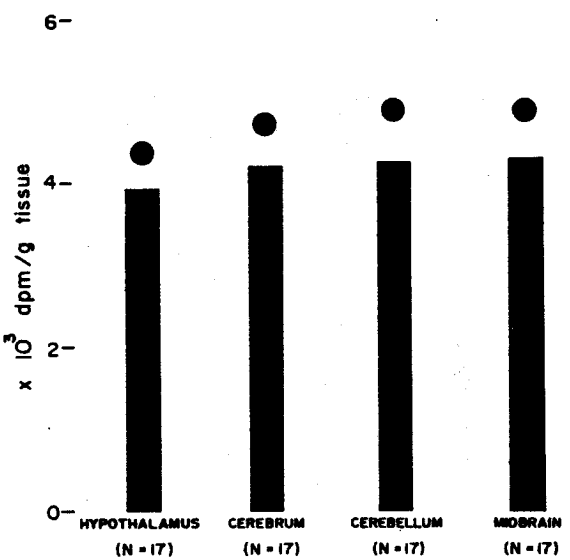

Figure 2. ^{3}H-Melatonin uptake in various brain regions after ^{3}H-melatonin infusion during the PM period. Each bar represents $\bar{X} \pm$ SE.

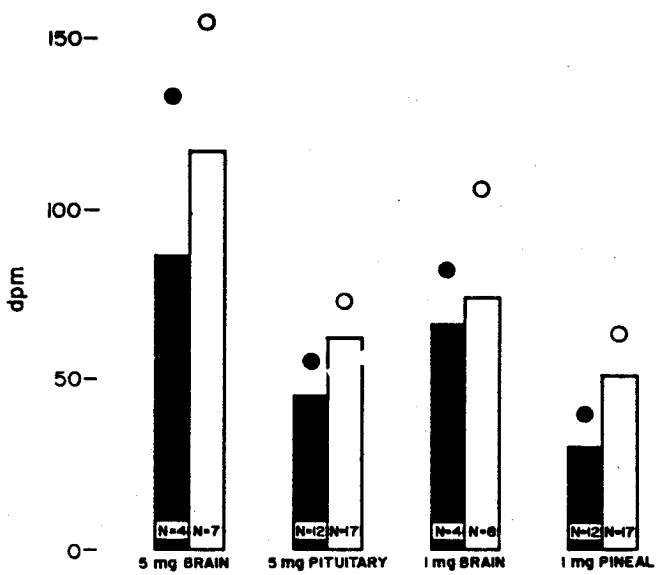

Figure 3. ^{3}H-melatonin uptake in the 5 mg anterior pituitary and the 1 mg pineal after the AM (■) and the PM (□) ^{3}H-melatonin infusion, compared to that of 5 mg and 1 mg brain tissue, respectively. Each bar represents $\bar{X} \pm$ SE.

TABLE 1. COMPARISON OF MELATONIN RADIOACTIVITY BETWEEN THE AM INFUSION (AM) AND THE PM INFUSION (PM) GROUPS IN VARIOUS REGIONS OF BRAIN.

	AM	PM	P
HYPOTHALAMUS	218 ± 58(12)*	194 ± 22(17)	NS
CEREBRUM	98 ± 13(13)	210 ± 26(17)	0.05
CEREBELLUM	136 ± 24(13)	211 ± 31(17)	0.05
MIDBRAIN	81 ± 16(12)	214 ± 30(17)	0.01

*$\bar{X}$ ± SE. (N) dpm / 50 mg tissue
NS, non - signifcant

TABLE 2. Comparison of total and melatonin radioactivity in serum between the AM infusion (AM) and the PM infusion (PM) groups.

	AM	PM	P
TOTAL RADIOACTIVITY	1,860 ± 274(14)*	1,655 ± 19(17)	NS
MELATONIN RADIOACTIVITY	733 ± 108(14)	1,183 ± 14(17)	0.01

*$\bar{X}$ ± SE. (N) dpm / 0.5 ml serum
NS, non - significant

antigonadotropic effect by interacting within the cerebrum, the cerebellum, the midbrain and possibly other brain regions. This mechanism however, does not have much support from previous work. Secondly, the hypothalamus might be a primary site of melatonin action but it also requires interactions between melatonin and other brain areas as a "permission" for its full antigonadotropic effect. This permissive effect could be mediated by some direct or indirect neuronal circuit between the hypothalamus and other areas. Direct neuronal pathways between the hypothalamus and midbrain are well established; melatonin administration resulted in increased serotonin level in the hypothalamus[21] accompanied by increased its binding to the hypothalamus, midbrain, and cerebral cortex[22]. Therefore, this second mechanism sounds possible and logical. To prove this, it must be further shown that a selective blockade to this permissive pathway(s) would abolish the effect of exogenous melatonin during late light phase.

Although the lower ^{3}H-melatonin uptake in the anterior pituitary and pineal compared to that of the brain tissue, does not support melatonin site of action in these structures, it does not rule out the possibility of melatonin action in these organs either. Melatonin action within the pituitary gland has been occasionally reported, especially that by Martin[23] who demonstrated melatonin binding in pituitary cell culture of neonatal hamsters.

The difference in serum ^{3}H-melatonin levels between the AM and the PM group despite of the comparable level in its total radioactivity is probably due to higher melatonin degradation in the morning. The fact that total radioactivity was of the same levels in both groups indicates that the same dose of radioactivity was given. And because they were from the same lot, difference in ^{3}H-melatonin percentage is very unlikely. Melatonin is transformed to 6-hydroxy melatonin, conjugated with glucuronic acid and sulfate salt in the liver, and then excreted by the kidney[24]. The metabolism occurs in liver microsome. It is possible that a large portion of ^{3}H-melatonin was bound to the brain tissue in the PM group and, thus, rendered free ^{3}H-melatonin less available to be degraded in the liver. Another possibility is that endogenous melatonin fluctuates in a day and, in general, is low during the day and high during the night. Anticipatory rise of serum melatonin during the first few hours before the onset of darkness was reported[25]. This means that the endogenous melatonin could be higher in the PM serum than in the AM serum. Endogenous melatonin can, enzymatically, compete with exogenous ^{3}H-melatonin for the degradation process. This would, therefore, result in less ^{3}H-melatonin degraded in the PM group and, therefore, higher serum ^{3}H-melatonin level. Both mechanisms, the increased melatonin binding in brain and the competition against endogenous melatonin, may be combined and accounted for the higher ^{3}H-melatonin in the afternoon serum.

References

1. Lerner, A.B., Case, J.D., Tabahshi, Y., Lee, T.H. and Mori, W. (1958) Isolation of melatonin, the pineal factor that lightens melanocytes. *J. Am. Chem. Soc.* **80**, 2587.
2. Reiter, R.J., (1980) The pineal gland and its hormones in the control of reproduction in mammals. *Endocrinol Rev.* **1**, 109
3. Johnston, P.G. and Zucker, I. (1980) Antigonadal effects of melatonin in white-footed mice *(Peromyscus leucopus). Biol. Reprod.* **23**, 1069.
4. Petterborg, L.J. and Reiter, R.J. (1980) Effect of photoperiod and melatonin on testicular development in the white-footed mouse, *Peromyscus leucopus.* *J. Reprod. Fert.* **60**, 209.
5. Glass, J.D. and Lynch G.R. (1981) Melatonin : Identification of sites of antigonadal action in mouse brain. *Science* **214**, 821.
6. Margolis, D.J. and Lynch, GR (1981) Effects of daily melatonin injections on female reproduction in the white-footed mouse, *Peromyscus leucopus. Gen. Comp. Endocrin.* **44**, 530.
7. Mieno, M., Yamashita, E.R., Ilmori, M. and Yanashita, K. (1978) An inhibitory effect of melatonin on the luteinizing hormone releasing activity of luteinizing hormone releasing hormore in immature male dogs. J. Endocrinol **78**, 283.
8. Macphee, A.A., Cole, F.E. and Rice BF (1975) The effect of melatonin on steroidogenesis by the human ovary in vitro. *J.Clin. Endocrinol. Metab.* **40**, 688.
9. Lerner, A.B. and Nordlund, J.J. (1977) The effects of oral melatonin on skin color and on the release of pituitary hormones. *J. Clin. Endocrinol. Metab.* **45**, 768.
10. Tamarkin, L., Westrom, W.K., Hamill, A.I. and Goldman BD (1976) Effect of melatonin on the reproductive systems of male and female hamsters : a diurnal rhythm in sensitivity to melatonin. *Endocrinology* **99**, 1534.
11. Reiter R.J. Rudeen, P.K. Sackman, J.W., Vaughan, M.K., Johnson, L.Y. and Little J.C. (1977) Subcutaneous melatonin implants inhibit reproductive atrophy in male hamsters induced by daily melatonin injections. *Endocrinol. Res. Commun.* **4**, 35.
12. Goldman, B., Hall, V., Hollister, C., Roychoudhury, P., Tamarkin, L. and Westrom, W. (1979) Effects of melatonin on the reproductive system in intact and pinealectomized male hamsters maintained under various photoperiods. *Endocrinology* **104**, 82.
13. Trakulrungsi, C., Reiter, R.J., Trakulrungsi, W.K., Vaughan, M.K. and Waring-Ellis, P.J. (1979) Interaction of daily injections and subcutaneous reserviors of melatonin on the reproductive physiology of female Syrian hamster. *Acta Endocrinol.* **91**, 59.
14. Richardson, B.A., Vaughan, M.K., Brainard, G.C., Huerter, J.J., Santos, R. and Reiter, R.J. (1981) Influence of morning melatonin injections on the antigonadotrophic efficts of afternoon melatonin administration in male and female hamsters. *Neuroendocrinology* **33**, 112.
15. Chen, H.J. (1981). Melatonin : Failure of pharmacological doses to induce testicular atrophy in the male golden hamster. *Life Sci.* **28**, 767.
16. Blask, D.E., Nodelman, J.L. (1979) Antigonadotrophic and prolactin-inhibitory effects of melatonin in anosmic male rats. *Neuroendocrinology* **29**, 406.
17. Szentagothai, B., Flerko, B., Mess, B. and Halasz, B. (1968) Hypothalamic *Control of the Anterior Pituitary (3 rd ed).* Akademiai Kiado, Badapest.
18. Klein, D.C. and Notides, A. (1969) Thin-layer chromatographic separation of pineal gland derivatives of serotonin - 14 *C. Anal. Biochem.* **31**, 480.
19. Niles, L.P., Wong, Y.W., Mishra R.K. and Brown G.M. (1979) Melatonin receptors in brain, *Eur. J. Pharm.* **55**, 219.

20. Cardinali, D.P., Vacas M.I. and Boyer, E.E. (1979) Specific binding of melatonin in bovine brain. *Endocrinology* **105**, 437.
21. Anton-Tay, F., Chou, C., Anton, S. and Wurtman R.J. (1968) Brain serotonin concentration elevation following intraperitoneal administration of melatonin. *Science* **162**, 227.
22. Anton-Tay, F. and Wurtman, R.J. (1969) Regional uptake of ^{3}H-Melatonin from blood or CSF by rat brain. *Nature* **221**, 475.
23. Martin, J.E., Engel, J.N. and Klein, D.C. (1977) Inhibition of the *in vitro* pituitary response to luteinizing hormone releasing by melatonin, serotonin, and 5-methoxytryptamine. *Endocrinology* **100**, 675. (1961).
24. Kopin, I.J.,·Pare, M.B., Axelrod, J. and Weissbach, H. (1961) The fate of melatonin in animals. *J. Biol. Chem.* **236**, 3072.
25. Withyachumnarnkul, B. and Knigge, K.M. (1980) Melatonin concentration in cerebrospinal fluid, peripheral plasma, and plasma of the confluens sinuum. *Neuroendocrinology* **30**, 382.

บทคัดย่อ

เมลาโทนิน (melatonin) เป็นฮอร์โมนตัวหนึ่งของต่อมไพเนียล (pineal gland) ซึ่งทำหน้าที่ลดประสิทธิภาพในการสืบพันธุ์ของสัตว์เลี้ยงลูกด้วยนมบางชนิด โดยเฉพาะพวกที่สืบพันธุ์เป็นฤดู เช่น หนู กระรอก การฉีดเมลาโทนินที่จะให้ได้ผลดังกล่าว ต้องฉีดในช่วงเวลาบ่าย ถ้าฉีดเวลาอื่นจะไม่ได้ผล ยังไม่มีผู้ใดรายงานการค้นคว้าเหตุผลของปรากฏการณ์อันนี้ ผู้ทำการทดลองจึงได้ตั้งทฤษฎีว่า ความแตกต่างในผลของการให้เมลาโทนิน ในช่วงเช้า และบ่าย อาจเกิดจากความแตกต่างในการจับเมลาโทนิน ของสมองในช่วงเช้าและช่วงบ่าย โดยเฉพาะอย่างยิ่งบริเวณ ไฮโปทาลามัส (hypothalamus) เพราะเป็นที่ทราบกันค่อนข้างแน่นอนว่า เมลาโทนินออกฤทธิ์ที่บริเวณไฮโปทาลามัส จึงได้ทำการทดลองฉีดเมลาโทนินชนิดกัมมันตรังสีเข้าสู่สมองโดยตรง โดยเข้าทาง common carotid artery ด้านขวา และวัดปริมาณของสารนี้โดยแยกวิเคราะห์ทางชีวเคมีออกจากส่วนต่าง ๆ ของสมอง เปรียบเทียบระหว่างหนูที่ได้รับฮอร์โมนในช่วงเช้า และที่ได้รับในช่วงบ่าย ในช่วงเช้าปริมาณเมลาโทนินในไฮโปทาลามัส สูงกว่าบริเวณอื่นของสมองเป็นนัยสำคัญทางสถิติ แต่ในช่วงบ่ายปริมาณของสารนี้กลับเท่ากันหมด โดยที่ปริมาณเมลาโทนินในสมองส่วนอื่นได้แก่ cerebrum, cerebellum, และ midbrain มีระดับสูงกว่าระดับของมันเองในช่วงเช้า แต่ระดับของไฮโปทาลามัสในช่วงเช้าและช่วงบ่ายเท่ากัน ปริมาณของเมลาโทนินในต่อม pituitary และต่อม pineal ต่ำกว่าปริมาณที่พบในสมองโดยเฉลี่ย นอกจากนั้นปริมาณของเมลาโทนินที่พบในน้ำเลือดในช่วงเช้า ก็ต่ำกว่าที่พบในช่วงบ่ายเป็นนัยสำคัญทางสถิติอีกด้วย จากข้อมูลที่ได้ทั้งหมดนี้ส่อให้เห็นว่า เมลาโทนินออกฤทธิ์ที่ไฮโปทาลามัส และการออกฤทธิ์นี้จะได้ผลสมบูรณ์ก็ต้องอาศัยปฏิกิริยาระหว่างเมลาโทนิน และสมองส่วนอื่น ๆ เป็นตัวช่วยด้วย ปฏิกิริยานี้จะเกิดขึ้นในช่วงบ่ายมากกว่าช่วงเช้า การที่เมลาโทนินถูกจับตัวไว้ในสมองมากในช่วงบ่าย ก็จะทำให้เมลาโทนินถูกทำลายในตับน้อยลง.