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NEUROPHARMACOLOGICAL EVALUATION OF HORMONES IN MICE AND INSILICO ANALYSIS OF MELATONIN AND SOMATOSTATIN RECEPTORS

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Abstract

Spatial learning and memory consolidation are important aspects of human cognition. The test for motor co-ordination in mice is to characterize the motor phenotype of animals and also in relation to human beings. It is among the most fundamental aspects of everyday life as each physiological process that must be performed in order to achieve movement. In this study, depressive effect and effect on learning and memory of the two hormones, melatonin and somatostatin on animals was evaluated using the loco-motor activity test, rotarod and rectangular maze. The effect of Melatonin and Octreotide acetate alone and in combination with each other were estimated in Swiss albino mice. Melatonin was administered dose dependently and time dependently. Octreotide acetate was administered dose dependently. We compared the combined effect of these hormones for motor co-ordination, loco-motor activity and for the memory and learning behavior of animals. As per the present study, we found significant changes in the motor co-ordination, learning, and memory and depressive effect in animals and there was potentiated depression and attenuated learning and memory when the hormones were used in combination. In-silco analysis was conducted using Patchdock software and molecular docking of the ligands to their respective receptors conducted.

Key Words: Melatonin; Octreotide; Locomotor; Memory; Depression.

Introduction

Melatonin (MLT) is a naturally occurring hormone released from the pineal gland and also present in the gastrointestinal tract [1] found in most animals, including humans, and some other living organisms, including algae. Cloning studies have revealed two recombinant mammalian melatonin receptors - Mel1a and Mel1b, now termed MT1 and MT2 [2]. MT1 receptors signal via inhibitory G proteins ($G\alpha_i$ and $G\alpha_o$) leading to adenyl cyclase inhibition and possibly inositol phosphate stimulation in recombinant systems [3, 4, 5]. Melatonin-induced cytosolic calcium mobilization via PTX-insensitive G proteins was confirmed in primary cultures of ovine pars tuberalis cells endogenously expressing Mel 1a receptors. Recombinant Mel 1a receptors were shown to activate potassium ion channels, to potentiate PGF2a-promoted stimulation of phospholipase C, and to modulate protein kinase C (PKC) and phospholipase A_2 via Gbg-subunits liberated during $G_{i/o}$ protein activation [6]. Circulating levels vary in a daily cycle, and the role of endogenous melatonin in circadian rhythm disturbances and sleep disorders is well established. Melatonin receptors appear to be important in mechanisms of learning and memory in mice, and melatonin can alter electrophysiological processes associated with memory, such as long-term potentiation (LTP) [7]. The first published evidence that melatonin may be useful in Alzheimer disease was the demonstration that this neurohormone prevents neuronal death caused by exposure to the amyloid beta protein, a neurotoxic substance that accumulates in the brains of patients with the disorder [8]. Melatonin also inhibits the aggregation of the amyloid beta protein into neurotoxic micro aggregates which seems to underlie the neurotoxicity of this protein, causing death of neurons and formation of neurofibrillary tangles, the other neuropath logical landmark of Alzheimer disease. Melatonin has been shown to prevent the hyperphosphorylation of the tau protein in rats. On the other hand, studies in rats suggested that melatonin may be effective for treating Alzheimer's disease [8, 9].

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On the other hand, Octreotide acetate (OA) is a long-acting octapeptide with pharmacologic actions mimicking those of the natural hormone somatostatin. It is an even more potent inhibitor of growth hormone, glucagon, and insulin than somatostatin. Octreotide and melatonin have been tried in rats for spinal cord injury in which melatonin was found to be superior to octreotide with respect to the prevention of congestion, edema, axonal degeneration and necrosis [10].

Receptors of both these hormones come under the classification of G-protein Coupled Receptors. Melatonin receptors belong to class 1 or family A of GPCR. GPCR are involved in wide variety of physiological process. Their functions are extremely diverse as they regulate many physiological process related to neurological and neurodegenerative functions and metabolic control. The receptors in the mammalian brain bind several different neurotransmitters, including serotonin, dopamine GABA, opioid peptides, vasopressin, melatonin and glutamate. Melatonin may also affect clock gene protein levels in the adrenal cortex and influence adrenal functions [11]. Via its action upon two receptor subtypes called Mel1a (MT1) and Mel1b (MT2) receptor, melatonin may provide a feedback loop to the circadian clock in the SCN [11].

Keeping these considerations in mind, the primary objective of this study was to evaluate the effect of Melatonin on the motor co-ordination, loco-motor activity and memory and learning of animals. The secondary objectives were to evaluate the effect of Octreotide acetate on the motor co-ordination, loco-motor activity and memory and learning of animals and to evaluate the combined effect of Melatonin and Octreotide acetate with each other for physiological effects such as motor co-ordination, loco-motor activity and memory and learning behavior of animals. The novelty of this study is the usage of the combination of the above mentioned drugs. The final objective of the present study was to evaluate model of structure of receptors which are not available in the Protein Data Bank (PDB) and to dock the structures of the receptors with the ligands (hormones).

Materials and Methods

Experiments were performed on female Swiss albino mice weighing 25-35g. They were obtained from Institutional Animal Centre of Christian Medical College, Vellore, India. Animals were divided into groups of 6-8 and kept in separate polypropylene plastic cages under hygienic conditions, lined with paddy-husk bedding. These animals were housed in a colony room under controlled temperature (25±1ºc), relative humidity of (60±2%) and were exposed to a 12-h dark cycle, with food and water available *ad libitum*. All experiments were conducted during the light phase between 9.00 a.m. and 4.00 p.m. The experimental protocol was approved by the Institutional Review Board (IRB) and care of animals was taken as per guidelines of CPCSEA, Department of Animal Welfare, Government of India.

Drugs

Melatonin Tablets were obtained from Aristo Pharmaceuticals Pvt. Ltd., Nani Daman, Daman (U.T) and Octreotide acetate (OTIDE) injection was obtained from United Biotech (P) Ltd., Bagbania, Solan, H.P., India.

Methods

(A) Rota-rod Test (Motor Co-ordination in mice): Rota-rod test is often used with the apparent assumption by the experimenters that it is a straightforward and simple assay of coordination. A Rota-rod tread mill device (Inco, India) was used for the evaluation of the effect of drugs on the motor coordination at speed of 20 rotations per minute .Thirty minutes after administration of melatonin (180 µg/kg, 360 µg/kg, 540 µg/kg) i.p and Octreotide acetate (0.1 µg/kg, 0.2 µg/kg, 0.3 µg/kg) i.p. Each mouse was placed on the rotating rod for 5 min (300 secs). The endurance time for each mouse on the Rota-rod was noted.

(B) Rectangular Maze Test: Maze studies helped uncover general principles about learning that can be applied to many species, including humans. Mazes are used to determine whether different treatments or conditions affect learning and memory in rats. Working concentration of the drugs i.e. Melatonin (540 µg/kg), and Octreotide acetate (0.2 µg/kg) was injected intraperitoneally and time taken to reach the chamber B of the rectangular maze was recorded.

(C) Loco-motor Activity Test: The loco-motor activity of albino mice weighing between 25- 35 g were evaluated in an open field loco-motor box made of wood with dimensions (24×24×5) inch and comprising of 16 squares. Loco-motor activity was estimated visually counting the no. of squares the animals crossed in 6 mins. Mice were injected with one of the test drugs or its vehicle and were place in holding cage for 30 minutes for Melatonin and Octreotide acetate before testing. Each animal was tested once for the loco-motor activity [12].

Protocol of Experiments:

Swiss albino mice were divided into four groups. Six in each group were employed in dose dependent and time dependent studies. The control and the test groups

of mice (n=6) were injected intraperitoneally (i.p.) either with saline (0.9% NaCl) or the drugs of graded doses respectively.

Group 1: Served as control and was treated with normal saline. Equivolume of 0.9% NaCl was administered i.p.

Group 2: Melatonin (180µg/kg, 360 µg/kg, 540 µg/kg) was injected i.p. to eighteen mice for the study of dose dependent effect. Melatonin (540 µg/kg) was injected (I.P) to six mice to find the alteration of motor co-ordination on rota-rod test was recorded after 15, 20, 30 minutes of time and time dependent effect of melatonin was evaluated.

Group 3: Octreotide acetate (0.1 µg/kg, 0.2 µg/kg, 0.3 µg/kg) was injected (I.P) to eighteen mice for the study of dose dependent effect.

Group 4: Combination of Drugs: Octreotide acetate (0.2 µg/kg) + Melatonin (540 µg/kg) was injected (I.P) to six mice. After 30 minutes of Octreotide acetate injection, melatonin was administered and the fall off time of mice was recorded.

***In-Silico* Analysis:** Certain *in-silico* studies were done to check for the binding affinity of the receptors with the ligands. The 3D structures of the ligands were generated using CORINA software by obtaining the SMILES string from PUBCHEM. Then the receptors protein sequence was taken from SWISSPROT for the modeling using 3D JIGSAW and SWISSMODEL software. The docking of the 2 structures was done using PATCHDOCK.

Statistical Analysis: For statistical evaluation of results and significance testing of group differences, the nonparametric Mann-Whitney U-test and Wilcoxon W test was performed. Results were considered to be of statistical significance at $P \leq 0.05$ (95% confidence interval). Data are presented as Means ± S.E.M.

Results

Results have been evaluated as percentage change in the various parameters of mice by comparison of pre-drug values with post-drug values.

A. ROTA-ROD ANALYSIS

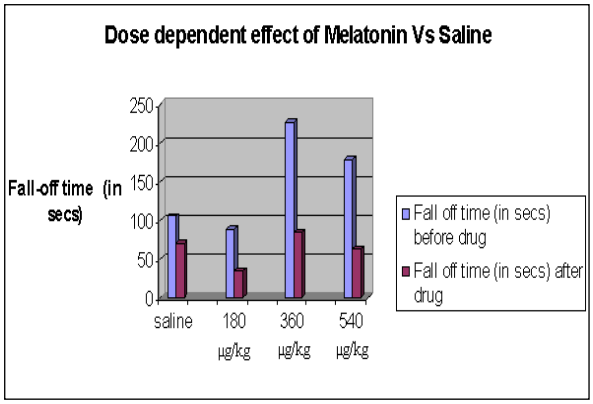


Figure 1. Dose dependent effect of Melatonin compared with Saline (0.9% NaCl)

Table 1. Time dependent study of Melatonin

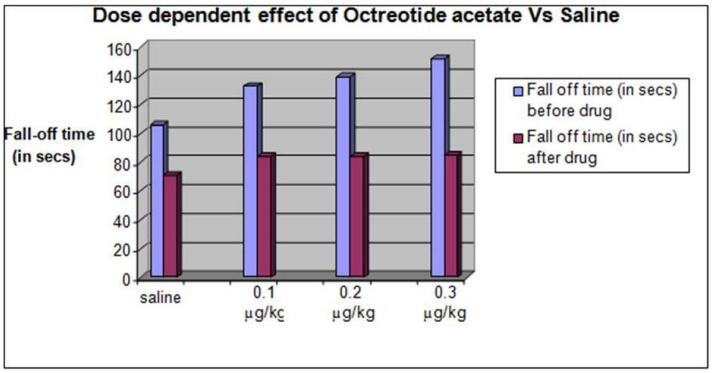
Pretreatment time Melatonin	Fall off time (in secs)		Percent decrease
	Before drug	After drug	
15 mins	101.6	46	54.72%
20 mins	162.9	86	47.20%
30 mins	98.6	73.1	25.86%

(a) Effect of Melatonin on Rota-rod Test: The Dose dependent effect of the hormone has a highest percent decrease in fall-off time for the working concentration of 540µg/kg and it was noted to be 64.66% (figure 1). The time dependent effect was also depicted in table 1 and it is reported to be decreased by 54.72% for 15 mins of pre-treatment time.

(b) Effect of Octreotide acetate in Rota-rod Test: With increase in dosage, the fall-off time has decreased significantly for 0.3µg/kg and noted to be 44.01%. From the figure 2, it is clearly seen that the fall-off time has decreased apparently among the experimental group and increased when compared with saline.

(c) Effect of Combination of Hormones on Rota-rod Test: Octreotide acetate and Melatonin combination had a greater percent increase of 59.73% on the motor co-ordination of the Rotarod activity test over the other combinations.

Figure 2. Dose dependent effect of Octreotide compared with Saline (0.9%NaCl)



(B) Rectangular Maze

Table 2. Results of Rectangular maze

S. NO	DRUG	DOSAGE	MEAN ± SEM
1	Saline	0.2 ml	55.33 ± 3.938
2	Melatonin	540 µg/kg	70.00 ± 10.739 ; P< 05
3	Octreotide acetate	0.2 µg/kg	71.33 ± 23.489 ; NS
4	Melatonin + Octreotide acetate	540 µg/kg + 0.2 µg/kg	68.00 ± 8.181 ; NS

NS= Not significant, P>.05

(C) Locomotor activity

Table 3. Results of Loco-motor activity

S. NO	DRUG	DOSAGE IN ML	MEAN ± SEM
1	Saline	0.2 ml	110.17 ± 28.579 ;
2	Melatonin	540 µg/kg	97.17 ± 2.892 ; NS NS
3	Octreotide acetate	0.2 µg/kg	95.83 ± 26.872 ; NS
4	Melatonin + Octreotide acetate	540 µg/kg + 0.2 µg/kg	50.33 ± 8.106 ; P<.05

NS= Not significant, P >.05

(a) Effect of Melatonin on loco-motor activity and memory and learning behavior of animals: The time taken to reach chamber B has significantly increased from 55.33 ± 3.938 sec to 70 ± 10.739 sec (Table 2) when compared with saline and the memory and learning process has been probably lowered down due to the sleeping effect caused by the drug melatonin. It is also noted that the loco-motor activity has been has decreased (Table 3) using the drug on comparison with saline from 110.17 ± 28.579 sec to 97.17 ± 2.892 sec moves in 6 min. The statistical significance of values obtained is shown in Table 2 and 3

(b) Effect of Octreotide acetate on loco-motor activity and learning behavior of animals: As reported in Table 2, time taken to reach chamber B has a significant increase over saline from 55.33 ± 3.938 sec to 71.33 ± 23.489 sec. The memory and learning activity is said to be apparently decreased with increase in time. It is seen that there is an apparent decrease in the no. of moves of the animal from 110.17 ± 28.579 sec to 95.83 ± 26.872 sec (Table 3). The statistical significance of values obtained is shown in Table 2 and 3

(c) Effect of combination of hormones on loco-motor activity and learning behavior of animals: The time taken to reach chamber B for the animals administered with the combination of hormones was found to be increasing among the group in comparison with saline as reported in Table 2 for the memory and learning activity. The loco-motor activity has also seemed to vary among the group either decrease or increase in the no. of moves. It showed significant decrease in the administration of Octreotide acetate + Melatonin combination when compared with saline (Table 3).

***In-silico* analysis:** The score for the docked structures of the receptors with their ligands (hormones)

using the Patch-dock software was calculated. In the same way, Melatonin MT1 receptor binds the ligand Melatonin with a maximum score of 4598. Somatostatin receptor subtype 1 (SSTR1) has the highest score of 7940 on binding Octreotide acetate, a potent analog of the growth hormone inhibitor Somatostatin. Another analog of Somatostatin named Lanreotide, has the maximum score for docking with the Somatostatin receptor subtype 3 (SSTR3) of 8438.

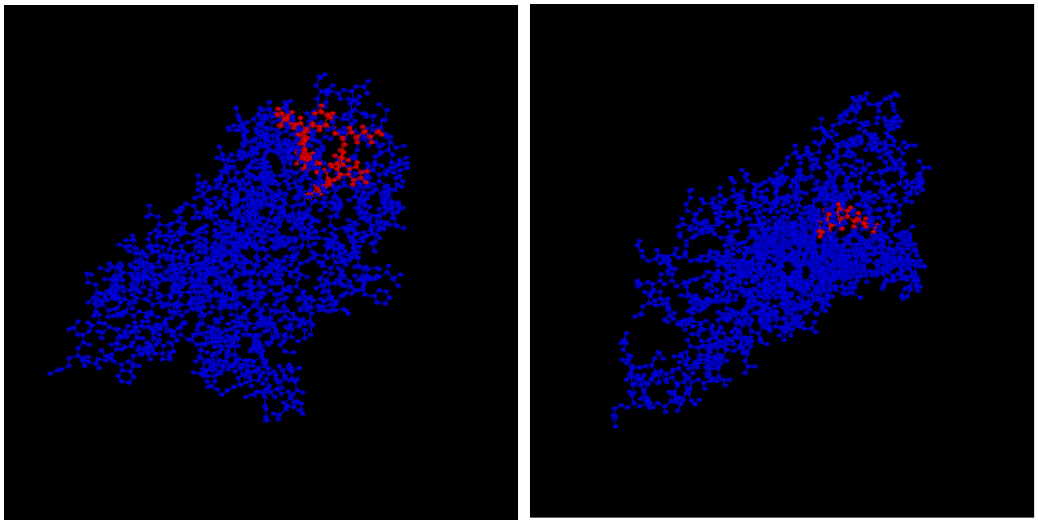


Fig 3. (A) Docked structure of somatostatin receptor with octreotide (B) Docked structure of melatonin receptor with melatonin

Discussion

The present study was conducted to ascertain the effect of the two hormones on neurobehavioral parameters. The results of the present study suggest that the combination of hormones has increased effect on the neurophysiological processes and increase the stability on the motor co-ordination and potentiated the depressive effects with both melatonin and octreotide the fall of time for rotarod was substantially decreased.These effects suggest their significant effects on the central nervous system. These results are in accordance to Shaji and Kulkarni [13] melatonin has been shown to have central nervous system depressant activities. There was marginal decrease in locomotor activity produced by melatonin and learning and memory was also reasonable reduced. These effects may be due to the prominent central nervous system effects of melatonin because melatonin receptors are present in the central nervous system [14]. MLT exert its physiological activities through the activation of series of G protein coupled receptors, two of which (MT1 and MT2) have been found in mammals, including humans and subsequently cloned. MT1 and MT2 receptors are expressed both centrally (SCN, cortex) and peripherally (kidney, adipocytes, retina, blood vessels). There is evidence that MT1 receptors might be implicated in sleep promoting effects of MLT.

These hormones bind to their respective GPCRs and stimulate the production of cGMP on the receptor region, which acts as a secondary messenger in the cascade thereby activating the G-proteins. The activation results in a cascade of signal transduction events such as stimulation or inhibition of adenylyl cyclase and activation of phospholipase C. These G-proteins in turn help in the production of nitric oxide (by endothelial NOS) which brings about vasodilation of the blood vessels and thus increasing the blood flow and relieving the depression and increasing the heart rate of the organism. They also stimulate the microtubules and change the physiological effect of the animal. On the other hand, when nitric oxide is produced by the inducible nitric oxide synthase (iNOS) by the cardiovascular system which is calcium independent, reverts the effect and induces depression and impairs motor co-ordination in animals. In Mt1-CHO cells, short melatonin exposure inhibited the cAMP-dependent signal transduction pathway, whereas after withdrawal from physiological melatonin exposure, a super sensitization of forskolin-induced cAMP formation, PKA activation, and phosphorylation of CREB were observed. Super sensitization of cAMP-dependent signaling cascades after agonist exposure has been shown for other inhibitory G protein-coupled receptors as well. The results of von Gall et al. [15] indicating that melatonin MT1-receptor knockout in the pars tuberalis (PT) has a blunting effect on the expression of PER1 protein suggest that the melatonin MT1-receptor has a major rhythm-supporting role in this particular tissue, but obviously not, or not to the same extent, in pancreas and liver.

Melatonin GPCR signaling is generally a negative regulator of cell function via reduction of intracellular second messengers such as cAMP, Ca2+, and cGMP via Gαi/o subunits. The MTR 1/2 receptors activate G-protein alphaq (Gαq) subunits that stimulate phospholipase C, leading to DAG and IP3 actions. Melatonin regulates cell processes via nuclear signaling via transcription factors, RZR/ROR (RZR alpha and beta). Genes regulated by RZR/ROR include 5-lipoxygenase, p21WAF1/C1P1, apolipoprotein A-1, N-myc and Purkinje cell protein 2 [16].

On the other hand , Octreotide also produced a similar profile on locomotor activity and learning and memory process. When given together both melatonin and octreotide produced a synergistic effect on locomotor activity, learning and memory process. These actions of octreotide a surrogate of somatostatin may be attributed to the prominent CNS expression of somatostatin receptors and its effects on learning memory process [17]. The ligand binding domain for SST ligands is made up of residues in TMs111-V11 with a potential contribution by the second extracellular loop. SSTRs are widely expressed in many tissues including the central nervous system, frequently as multiple subtypes that coexist in the same cell. The five receptors share common signaling pathways such as the inhibition of adenyl cyclase, activation of phosphotyrosine phosphatase (PTP), and modulation of mitogen-activated protein kinase (MAPK) through G-protein-dependent mechanisms. Some of the subtypes are also coupled to inward rectifying K^+ channels (SSTR2, 3, 4, 5), to voltage-dependent Ca^{2+} channels (SSTR1, 2), a Na^+/H^+ exchanger (SSTR1), AMPA/ kainate glutamate channels (SSTR1, 2), phospholipase C (SSTR2, 5), and phospholipase A_2 (SSTR4) [18]. SSTRs block cell secretion by inhibiting intracellular cAMP and Ca2+ and by a receptor-linked distal effect on exocytosis. Some of the effects of somatostatin may attributed to its interaction with other neurotransmitters. Somatostatin receptors and dopamine receptors are co localized in neuronal subgroups, and somatostatin is involved in modulating dopamine-mediated control of motor activity [19]. Dopamine, somatostatin and angiotensin II receptors are negatively coupled to adenylate cyclase in anterior pituitary cells. Dopamine and somatostatin also directly modulate voltage-dependent calcium channels, perhaps through a direct coupling with potassium channels. It is also proved that the dopamine and somatostatin receptors appear coupled to various transduction mechanisms through pertussis-sensitive G proteins in anterior pituitary cells [20]. Since both the melatonin and somatostain are GPCRs its important to study their possible therapeutic actions. GPCRs have also been validated as therapeutic targets for a number of diseases based on the finding that naturally occurring mutations of the receptor can result in either a gain or loss of function of the receptor leading to an association with a disease state such as rhodopsin (retinitis pigmentosa), cholecystokinin-2 receptor (gastric carcinoid tumor), KSHV-GPCR (Kaposi's sarcoma), chemokine receptor US28 (atherosclerosis), thyroid-stimulating hormone receptor (hyperthyroidism), luteinizing hormone receptor (precocious male puberty), parathyroid hormone receptor (dwarfism, hypercalcemia, hypophosphatemia), and calcium-sensing receptor (hypocalcemia). Thus, the importance of these hormones in neurobehavioral actions can be speculated based on our results. The combination Melatonin and Octreotide acetate significantly increased the time to reach chamber B, thereby impairing the memory and learning behavior of animals (Table 2) and potentiated the depressive effect by decreasing the loco-motor activity of the animals (Table 3).

The in-silico studies clearly suggest the good affinity of Octreotide and melatonin to its respective receptors. Molecular docking results suggest good affinity of the respective hormones to their respective receptors.

Conclusion

The results suggest that the motor co-ordination of animals is stabilized to a higher extent on administration of combination of hormones at the working concentration in the Rota-rod test. The loco-motor activity test conducted to check the depressive effect of the hormones also produced significant results proving that the hormones play a major role in the central nervous system acting as a depressant. The spatial learning and memory considered as the social behavior of animals has significantly decreased among the experimental mice group where the combination of hormones is administered.

Melatonin has been shown to regulate intracellular concentrations of second messengers such as cAMP, calcium and Arachidonic acid. Under pathophysiological conditions such as inflammation, fever and immune reactions, however, melatonin, an endogenous negative regulator of $cPLA_2$ (cytosolic phosphplipase A_2), and its synthetic analogues may be explored as pharmaceutical agents to reduce abnormal activation of $cPLA_2$.

As reported that the somatostatin receptor subtypes 1–5 (sst1–sst5) exhibit different intracellular trafficking and endosomal sorting after agonist exposure, they are highly expressed in neuronal as well as in non-neuronal tissues and tumors. They have complex overlapping patterns of expression. This differential expression of

SSTRs in tumors and their ability to undergo internalization and desensitization paves the way for the clinical use of Somatostatin analogs in the near future.

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