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Effects of Melatonin on the Isolated Scale Melanophores of an Indian Major Carp *Labeo rohita* (Ham.)

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Abstract: Melatonin is mainly involved in the maintenance of the circadian sleep-wake cycle, seasonal rhythms, body colouration, etc. *In vitro* conditions of the melanophore-melanosomes system proves to be outstanding in delivering the considerable responses in a short instant of time. This study involves the application of melatonin as an agonist. The hormone melatonin in a wide dose-range of 4.31×10^{-16} M to 4.31×10^{-4} M has consistently induced aggregation (MSI 5.11 ± 0.43 to 1.22 ± 0.16) in a dose-dependent manner in the isolated scale melanophores of *Labeo rohita* (Ham.). The results indicate that the typical receptors for melatonin are also reliant upon the stimulation of several other receptors with the requirement of Ca^{2+} cations in illustrating their absolute cellular expression of the melanophore aggregation. A complete inhibition of the melatonin response was observed when the melanophores were pretreated with prazosin, luzindole and the antibiotic neomycin, while K185 was found to be weak but significant in eliciting such inhibitory actions. Hence there is a clear indication of the presence of all the three subtypes of melatonin receptors viz. MT_1, MT_2 and MT_3 in this fish melanophores which can serve as the best working model for pharmacological investigations.

Key words: Aggregation · Denervation · Melanophores · MSI · Physiological · Receptors

INTRODUCTION

The hormone melatonin was discovered in 1958 by Lerner and his coworkers from the bovine pineal gland [1] and was named melatonin because of its lightening effect on the skin colour of the frogs causing the aggregation of the melanin granules towards the nuclei of the melanophore cells present in the skin [2,3]. Studies of the effects of melatonin on the fish melanophores started with the fish goby, *Chasmichthys gulosus* [4].

Melatonin shows its photoperiodic, circadian effects and a number of chronobiological processes through the pharmacologically specific, high-affinity receptors [5-12]. Functional roles of melatonin have been discovered in man as well as in subhuman species [13]. Generally synthesized at the night hours, melatonin may act as a signal of darkness to the body [14-16].

Reiter and coworkers [17] successfully found that the tart cherries (*Prunus cerasus*) contain substantial amounts of melatonin, at the levels higher than that normally found in human blood. Montmorency cherries contain about 13.5 ng/g of melatonin [18]. The hormone melatonin does exist in bacteria, algae, fungi, plants, insects and almost all the vertebrates [19]. Melatonin is also responsible for translocating the pigments towards cell centre by interacting with the receptors present on the surface of the melanophores in the Atlantic cod [20]. The occurrence and physiological roles of melatonin in the plants have been brilliantly described in an article [21] and also other facts have been highlightened in the other [22].

The physiological colour changes in teleosts are controlled by the neuronal or hormonal or by both the mechanisms [23, 24]. MT shows its aggregatory effect on the melanophores of *Chasmichthys gulosus* [4], in *Scardinius erythrophthalmus* [25]. *Carrassius auratus* [26], in *Phoxinus phoxinus* [27] and in *Salmo gairdneri* [28, 29], in *Channa gachua* [30], etc. However, a weak or negative response has been observed in the melanophores of *Fundulus heteroclitus* [31], in *Potamotrygon reticulatus* and *Lepidosiren paradoxa* [32]. Anomalous findings do exist in literature such as: melatonin disperses the melanophores responsible for night colouration in *Nannostomus beckfordi anomalus* [33]; existence of "β-melatonin" receptors causing dispersion of the melanosomes in the melanophores of *N. beckfordi* [34], in *N. trifasciatus* [35] and in

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Pagrus pagrus [36]. Melatonin has led towards all the three effects of aggregation, dispersion and no responses in the fish *Cyprinus carpio communis* [37] and also it has shown aggregatory response in the band region melanophores of *Rasbora daniconius*, while in the dorsolateral region, there were both aggregatory as well as dispersal responses [38]. Effect of melatonin has been found to vary with the seasons in *Zacco temmincki* [39].

The fish melanophore sets an example of microtubule-actin based organelle transport system. Stimulation of the minus-end motors leads to an aggregation of granules at the cell center, while stimulation of the plus-end motors is predicted to drive the granules towards the cell margin causing the dispersion [40, 41]. The melanophores, belonging to a group of pigment cells called chromatophores, originate from the embryonic neural crest cells during the developmental stages [42]. The translocation of the melanosomes within the melanophores requires a threshold dose of the neurotransmitters and the hormones [43]. The precise involvement of the melatonin receptor subtypes have been explored in this study through the application of specific and unspecific melatonin receptor blockers.

MATERIALS AND METHODS

Experiments were carried on the young fishes of *Labeo rohita* (Ham.) of both the sexes. Fishes selected were of uniform size and body weight. All the fishes used were about 8 to 12 cm in length having 15 to 20 g of body weight. The fishes, brought from the local fish farms, were acclimatized in the laboratory conditions in the temperature range of 20°C to 30°C for at least 48 hours prior to the experiments. Nutritional care of the fishes which are herbivorous in general [44] were taken by providing them the commercial fish food twice or thrice a week. Constant care was taken to maintain the fishes in healthy conditions. Those fishes which had infections or showed slight sluggishness were immediately discarded. The cycloid scales of the fish were removed from the dorso-lateral region and were immediately immersed in the freshly prepared fish Ringer solution which is basic in nature having a pH in the range of 7.5 to 8.5, in transparent glass petridishes, as this solution provided the best result in comparison to other physiological salt solutions for the isolated scale melanophore preparations [45-47]. The scales were removed according to a specific protocol [48] and were equilibrated in fish Ringer for 15-30 min with frequent shakings. For drug treatment, 1 ml Ringer solution was removed from each petridish and the drug in equal volume was added so that the final volume of the solution in the petridish may not exceed 10 ml. Weighing of the salts and drugs were done through the pan balance (Dhona, calibrated for weighing from 0.1 mg upto 150 g) and also through the electronic digital balances for the quantities in μg.

Drugs were immediately dissolved in their solvents first [49] and then their serial dilution of the desired concentrations were made with the Ringer solution before use. In each petridish only one concentration of the drug was tested. Incubation time of the scales in the drug solution was 10 minutes. When antagonists were employed along with agonists, scales were first treated with antagonist for 10 min and then the agonist was added. The incubation time for the agonist was also 10 min. For observation under a compound microscope the control as well as treated scales, 3 to 4 in number, were placed on a glass slide with dermal side down with a little incubation medium and covered with a glass cover-slip.

Measurement of the actual size of the diameter of the melanophores under observation field was done by using an ocular micrometer (Erma, Japan), calibrated with 10 x 10 magnification of the microscope [50]. The maximum vertical and horizontal diameters of the five melanophores on a scale were measured and the Melanophore Size Index (M.S.I.) was recorded as:

(Vertical Diameter x Horizontal Diameter)/100:

The standard error (±) of the mean values of M.S.I. and the level of significance, wherever necessary, was calculated using the standard methods of statistical analysis [51]. Based on these calculations, line graphs and histograms were plotted. In the present study only dermal melanophores were taken under observation. The experiments were carried out at the room temperature ranging between 20 °C to 30°C. Experiments were avoided in the extreme cold, i.e. when the mercury dipped below 15°C and also in the hot days of above 35 °C.

Drugs Used:

- K185 = N-Butanoyl 2-(5,6,7-trihydro-11-methoxybenzo[c]cyclohept[2,1-a]indol-13-yl)ethanamine (Sigma, USA)
- Luzindole = N-Acetyl-2-benzyltryptamine (Sigma, USA)
- Melatonin (Sigma, USA and Aristo pharma, Mandideep)
- Neomycin sulphate (Himedia, Mumbai)
- Prazosin HCl (Sigma, USA)

All the drug solutions were prepared freshly before use. The drugs were added in the petridishes by a tuberculine syringe. In the present study, the final concentration of the drugs in petridishes is expressed in molar concentration. Statistical analyses were performed using the't' test. The solvents and the solubility of the chemicals used were employed according to the available handbooks [49].

RESULTS

Isolated scale melanophores of the fish *Labeo rohita* (Ham.) were of typical branched star shaped structures, most of which were showing the dendritic processes as well. Both dermal as well as epidermal melanophores were seen in the scales. The scales showed a rich abundance of dermal melanophores, while the epidermal melanophores were few in number. Hence, our investigations in the present study have been made solely on the dermal melanophores. Melanophores of *L. rohita* under control are depicted in photomicrograph 1.

Melatonin (MT) in a wide dose-range of 4.31×10^{-16} M to 4.31×10^{-4} M has induced aggregation (MSI 5.11 ± 0.43 to 1.22 ± 0.16) in the isolated scale melanophores (photomicrographs 2 and 3) of the dorso-lateral region of the fish *Labeo rohita*.The aggregation of the fish melanophores clearly shows a dose-dependent relationship from 4.31×10^{-16} M to 4.31×10^{-4} M (Figs.1, 2 and 3). In all the experiments related with MT it was found that the aggregatory effect of MT was not prevailing much over the epidermal melanophores which were under the static state of either dispersion, i.e. their MSI much larger than that of the dermal melanophores of the scales taken out after incubation in the Ringer medium, or they were already in such an aggregated state that MT's action was unable to make them further aggregated. Therefore, it is concluded that the effect of MT on epidermal melanophores of this fish were greatly variable, inconsistent and mostly inconclusive.

Experiments conducted with luzindole (1.71×10^{-5} M) which is a non-selective MT receptor antagonist, showed a profound blocking effect of MT in all of its concentrations employed except one at the lower concentration of 4.31×10^{-16} M (Fig. 1). Most of the inhibitory effects by luzindole were significant ($p < 0.01$). Blocking effect upto 143.93% has been recorded.

Another MT receptor (type II, i.e. MT_2) antagonist, N-Butanoyl 2-(5,6,7-trihydro-11-methoxybenzo [c]cyclohept[2,1-a]indol-13-yl)ethanamine, i.e. K185 (2.66×10^{-7} M), employed showed the inhibitions of MT at the three stages viz. 4.31×10^{-13} M, 4.31×10^{-11} M to 4.31×10^{-8} M and 4.31×10^{-6} M to 4.31×10^{-4} M (Fig. 2). Blocking was seen as significant ($p < 0.01$) with a maximum blockage of 79.6%.

To further analyze the effects with other antagonists we selected twelve to thirteen concentrations of the agonist (MT) and one concentration of each antagonist. It was observed that each of these antagonists has induced it's *per se* effect of either aggregation or dispersion in varying degrees or even no effect at all. One of the most interesting observations made in our experiments was that the α_1 adrenoceptor specific antagonist prazosin (1.19×10^{-5} M) which is also a melatonin receptor (type III, MT_3) antagonist, showed a good blocking effect, 7% to 231% ($p < 0.05$, $p < 0.01$), from the lower dose (4.31×10^{-14} M) to the higher dose (upto 4.31×10^{-4} M) of MT (Fig. 3). This indicates that MT_3 receptors are richly present in this fish melanophores.

Photomicrograph No. 1: Untreated control melanophores of *L. rohita* scales. Melanophores are in an intermediate state of neither aggregation nor dispersion. Magnification: 10 x 10. Photomicrograph No. 2: Effect of melatonin (4.31×10^{-4} M). Aggregation of the melanophores in an advanced stage is apparent. Magnification: 10 x 10. Photomicrograph No. 3: Effect of melatonin (4.31×10^{-4} M) in higher magnification: 10 x 40. The cell is in a higher degree of aggregation.

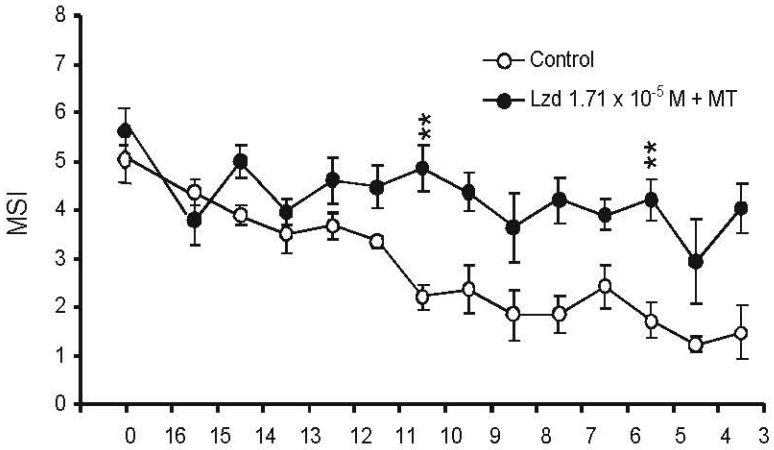


Fig. 1: Graph showing the concentration-response curves of melatonin (MT) in absence and in presence of Luzindole (Lzd) on the isolated scale melanophores of the dorso-lateral region of the fish *Labeo rohita*. Abscissa: Molar concentrations of MT. Ordinate: Responses of the melanophores as melanophore size index (MSI). Each point is the mean ± SE (vertical bars) from five experiments on different fishes. Absence of the vertical bars indicates that SE lies within the symbol. P values were calculated between concentration-response curves of melatonin in absence and in presence of melatonin.

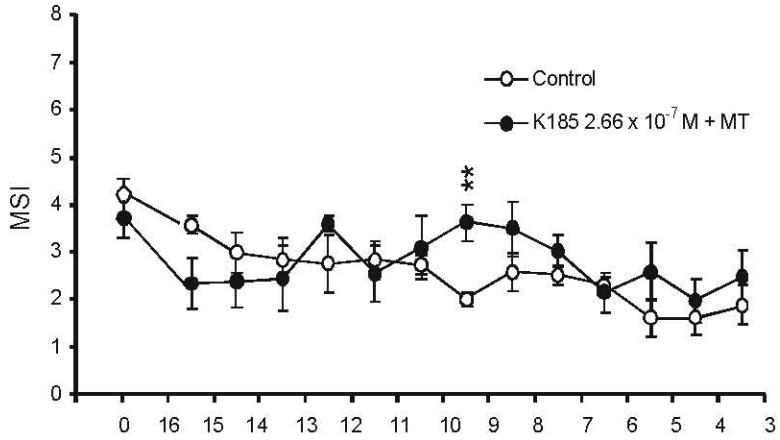


Fig. 2: K185 + MT.

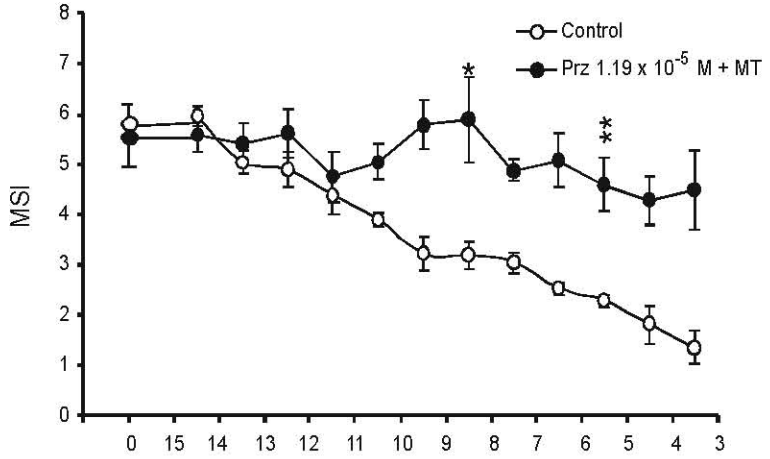
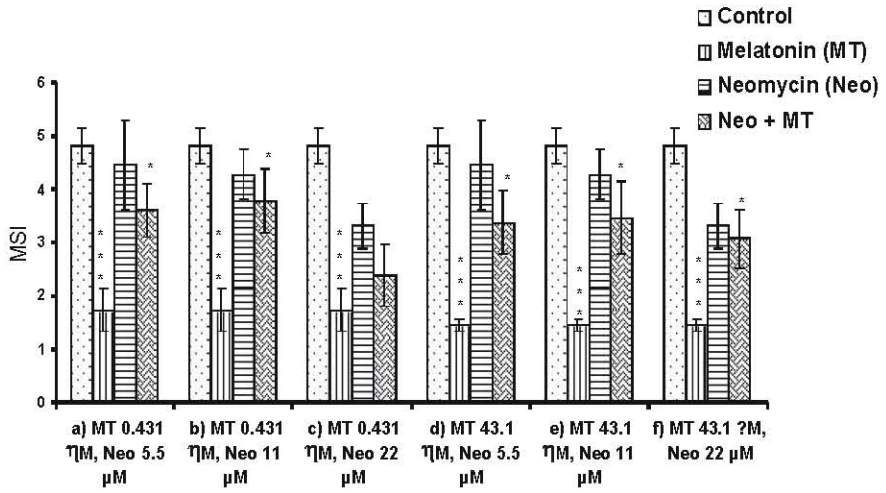


Fig. 3: Luzindole (Lzd) + MT.



Figs. 4a-f: Histograms showing the effects of Neomycin (Neo) + MT.
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

In order to find out the interaction with neomycin which is an antimicrobial agent and blocks the voltage sensitive Ca^{2+} channels without affecting the Na^{+}/Ca^{2+} antiporter in neurons, we applied three concentrations of neomycin (5.5×10^{-6} M, 1.1×10^{-5} M and 2.2×10^{-5} M) against the two concentrations of MT viz. 4.31×10^{-10} M and 4.31×10^{-8} M (Fig. 4a-f). The *per se* effects of the two concentrations of neomycin have been found to be about the MSI value of the control (MSI 4.82 ± 0.34), but the third concentration of neomycin, i.e., 2.2×10^{-5} M has led towards a slight aggregation (MSI 3.32 ± 0.42). We found that all the three concentrations of the antagonist have significantly blocked the two concentrations of the agonist from inducing its aggregative effects with a maximum inhibition of 136.98% ($p < 0.05$, $p < 0.001$) except in Fig. 4-c where only a feeble blockage of the MT induced aggregation by neomycin has been observed.

DISCUSSION

Melatonin (MT), in the present studies on *Labeo rohita* melanophores, has consistently induced a concentration related aggregation. In the earlier reports from our laboratory on *Cirrhinus mrigala* [52], *Oreochromis mossambica* or *Tilapia mossambica* [53], *Rasbora daniconius* [38] and *Channa gachua* [54] etc., MT was found to induce a steady aggregatory effect. The response of melatonin was found to vary with the seasons [38]. The brink dose to induce a discernible response has been found to fluctuate among the different fish species. In our study it was 4.31×10^{-16} M for MT.

In order to evaluate the site of action of MT on *Labeo rohita* melanophores *in vitro* and the nature of receptors involved in its effects, we employed quite a few specific and some non-specific antagonists [55]. A significant blocking of the MT induced aggregations by luzindole reveals that there is an active role of MT_1 and MT_2 melatonin receptors in presenting the effect of the ligand. Thus, it supports the findings [56] about the antagonizing effects of luzindole on the pigment aggregation of melanophores in *Xenopus laevis* under the effect of MT. Our result goes in agreement with a finding that of luzindole absolutely depletes the cAMP formation within the melanophores under the effect of melatonin leading to the aggregation of the pigments [57]. Also it has been found that luzindole and K185 are the perfect inhibitors of the MT receptors in both dorso-lateral as well as band regions of the fish *Rasbora daniconius* [38].

K185 has shown blocking effect which was absent in the lower doses of MT. Hence, it is confirmed that all the three melatonin receptor subtypes viz. MT_1, MT_2 and MT_3 are simultaneously activated in the MT mediated aggregatory effects. Other than the melanophore model system, this result agrees with the reports that K185 significantly antagonized the reversal effect of melatonin on the expression of morphine-induced conditioned place preference in mice [58, 59].

Recently, prazosin was found to be an effective MT_3 receptor antagonist [10]. Thus, the blocking effect by prazosin indicates that the MT_3 receptors, along with the α_1 adrenoceptors, are present in this fish melanophores through which the aggregation induced by MT was

mediated. The present result may be unique and for the first time the presence of MT_3 receptors have been indicated in any fish species melanophores. Our finding favours the report [60] that the melatonin receptor subtype MT_3 appears to mediate the reduction in intraocular pressure in rabbit's eye as the pretreatment with prazosin evoked an increase in intraocular pressure.

Our result with neomycin shows that there should be a free shipment of the Ca^{2+} cations for the MT mediated aggregatory phenomenon to be effectual. This result is supported from the findings related to the erythrophores of a freshwater crustacean, *Macrobrachium potiuna*, a shrimp, revealing that neomycin sulphate has decreased the responses to pigment-concentrating hormone (PCH) through the inhibition of Ca^{2+} channels [61]. Our result also agrees with a finding [62] that neomycin has significantly blocked the MCH mediated aggregatory effect in the melanophores of the fish *R. daniconius*.

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