Melatonin Induced Pigment Translocations in Channa punctatus (Ham.) Melanophores May be Mediated Through Specific Receptors

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ABSTRACT
Background: The pigment translocations involved in the fish Channa punctatus under the influence of the pineal hormone melatonin have been studied. The subtypes of melatonin (MT) receptors and their mode of actions were investigated. Materials and Methods: The agonist and antagonists responses were recorded through the Melanophore Size Index (MSI). The dermal melanophores were studied from the isolated scales which were incubated in different drug solutions. Results: The pineal hormone MT has shown a dose dependent aggregation in the isolated scale melanophores of the fish Channa punctatus (Ham.). The fish melanophores were found refractory to MT after denervation. A free flux of Ca2+ ions is needed in the higher dose of MT induced aggregation. An ancillary role by α2 adrenoceptors during MT induced aggregation is implied. Also the higher dose MT effects have been perfectly blocked by yohimbine, indicating the release of neurotransmitters specific for α2 adrenoceptors. Inhibition by luzindole, K-185 and prazosin strongly support the occurrence of all the three subtypes of MT receptors viz., MT1, MT2 and MT3 in this fish melanophores. Blocking of MT effects by okadaic acid was found to be diminutive. Conclusion: Species variation in responses of the fish melanophores to different concentrations of agonists and antagonists is being signified.

Keywords: Channa punctatus, melanophores, melatonin, okadaic acid, verapamil

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INTRODUCTION
The vertebrate pigment cells: Melanophores, xanthophores and iridophores, contain pigmented granules, respectively, as melanosomes, xanthomes and reflecting platelets. Various pigment cells are derived from a stem cell that contains a primordial organelle of endoplasmic reticular origin. This primordial organelle can differentiate into any of the known pigmentary organelles. This is actually the neural crest tissue which, besides giving rise to the pigment cells, also forms most of the peripheral nervous system and a variety of ectomesenchymal cell types (Bagnara and Hadley, 1973). Melanophores, synthesizing melanin, are the most striking chromatophores. The dispersion of melanosomes having the melanin into the dendritic cells processes results in skin darkening, whereas granule aggregation around the perinuclear region leads to skin lightening (Sherbrooke et al., 1988; Viscanti and Castrucci, 1993). The pineal secretions appear to be most physiologically significant in the control of vertebrate pigment patterns. Melatonin (N-acetyl 5-methoxytryptamine) was first identified by Lerner and co-workers some 55 years ago, as a secretary product of the bovine pineal gland. The hormone was isolated and characterized as a causing agent in the process of amphibian skin paling (Lerner et al., 1959). Melatonin exhibits its peak concentration during the dark phase and usually induces lightening of teleosts, amphibians and reptiles skin (Filadelfi and Castrucci, 1994).

In amphibian species, melatonin induces a strong skin lightening effect. In teleostean fishes and reptiles, melatonin mediated effects may vary with species, developmental stages and the location of pigment cells (Filadelfi and Castrucci, 1994). Fujii (1961) first reported that melatonin strongly aggregated the melanophores of the goby Chaenarchus gulosus. Since, then a number of report appeared about the effect of melatonin on different fish species melanophores in vitro and in vivo which state that the hormone exerts from very weak to strong aggregating effects on different fishes. The literatures reveal that some fish melanophores are refractory to the melatonin effect (Fujii, 1993, 2000; Aspengren et al., 2009).
Besides the classical effects described above, Nishi and Ryozo (1992) demonstrated the dispersal effects of melatonin on the pencil fish Namatomaus beckfordi. The dispersal effects of the fish melanophores were attributed to be mediated through the specific novel melatonin receptors, termed as “β-MT receptors”. Again the “β-melatonin” receptors were identified by Masagaki and Fuji (1999) in the species of threespine pencilfish, Namatomaus trifasciatus. In comparison to this, the term “a-MT receptors” was suggested to be the conventional pigments aggregating melatonin receptors (Fuji, 2000). The pineal hormone melatonin exerts its biological effects through specific, high affinity G-protein coupled receptors (Pickering et al., 1996).

According to the Nomenclature Committee of IUPHAR (Dubocovich et al., 1998; The and Sugden, 1998) Melβ corresponds to ‘mt’ and Melα as ‘MT’. The Melα subtype has not been included in this nomenclature system as it is not expressed in the mammals (Teh and Sugden, 1999). Masana and Dubocovich (2001) simplified further and reported that melatonin mediates its effects through the G protein-coupled receptors viz., MT₁ (previously known as Mel 1a), MT₂ (previously known as Mel 1b) and possibly through. Thus it can be convenient to categorize the melanin receptors into four types: MT₁, MT₂, MT₃, and Melα.

Present investigation was carried out in vitro with the isolated scale melanophores in order to understand the colour change mechanisms involved in the fish Channa punctatus under the influence of the pineal hormone melatonin (MT). Identification of the subtypes of MT receptors has been attempted with the aim of tracing the evolutionary lineage of such receptors in this fish. The direct or indirect mode of action of the hormone MT has also been unearthed.

MATERIALS AND METHODS

The fishes Channa punctatus (Ham.) of either sex, 12-15 cm long and 25-35 g in weight were procured from the local fish markets. The fishes were transported alive to the laboratory and acclimatized in it for at least 48 h with normal day and light cycle of the prevailing season at room temperature (25°C). Care was taken to maintain the fishes in a healthy condition. Those fish which had infection or which showed slight sluggishness were immediately discarded. Fishes were fed every alternate day with minced goat liver. Water was changed twice a week. The fishes of approximately uniform size and weight were considered for each set of experiments.

Scales isolation: Scales were removed from both the dorso-lateral regions of the fish and immediately immersed in fish Ringer solution (Ovais et al., 1993). The scales were removed according to the method of Spench (1913). Care was taken to hold the scales from their non-pigmented sides. The fish scales from the above mentioned sites were found to have a uniform population of melanophores. The scales were equilibrated in fish Ringer solution for 30 min with frequent shakings. Scales (3-5) were transferred in glass Petri-dishes containing 10 mL fish Ringer solution. For drug treatment, 1 mL Ringer solution was removed from Petri-dishes and drug in equal amount was added so that the final volume may not exceed 10 mL. Contact time of the agonist, melatonin (MT), with the scales was 10 min. When the antagonists were used, the scales were first incubated in it for 10 min followed by the addition of melatonin and further incubated for 10 min. The control as well as treated scales were placed on a glass slide with a little incubation medium and covered with a glass cover slip with the dermal side down.

Analysis of melanophores: Individual melanophores were measured with an ocular micrometer (Erma, Japan) in low power microscope and mean melanophores size index was calculated according to the method of Bhattacharya et al. (1976). The increase or decrease from control value represents dispersion and aggregation of melanophores, respectively.

Denervation: Denervation of the fish melanophores was performed in vitro by reserpine treatment method of Katayama et al. (1990). The state of denervation of the melanophores was tested by KCl treatment of the scales for 10 min. No aggregatory response of the melanophores to KCl treatment was considered a positive denervation state. Statistical analysis was performed with the Student’s t-test.

The drugs used were: melatonin (Aristo Pharma, Mumbai, India), okadaic acid (Sigma-Aldrich, USA), verapamil (Samarth Life Sc., Mumbai, India); other chemicals were from Merck India Ltd.

RESULTS

Melanophores in the isolated scales of the air-breathing fish Channa punctatus were branched star shaped structures. The present study is wholly based on dermal melanophores as the epidermal melanophores were very few in number. It was found that the effect of melatonin (MT) was not consistent on the melanophores present on the apical part of the scales while the melanophores situated at the centre of the scales responded consistently. It was further noted that few melanophores were not responsive to MT while others responded normally. During excessive winter (Dec-Jan), melanophores were not distinctly visible and a slight mishandling of the fish during isolation of the
scales, disturbed most of the cells resulting in breaking up of dendrites and the release of pigment granules.

Melatonin (4.31×10^{-7} M-4.31×10^{-6} M) has induced concentration related aggregation in C. punctatus melanophores (Fig. 1). Within 10 min of incubation of the scales in melatonin (MT), the aggregatory responses were observed distinctly. Denervation of the fish melanophores, by an in vitro method as described in the material and methods, did not cause any significant change in the sensitivity of the melanophores to MT. This is evident from the overlapping graphs of the innervated and denervated melanophores (Fig. 1). To understand the mechanism and site of action of MT on C. punctatus melanophores, we employed the non-specific and specific antagonists to block the aggregatory responses of the melanophores. We selected one or two concentrations of MT, showing a good aggregatory

effect, from the concentration response curve to test the effects of antagonists. Verapamil (2.04×10^{-4} M), the Ca^{2+} channels blocker significantly inhibited the aggregation of the fish melanophores (Fig. 2a, b) induced by the higher concentration of MT (4.31×10^{-6} M). It was observed that yohimbine (Fig. 3a, b) has significantly blocked the higher-dose MT induced aggregation. Luzindole (Fig. 4a, b), K-185 (Fig. 5a, b), prazosin (Fig. 6a, b) and okadaic acid (Fig. 7a, b) all have elicited dispersion especially in their high dose of application. Luzindole and K-185, the MT_{1} and MT_{3} receptors antagonists, respectively, have exerted inhibitory effects on the MT induced aggregatory responses of C. punctatus melanophores. Prazosin, the MT_{3} receptors and α_{1} adrenoceptors antagonist, has effectively blocked the aggregation of this fish melanophores induced by
DISCUSSION AND CONCLUSION

Sheikh and Ovais (2007) have reported that in the denervated melanophores of Channa gachua, lower concentrations of melatonin (MT) were sufficient to cause the aggregatory response while the higher doses of melatonin failed to show any aggregatory response. Our results regarding the sensitivity of this fish C. punctatus melanophores to MT after denervation are fully in agreement with the results of Iga and Takabatake (1982) on Zaico temmincki where the fish melanophores were refractory to MT after denervation. This shows that in our fish MT mediates its effects directly through the activation of MT receptors.

In Rasbora daniconius, verapamil’s inhibition of MT induced aggregatory effect was found in the dorso-lateral region only (Srivastava, 2006). However, verapamil was unable to block the aggregatory effects of MT in Channa gachua (Sheikh and Ovais, 2007). In our case, the higher dose of MT was blocked by verapamil in dispensing the aggregatory effect. This shows that the lower dose of MT are binding directly with the typical MT receptors while the higher dose requires a free flux of Ca²⁺ ions through the specific channels in showing the aggregatory effect.

MT induced responses have been reported to be effectively and significantly inhibited by phentolamine, yohimbine, atropine and propranolol (Sheikh and Ovais, 2007). In the present investigation, blocking by yohimbine was found in the higher dose of MT application. In an earlier study on Cirrhinus mrigla melanophores (Gaur, 1994) it was found that yohimbine inhibited the aggregation of melanophores induced by
low-doses of MT only while the higher-doses MT induced aggregation was not inhibited. Thus, species related variations in responses of the fish melanophores to different concentrations of agonists and antagonists are indicated.

Luzindole and K-185 have been found to be perfectly blocking the MT induced aggregatory effect (Srivastava, 2006; Mubashshir et al., 2011a, b). However, in presence of K-185, melatonin incubation has led towards a further dispersion (Srivastava, 2006). The higher dose of MT getting blocked by luzindole indicates a dose specific binding nature of the MT receptors in C. punctatus. Further, a prominent blocking of both the doses of MT by K-185 indicates a rich population of the MT receptors on the melanophores of this fish.

Inhibition by prazosin supports the findings of Mubashshir et al. (2011a) that prazosin has completely inhibited the aggregating effect of melatonin. Studies reveal that besides this the aggregating effects of other MT receptor agonists like the bitter tastant cyclo (Leu-Trp) and the artificial sweetener saccharin have also been found to be blocked by prazosin (Mubashshir et al., 2011b, 2012). Hence, the existence of MT receptors in C. punctatus is strongly supported. Since prazosin is also the α1 adrenoceptors antagonist, thus the MT mediated release of neurotransmitters specifically binding with the α1 adrenoceptors must also have an additive role during MT induced aggregation in the fish C. punctatus.

The per se effect of dispersion by okadaic acid finds a resemblance with a work where okadaic acid has been reported to induce melanosomal dispersion in cultured Xenopus laevis melanophores in a way similar to that of α-MSH and can be reversed by melatonin treatment (Cozzi and Rollag, 1992; Sammaka et al., 1992). In our fish C. punctatus, okadaic acid was found to weakly block the MT induced aggregation. Similar findings have been observed upon the melanophores of African cichlid, Tilapia mossambica, where aggregation but not dispersion, is inhibited by okadaic acid (Thaler and Haimo, 1990).

In conclusion MT leads to aggregation in the melanophores of C. punctatus. The hormone shows its direct effect through the MT, MT, and MT3 receptors in the fish. This shows that the MT receptors made their successful existence long back in the evolutionary hierarchy. MT also mediates its effects indirectly through the transfer of Ca ions and also through the release of neurotransmitters that are binding with the α1 and α2 adrenoceptors.

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