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Differential Actions of Melatonin on Melanophores of the Threeline Pencilfish, *Nannostomus trifasciatus*

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ABSTRACT—The longitudinally striped pattern of the threeline pencilfish, *Nannostomus trifasciatus*, is observed only in the daytime, and changes into a different pattern with three dark spots at night. In this study, microscopic examinations and photoelectric measurements revealed that melanophores in the greater part of the integument respond to melatonin by the aggregation of melanosomes. By contrast, larger melanophores existing in dark spots respond to the amine by the dispersal of pigment. Physiological tests indicated that the nervous mechanisms controlling these melanophores are practically identical to each other, and also with those existing in many teleost species known hitherto. It is further shown that membrane-permeating analogues of 3',5'-cyclic nucleotides effectively disperse melanosomes in all these melanophores. We conclude that melanophores in the spots possess melatonin receptors that mediate melanosome dispersion, which we have recently described as "β-melatonin receptors" in some melanophores of another pencilfish species, *N. beckfordi*. Stimulation of the regulatory subunit of these receptors may be signaled via the G_s subunit, which activates the catalytic subunit, nucleotide cyclase, to increase the cytosolic concentration of cyclic nucleotide(s) as the second messenger. These results demonstrate that the threeline pencilfish affords an excellent model for studying signaling mechanisms through β-melatonin receptors.

INTRODUCTION

Pencilfish are rather popular tropical aquarium fish among hobbyists. Among them, the golden pencilfish, also called the Beckford pencilfish, *Nannostomus beckfordi*, frequently appears on picture books of aquarium fish, displaying its pigmentary pattern with a longitudinal dark stripe. In reality, this pattern is characteristic of the species only during the day, and Reed and his associates initially described that at night the stripe disappears and three round spots appear on the side (Reed, 1968; Ruffin *et al*., 1969). They further showed that the pineal hormone, melatonin (MT), may be involved in the circadian changes of the integumentary patterns in this species, and that, even in the daytime, the night pattern could be induced by treating fish with MT.

During our studies on the circadian phenomenon of the same species of fish, we noticed that distinct parts of the skin became unusually dark in response to MT, while other parts responded by becoming lighter as occurs in the skin of many other fishes (Nishi and Fujii, 1992). Analyses were then made on the characteristics of these melanophores, special attention being paid to receptors for MT. These subsequent studies showed that MT receptors of these melanophores mediate the dispersion of melanosomes, the direction of movement being reversed to that observed under the influence of the amine in common melanophores previously described (Fujii, 1993a; Fujii and Oshima, 1986, 1994). We named such receptors β-MT receptors (Nishi and Fujii, 1992).

It was extremely delicate work, however, to prepare suitable skin preparations from the common pencilfish to study signaling mechanisms of β-MT receptors, and therefore, we have tried to find a species of fish more appropriate for such purpose. Recently, we recognized that similar circadian changes of integumentary patterns took place more clearly and remarkably in another species of pencilfish, namely, the threeline pencilfish, *N. trifasciatus*. Areas that darkened in response to MT were larger, and could be observed more distinctly. Taking advantage of these merits, we have now further characterized the intracellular events associated with the melanosome-dispersing action of MT.

MATERIALS AND METHODS

Materials

Adult forms of both sexes, of the threeline pencilfish, *N. trifasciatus* (Lebiasinidae, Characiniformes), were used. They were obtained from local dealers and acclimatized in fresh water aquariums in our laboratory for at least one week before experimentation. Usually, the fish were reared under 12L-12D regime in a dark room.

Scales were carefully plucked from various parts of the body surface and soaked in physiological saline solution, which had the

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following composition (in mM): NaCl, 125.3; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.8; D-(+)-glucose, 5.6; Tris-HCl buffer, 5.0 (pH 7.3). Since they were not covered by epidermis, melanophores in the dermis on the inner surface of the scales were more easily accessible by chemicals and drugs than those of many other species. Such properties are similar to those described in some species of cyprinids, including the dark chub, *Zacco temmincki*, and the common minnow, *Z. platypus* (Iga and Matsuno, 1980; Hayashi and Fujii, 1993). Thus, scales of the threeline pencilfish could be employed as convenient and reliable specimens for quantitative measurements.

Observation of live fish

Chromatic states of live fish were examined by the naked eye, and were recorded photographically with a 35-mm camera (OM-4, Olympus, Tokyo) with a closeup lens (Zuiko Automacro, 50 mm, Olympus). Fish taken out of the aquarium were quickly placed on a Petri dish containing aquarium water sufficient to immerse the body, and were photographed. Color negative films (Agfacolor, XRG 100, ISO 100, Agfa-Gevaert, Leverkusen) were used. Color films and prints were processed by a reliable local photographic laboratory, while the black-and-white prints were developed by the authors.

Studies at the cellular level

The physiological and pharmacological methods used at the cellular level were fundamentally the same as those described in previous papers (Fujii and Miyashita, 1975; Oshima and Fujii, 1984). Motile responses of several melanophores on a scale were sometimes followed photomicrographically, using an ordinary transmission light microscope (Optiphot XT, Nikon, Tokyo) along with a photomicrographic apparatus (UFX, Nikon). Photographic materials and their processsing procedures were as described above.

More frequently, responses of a single melanophore were measured photoelectrically by a method fundamentally the same to that described in a previous report (Oshima and Fujii, 1984), but with some improvements were made for easier operation and higher stability (Fujii *et al.*, in preparation). In this study, light transmittance through a circular area of skin with a diameter of 150 μm was measured, and in many cases, this area included only the region occupied by a single melanophore.

In some experiments, a K^+-rich saline solution was employed for nervous stimulation of the cells, since an elevated K^+ concentration is known to act as a sympathetic stimulus via release of adrenergic transmitter from chromatic fibers (Fujii, 1959). In the present study, a 50-mM K^+ solution was used, in which the concentration of Na^+ was compensatorily decreased to have the same osmolarity as the primary saline. Namely, the K^+-rich saline employed had the following recipe (in mM): NaCl, 78.0; KCl, 50.0; $CaCl_2$, 1.8; $MgCl_2$, 1.8; D-(+)-glucose, 5.6; Tris-HCl buffer, 5.0 (pH 7.3).

All physiological and pharmacological measurements were performed at room temperature (20-25°C).

Chemicals used

Some substances known to have physiological roles in regulating the fish melanophores were tested for their effects on melanophores of this species. They included melatonin (MT, Sigma Chemical, St. Louis, MO), norepinephrine (NE, hydrochloride salt, racemic modification, Sankyo, Tokyo), and melanin-concentrating hormone (MCH, Peninsula Lab., Belmont, CA). Pharmacological drugs used were scopolamine hydrobromide (Yamanouchi Pharmaceutical, Tokyo), phentolamine mesylate (Ciba-Geigy, Basel), propranolol hydrochloride (Sumitomo Pharmaceuticals, Osaka), yohimbine hydrochloride (Nacalai Tesque, Kyoto) and prazosin hydrochloride (Sigma). In addition, some agents for signal transduction studies were also employed to analyze the role of second messengers in the motile responses of melanophores. In addition to common cyclic nucleotides, namely, adenosine 3',5'-cyclic nucleotide (cAMP) and guanosine 3',5'-cyclic nucleotide (cGMP), their membrane-permeating analogues, 8-bromo-adenosine 3',5'-cyclic nucleotide (8-Br-cAMP) and 8-bromo-guanosine 3',5'-cyclic nucleotide (8-Br-cGMP) were also employed, all from Sigma. As drugs pertinent to these analyses, the following chemicals were also used: adenylyl cyclase activator, forskolin (Wako Pure Chemical Industries, Osaka) and a selective inhibitor of NO-sensitive guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris Cookson, Bristol, UK). Working solutions of these substances were prepared by diluting their stock solutions with physiological saline immediately before use. At the end of each series of measurements, the maximal level of melanosome aggregation for reference was usually induced by 2.5 μM NE.

RESULTS

Circadian changes of pigmentary patterns

Visual observations of live specimens of threeline pencilfish revealed that they exhibited a pigmentary pattern of three longitudinal dark stripes or fasciae, identical to that described in picture books of aquarium fish (Fig. 1A). Actually, this pattern is characteristic of the species but observable only during the day. At night or in the dark, the stripes faded out, and three spots or traversing thick bands appeared on the sides (Fig. 1B).

Comparing these two patterns, areas of skin that became pale during the day but turned dark at night could be recognized as denoted by asterisks in Figure 1C. In view of previous reports, the presence of areas that darken during the night is rather unusual.

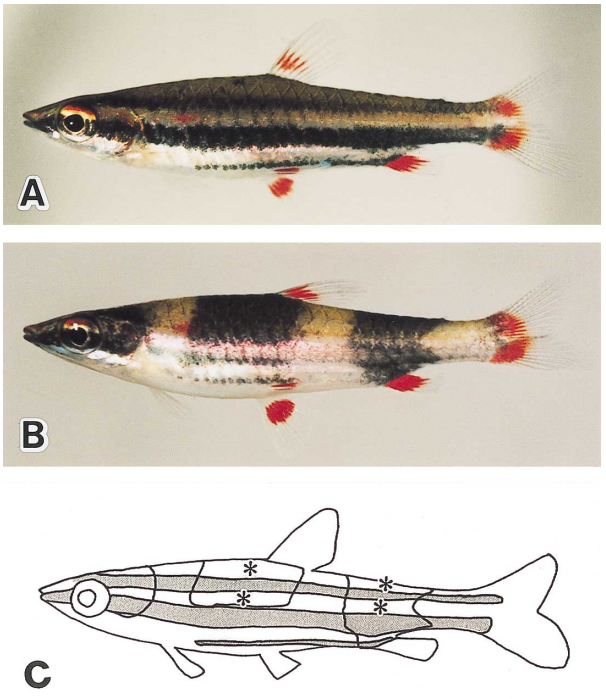

Fig. 1. Pigmentary patterns of the threeline pencilfish, *N. trifasciatus*. (**A**) Photograph of a fish displaying the daytime pattern; the characteristic three longitudinal stripes are seen. (**B**) Photograph of a fish displaying the nighttime pattern; three traversing thick bands or spots are observed. (**C**) Diagram showing both daytime and nighttime patterns of the integument overlapped; asterisks indicate areas where the skin is light in the daytime, but becomes dark at night.

Actions of melatonin on melanophores

Circadian changes in skin darkness in teleosts are now known to be due to the activity of the pineal body to secrete its hormone, melatonin (MT; Reed, 1968; Ruffin *et al.*, 1969; Nishi and Fujii, 1992). Therefore, melanophores on scales excised from various parts of the body surface of these pencilfish were tested for responses to MT.

Examination through a binocular dissecting microscope revealed that scales from the "day stripe" zones contained smaller melanophores of the darker type with many dense melanosomes. Such smaller melanophores were also distributed widely over the body surface. They responded to MT by aggregating melanosomes like many teleostean species previously described (Fujii, 1993a; Fujii and Oshima, 1986, 1994).

In addition to these smaller melanophores, skin on scales plucked from the "night spot" areas contained larger melanophores of two types; those of a darker type with many dense melanosomes, and those of a lighter type with smaller numbers of less dense melanosomes. As in melanophores of many teleosts, melanosomes in all melanophores of pencilfish became fully dispersed when excised scales were equilibrated in physiological saline (Fig. 2B, F).

The smaller type of melanophores responded to MT by aggregating their pigment (Fig. 2D, E). In contrast, both types of larger melanophores responded to the amine in an opposite manner, and dispersion of melanosomes took place (Fig. 2D, E). There was no fundamental difference in responsiveness between these two types of larger melanophores.

Action of MCH

We then tried to examine more precisely the pigment-dispersing effects of MT on the larger types of melanophores. As mentioned above, melanosomes in these melanophores were also dispersed, when equilibrated in physiological saline. To evaluate the pigment-dispersing effect therefore, melanosomes in the cells had to be aggregated beforehand. So, we looked for an appropriate pigment-aggregating agent that could induce sufficient and prolonged aggregation of melanosomes while present in the bathing medium. After testing several agents, we reached the conclusion that melanin-concentrating hormone (MCH) was most suitable for our purpose. At very low concentrations, such as 10 nM, MCH was able to induce the remarkable aggregation of melanosomes, which persisted as long as MCH was present in the perfusing

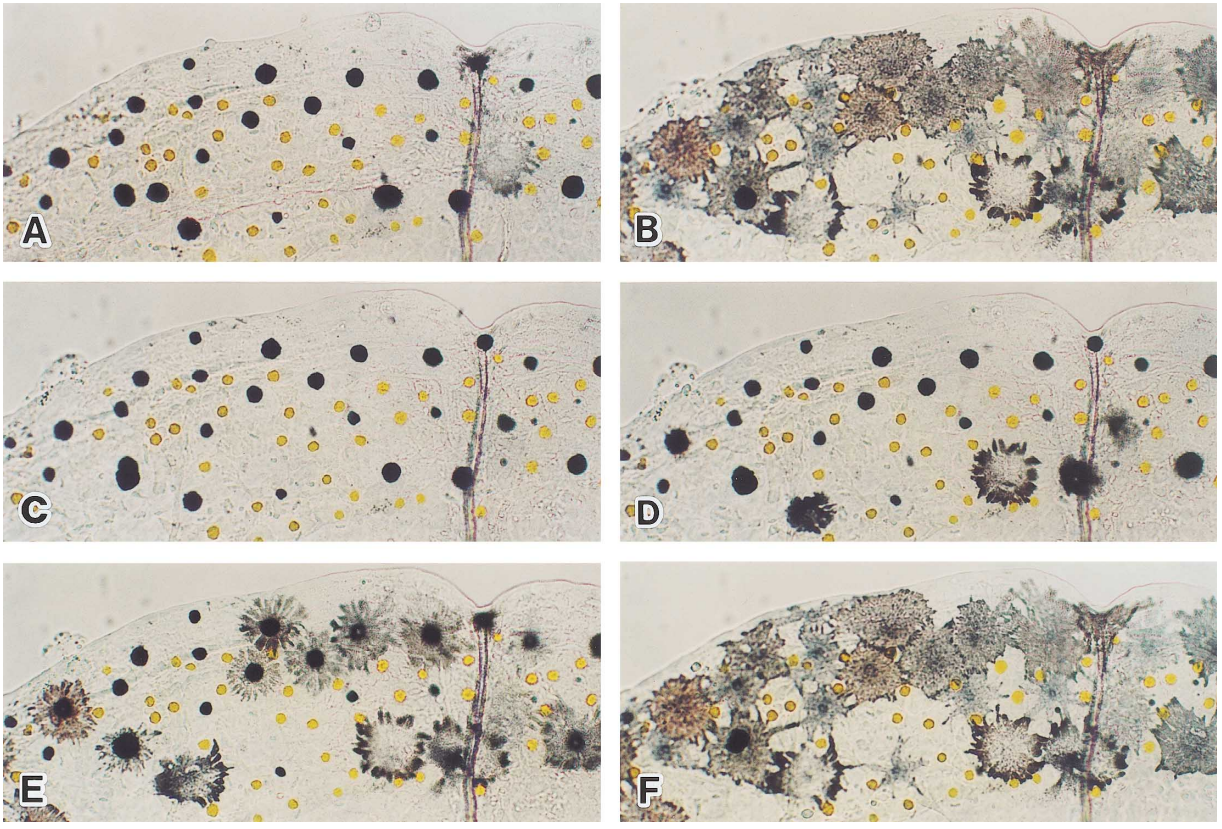

Fig. 2. Serial photomicrographs showing the motile responses of melanophores on a scale of a threeline pencilfish. This particular scale was plucked from an area of the skin where a day stripe and a night spot overlapped. The small round, yellowish cells are xanthophores. (**A**) 1.5 min after the application of 50 mM K^+ saline; melanosomes in melanophores are almost fully aggregated. (**B**) 20 min after equilibration in physiological saline; melanosomes are fully dispersed, the state being quite similar to that before application of the K^+-rich saline. (**C**) 10 min after application of 10 nM MCH; melanosomes are totally aggregated. (**D, E**) Five and 10 min after the application of 10 nM MT, respectively; melanosomes in the larger melanophores are gradually dispersing, while the pigment in smaller melanophores remains aggregated. (**F**) 25 min after equilibration in physiological saline, again; melanosomes are completely dispersed in the cells. ×150.

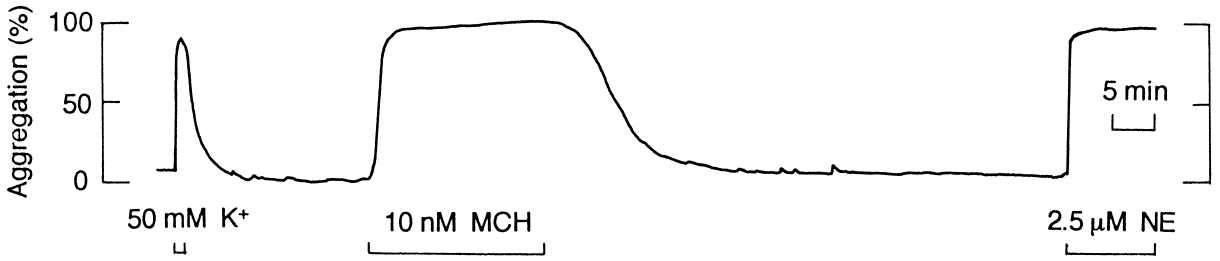

Fig. 3. Typical photoelectric recording showing the effects of MCH on motile responses of a single, larger melanophore on a scale plucked from a night spot area. First, 50 mM K^+ saline was applied to test the normal responsiveness of the cell. MCH effectively aggregated the pigment and the aggregated state was maintained as long as the hormone was present in the perfusing medium. Then, the medium was changed to physiological saline again. After a few min, the melanosomes began to disperse gradually. Finally, 2.5 μM NE was applied to induce maximal aggregation of melanosomes for reference. The K^+-rich saline and the NE solution were also employed in the photoelectric recordings that follow.

solution (Fig. 3). When the medium was changed to physiological saline, the aggregated state was maintained for a few min, but was then followed by the gradual dispersion of melanosomes.

Pigment-dispersing effects of melatonin

The effects of MT on larger melanophores of scales plucked from night spot areas were then examined more precisely. Scales were first treated with MCH to induce the aggregation of melanosomes within the cells. As exhibited in Figure 4, MT over wide range of concentrations typically induced the active dispersion of melanosomes.

Actions of nervous stimulation

As can be seen in the photomicrograph in Figure 2 (Panel A), and also in all photoelectric recordings displayed in this communication, elevation of the concentration of extracellular K^+ ions ($[K^+]_o$) always gave rise to the aggregation of melanosomes in all melanophores of this species. As already mentioned in the Materials and Methods section, an increase in $[K^+]_o$ acts as a sympathetic stimulus via the liberation of adrenergic neurotransmitter. Thus, positive responsiveness to increased $[K^+]_o$ indicates that the cell under study is normally innervated, in addition to the fact that it possesses normal cellular motility.

As shown in Figure 5, melanosome-aggregation elicited

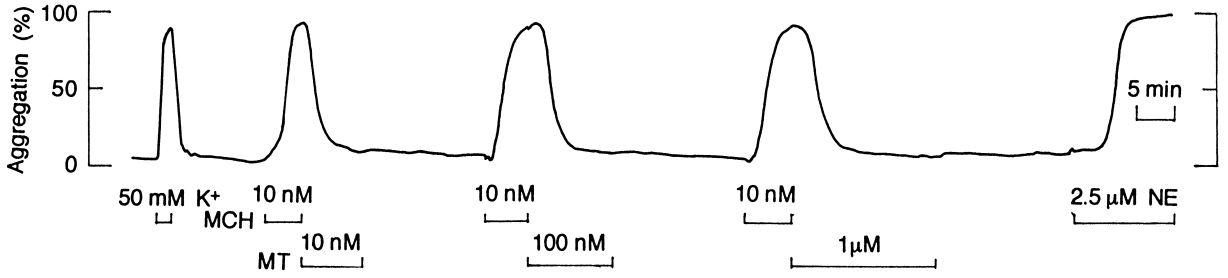

Fig. 4. Typical recording showing the effects of MT on a single, larger melanophore. A scale plucked from a night spot area was employed. Following treatment with 10 nM MCH to induce melanosome aggregation, MT solutions at three concentrations were applied. MT above 10 nM effectively induced the dispersion of melanosomes.

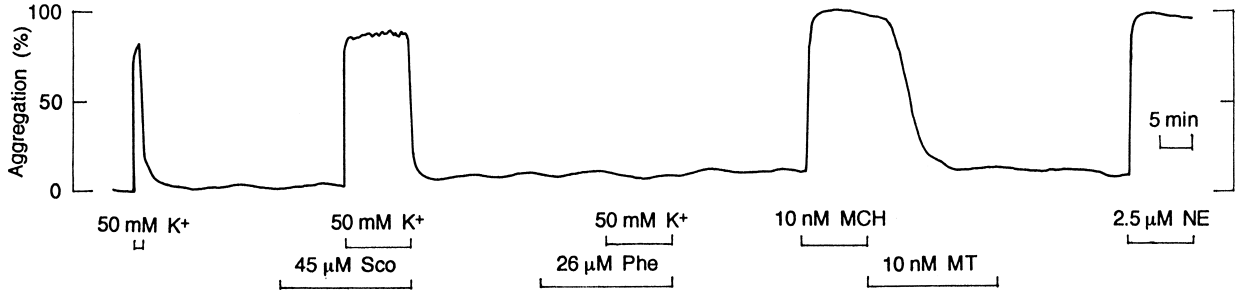

Fig. 5. Typical recording showing the effects on a single, larger melanophore of nervous stimulation elicited by increasing the concentration of K^+ ions, and the influence of two autonomic blocking agents, scopolamine (Sco) and phentolamine (Phe). A scale plucked from a night spot area was employed. Scopolamine did not antagonize pigment aggregation elicited by K^+, while phentolamine effectively blocked it. That the cell under examination was responsive to MT was confirmed by applying MT following treatment with MCH.

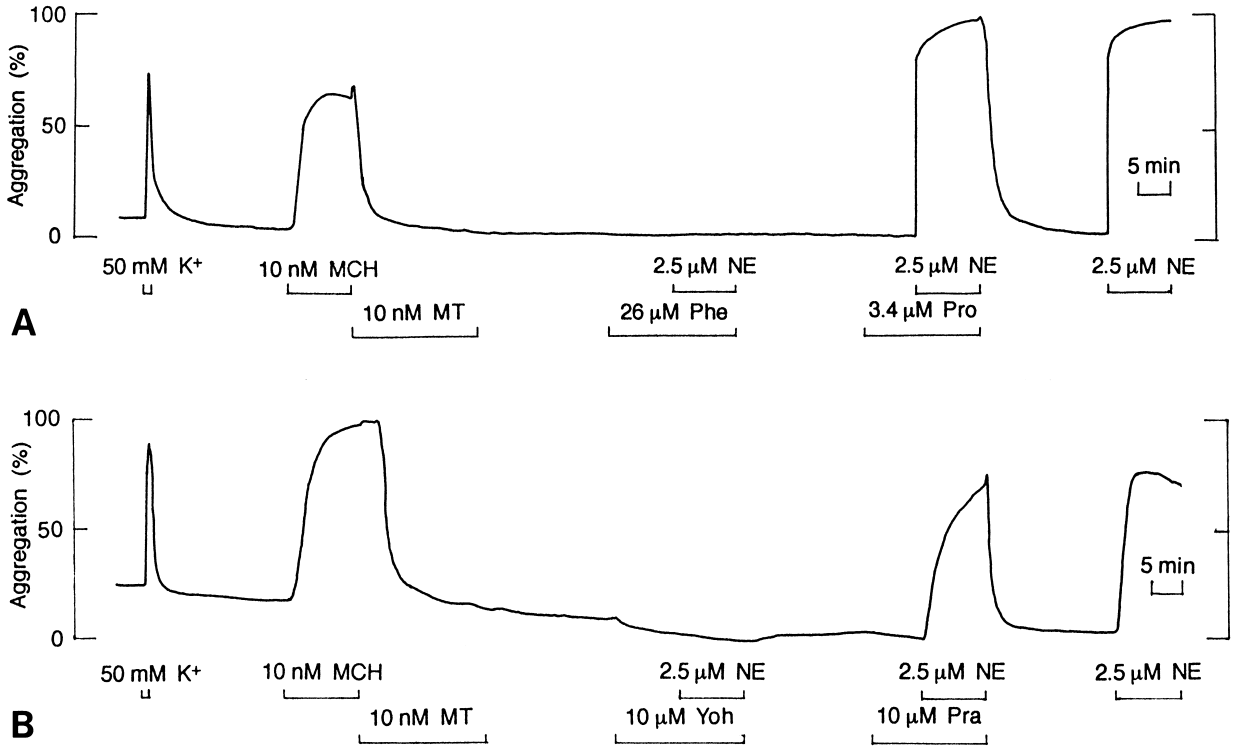

Fig. 6. Typical recordings showing the action of NE and the influence of several adrenergic blocking agents, on single, larger melanophores. Scales plucked from a night spot area were employed. (**A**) A non-selective α-adrenergic antagonist, phentolamine (Phe), blocked the aggregation caused by NE, while a β-adrenergic blocker, propranolol (Pro), did not. (**B**) A selective α_2-adrenergic antagonist, yohimbine (Yoh), effectively blocked the aggregation elicited by NE, but a selective α_1-adrenergic antagonist, prazosin (Pra), failed to do so. In both recordings, we confirmed that the cells were responsive to MT by melanosome dispersion, by applying the amine following the application of MCH.

by increased $[K^+]_o$ was not antagonized by a muscarinic cholinoceptor antagonist, scopolamine, but was effectively antagonized by a non-selective α-adrenoceptor antagonist, phentolamine. These observations clearly indicate that melanophores that react to MT by dispersing melanosomes receive common sympathetic innervation, similar to melanophores that respond by aggregating pigment.

Figure 6A further shows that phentolamine was also effective in blocking pigment aggregation caused by norepinephrine (NE), while an active β-adrenergic blocker, propranolol, did not. A selective α_2-adrenergic antagonist, yohimbine, blocked melanosome aggregation caused by NE, but a selective α_1-adrenergic antagonist, prazosin, did not (Fig. 6B). It was thus concluded that peripheral neurotransmission of the present material to larger melanophores is mediated by α_2-adrenoceptors. Namely, the nervous mechanism controlling the motile activity of these melanophores must be fundamentally the same as that existing in many other teleost species known hitherto.

Effects of cyclic nucleotides

Several cyclic nucleotides were tested for their effects on larger melanophores. Both cAMP and cGMP exerted only slight or negligible melanin-dispersing action on melanophores of this species. By contrast, 8-bromo analogues of these nucleotides showed remarkable melanin-dispersing action (Figs. 7, 8A, 8B).

Effects of nucleotide cyclase activator and inhibitor

A known adenylyl cyclase activator, forskolin, potentiated the pigment-dispersing effects of MT on larger melanophores (Fig. 9A). By contrast, a selective inhibitor of NO-sensitive guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) exhibited a delayed starting inhibitory effect on the pigment dispersion caused by MT (Fig. 9B).

DISCUSSION

Previous studies on the physiology of chromatophores in teleosts have indicated that melatonin (MT) always acts to aggregate pigmentary organelles in several types of chromatophores including melanophores, erythrophores and even light-scattering chromatophores known as leucophores (cf. Fujii, 1993a; Fujii and Oshima, 1986, 1994). Thus, our finding that some melanophores in the skin of the golden pencilfish, *N. beckfordi*, responded to MT by dispersing melanosomes was completely unexpected (Nishi and Fujii, 1992). Soon afterwards, we were able to add another example of such melanophores in the brown-tailed pencilfish, *N. eques*, that belongs to a different genus than *Nannostomus* (Hayashi *et al.*, 1994). Both species belong to the same family Lebiasinidae (order Characiniformes). Based on pharmacological analyses, we

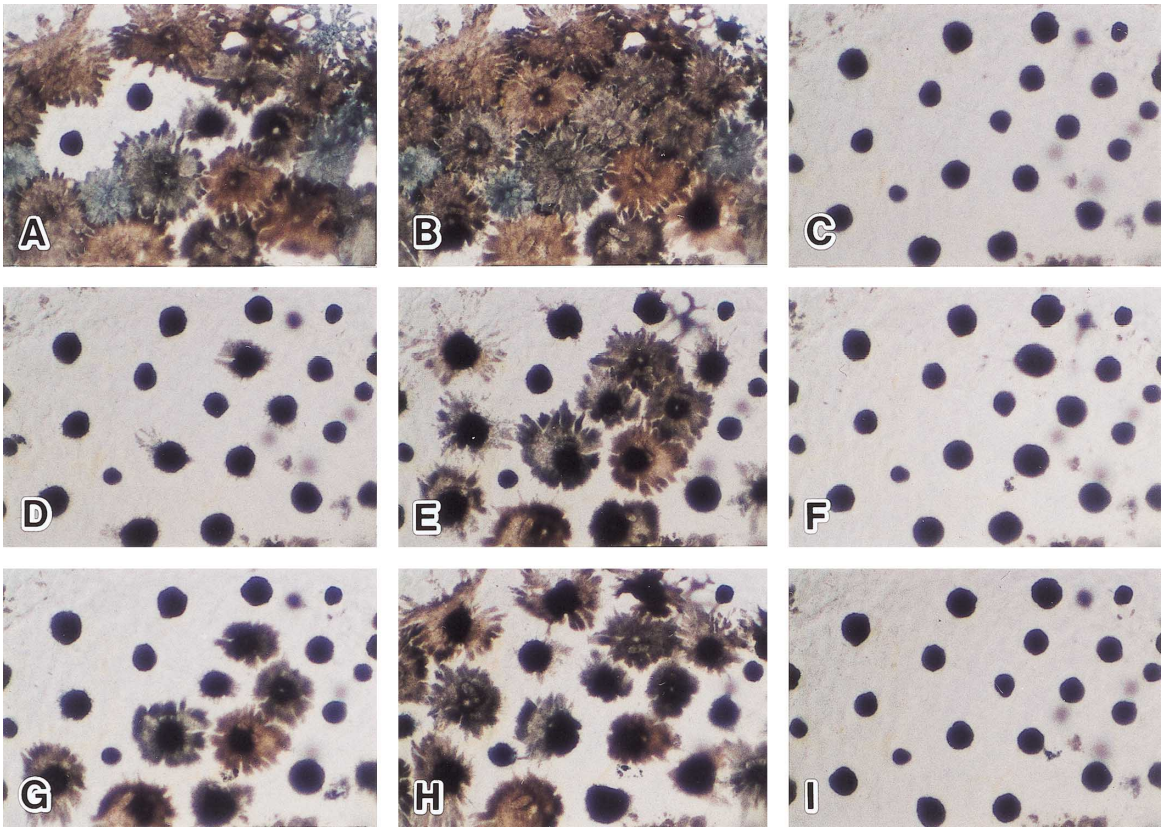

Fig. 7. Serial photomicrographs showing the motile responses of melanophores on a scale plucked from an area of the skin where the day band and the night spot overlapped. (**A**) 10 min after the application of 1 μM MT. The effects of MT on melanosomes were different among melanophores, and the melanosomes in some cells aggregated, while in others they dispersed. (**B**) After equilibration in physiological saline for 20 min, melanosomes are fully dispersed. (**C**) 10 min after the application of 100 nM MCH; melanosomes were totally aggregated in all melanophores. (**D, E**) 10 and 30 min after the application of 50 μM 8-bromo-guanosine 3',5'-cyclic nucleotide (8-Br-cGMP), respectively; melanosomes in some melanophores gradually dispersed in response to this nucleotide. (**F**) 10 min after the application of 100 nM MCH a second time; melanosomes were totally aggregated. (**G, H**) 10 and 30 min after the application of 8-bromo-adenosine 3',5'-cyclic nucleotide (8-Br-cAMP), respectively; melanosomes in many melanophores were gradually dispersed by the action of nucleotide. (**I**) 10 min after the application of 2.5 μM NE; melanosomes were completely aggregated in the cells. ×200

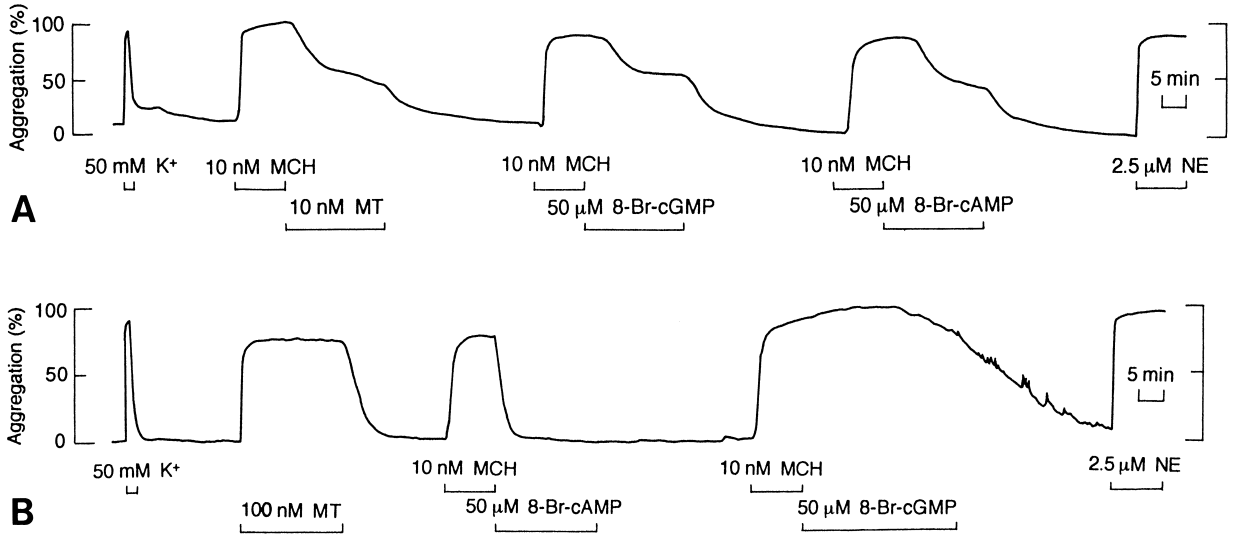

Fig. 8. Typical recordings showing the motile responses of individual melanophores to cyclic nucleotides. (**A**) A scale plucked from a night spot area, and MT dispersed the melanosomes. Both 8-bromo-guanosine 3',5'-cyclic nucleotide (8-Br-cGMP) and 8-bromo-adenosine 3',5'-cyclic nucleotide (8-Br-cAMP) showed remarkable melanin-dispersing action which was comparable with that induced by MT. (**B**) A scale plucked from a day band area; MT aggregated melanosomes. 8-Br-cAMP dispersed melanosomes strongly, but the action of 8-Br-cGMP was weak, if any.

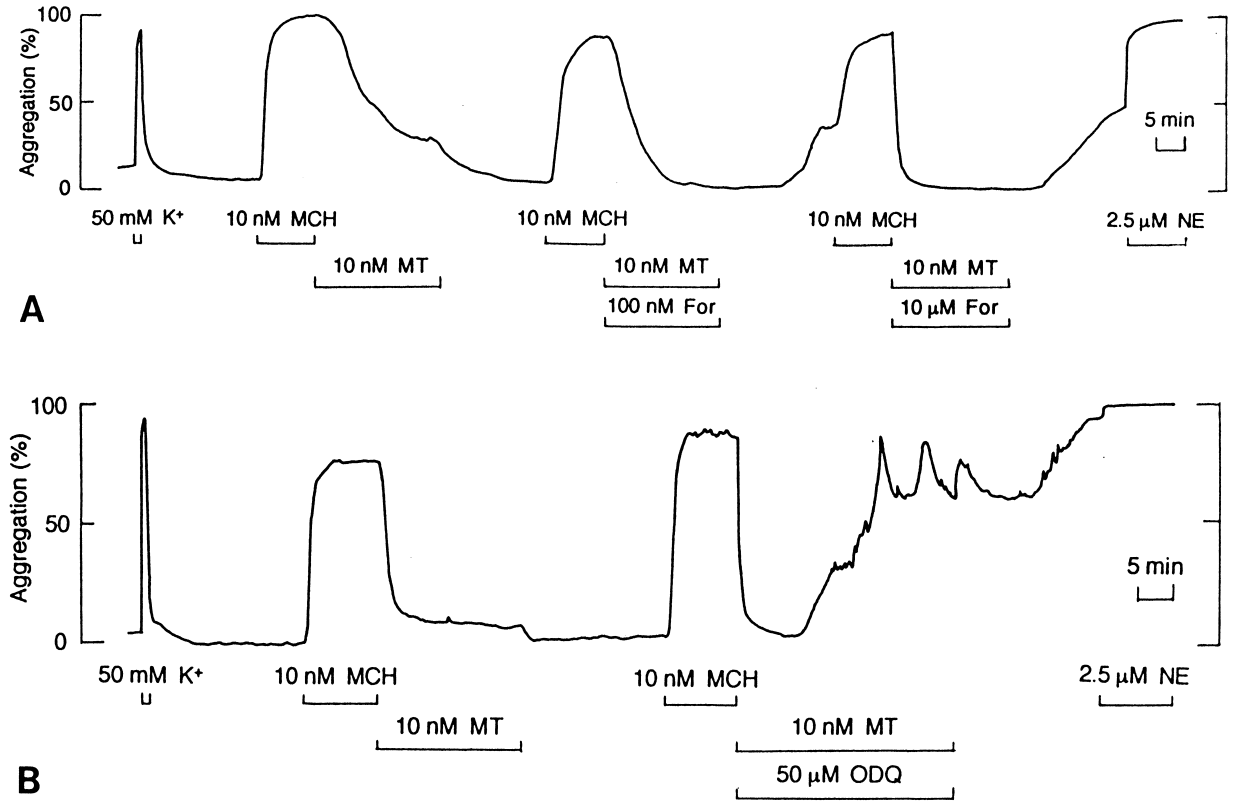

Fig. 9. Typical recordings showing motile responses of larger melanophores from scales plucked from night spot areas, to MT and the influence of substances known to influence the activity of nucleotide cyclases. (**A**) An adenylyl cyclase activator, forskolin (For), effectively potentiated the pigment-dispersing action of MT. (**B**) A selective inhibitor of NO-sensitive guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), was employed; rapid melanosome dispersion due to the action of MT was first recorded, but the effect was gradually reversed by the action of ODQ.

have concluded that melanophores possess receptors that mediate the dispersal of melanosomes when stimulated by MT. We designated these receptors "β-MT receptors", leaving conventional and common receptors for MT to be called "α-MT receptors" (Nishi and Fujii, 1992; Fujii, 1993a).

Using the threeline pencilfish, *N. trifasciatus*, we have described in this study a third instance of melanophores in which dispersal of pigment takes place in response to MT. The threeline pencilfish belongs to the genus *Nannostomus*, the same genus to which the golden pencilfish belongs. Upon careful examination of other species of pencilfish, namely, the Harrison's pencilfish, *N. harrisoni*, the brown-tailed pencilfish, and the one-lined pencilfish, *N. unifasciatus*, we have also recently found that some of those melanophores also responded to MT by pigment dispersion. It should be emphasized here however, that the present species may be especially appropriate for analysis of β-MT receptor action to MT and its analogues on melanophores, because areas in which these melanophores respond to MT by dispersing their pigmentary inclusions are much larger than those of the other pencilfish. That we can use isolated scales is another convenient feature of this species, because we can obtain appropriate skin specimens more easily. In the golden pencilfish incidentally, plucked scales were practically devoid of integumentary tissues that contain chromatophores, and special preparations of skin containing melanophores have to be excised. Namely, pockets of skin that housed individual scales had to be processed for this purpose (Nishi and Fujii, 1992).

Although still fragmentary, our results on the action of known pigment-motor substances show that the motile responses of larger melanophores are quite normally regulated. Namely, both MCH and the sympathetic neurotransmitter, NE, effectively aggregate pigmentary organelles. The sole difference is the action of MT: In contrast to the original name of this amine for its action to aggregate melanin, it dispersed the pigment in larger melanophores. Thus, our conclusion that receptors for MT possessed by these larger cells are different from those known hitherto may be justified.

In recent years, the mechanism of signal transduction in the normal, pigment-aggregating action of MT has been investigated, and the involvement of cyclic AMP and other mediators have been suggested (Fujii, 1993a). By contrast, intracellular events which occur during melanosome dispersion in response to MT have not yet been studied, although some assumptions were made (Nishi and Fujii, 1992; Fujii, 1993a). The present material has provided a good material for this purpose.

Melanophores existing over the greater part of the integu-

ment responded to MT by melanosome aggregation. This response should be mediated via MT receptors of the conventional type, to which we have given the tentative term of α-MT receptors (Nishi and Fujii, 1992). In the present study, we have actually found that 8-bromo analogues of cAMP and cGMP elicited dramatic melanosome dispersion in these melanophores, although the corresponding nucleotides, namely cAMP and cGMP, showed only slight or negligible melanin-dispersing effects. That unmodified, biomolecular nucleotides had only weak effects is probably due to their low permeability through the cell membrane. At this moment, we are not sure which cyclic nucleotide is physiologically responsible for dispersing pigment as the second messenger, although our data seem to favor the involvement of cyclic GMP. Further works are justly needed to clarify this issue.

Identical tests were also done on melanophores that responded to MT by dispersing pigment. Notwithstanding the fact that the effect was entirely reversed, practically identical results for the action of nucleotides were also obtained with these melanophores. In common melanophores therefore, the amine may act to decrease intracellular levels of cyclic nucleotide(s) via G_i protein to decrease the activity of adenylyl or guanylyl cyclase, while in melanophores that respond by dispersing melanosomes, the signal applied to the receptors may be transduced via G_s protein to activate nucleotide cyclase(s). The latter receptors that mediate the melanosome dispersion are classified as β-MT receptors, which we have already tentatively designated in the golden pencilfish melanophores.

The primary function of the daytime, striped pattern of this species should be recognition coloration or an aposematic one for conspecific individuals (cf. Fujii, 1993b). The functional significance of the spotted pattern observable at night still remains to be clarified, but might be for protective purposes. It is known that the present species is diurnal, and that they rest at night near the top of water. In addition, we know that the intensity of illumination often increases beyond 0.2 l×, when the moon is full. Predators may be able to recognize them as prey, and to avoid the attack by nocturnal predators that usually have good vision in darkness, hues and patterns that render prey inconspicuous against the background should naturally be advantageous.

It is known among hobbyists that characid fish species, including the neon tetra, *Paracheirodon innesi*, and the cardinal tetra, *P. axelrodi*, change their body colors and fade at night. Actually, Hayashi *et al.* (1993) reported recently that the red abdominal skin of these fish fades at night or in the dark. They have further discussed that this change in color of the abdominal skin during the night is primarily due to increased secretion of the pineal hormone, MT, that causes the aggregation of pigment in erythrophores. Concerning such changes of pattern, the phenomenon described in the present article is somewhat different from the case of tetra fishes. However, it is quite possible that the spotted pattern of pencilfishes during the night may function to increase their chance to survive in their natural habitat. By making a more appropriate pattern, β-MT receptors may be of use in increasing the rate of survival. Upon further correlation of ethological and physiological studies on varities of fish, we may be able to find interesting cases of color changes where the pineal hormone, MT, serves similar purposes.

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