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'FACILITATION' OF MELANOPHORE RESPONSES IN WINTER FLOUNDER *PSEUDOPLEURONECTES AMERICANUS*

BY D. BURTON AND M. P. O'DRISCOLL

Department of Biology and Ocean Sciences Centre, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1C 5S7

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Summary

Melanophores of winter flounder (*Pseudopleuronectes americanus*) subjected to repeated transfers between black and white backgrounds display pronounced enhancement of the rates of melanosome aggregation and dispersion. Melanosome aggregation in response to repeated injection of noradrenaline displays a similar enhancement. *In vitro* preparations of microscopic skin samples on scales removed during successive cycles of background changes also display increasing melanophore response rates to K^+ stimulation. It is concluded that a physiological 'priming' mechanism is involved peripherally at the melanophore level and it is proposed that this represents a form of facilitation. Also, it is deduced from these experiments that a modulation of the microtubular channels associated with melanosome translocation is probably the basis of the facilitation process.

Introduction

It is well established that changes in albedo, i.e. in the ratio of direct to reflected light received by the eye when teleosts are subjected to contrasting backgrounds, result in paling associated with melanosome aggregation or darkening associated with melanophore dispersion. In different teleosts varying relative degrees of neural and hormonal regulation are involved in these chromatic responses (Khokhar, 1971; Abbott, 1973), the regulation being predominantly neural in the winter flounder *Pseudopleuronectes americanus* (Burton, 1981). Neural regulation of teleost melanosome aggregation is sympathetic (Pouchet, 1876; von Frisch, 1911), noradrenaline being the neurotransmitter (Fernando and Grove, 1974; Visconti and Castrucci, 1981; Fujii and Oshima, 1986). *In vitro* integumentary preparations have been used extensively in physiological experimentation on teleost melanophores since the initial studies by Spaeth (1913). Such *in vitro* protocols include the induction of melanosome aggregation and dispersion by incubation in K^+- and Na^+-rich media, respectively. Fujii and Oshima (1986) have reviewed the increasing evidence that the melanosome-aggregating action of K^+ in teleosts is mediated through noradrenaline release from sympathetic nerve endings in the integument.

Key words: melanophores, response enhancement, *Pseudopleuronectes americanus*, flounder.

It has been reported (Sumner, 1911; Mast, 1916; Osborn, 1939) that flatfish, including winter flounder, respond with increasing rapidity to repeated changes of black and white backgrounds. These accounts lack both quantitative cellular data and experimental analysis of the physiological level at which the phenomenon is localised. The purpose of the current experiments was to obtain such information for the neuro-melanophore system of *Pseudopleuronectes americanus* and to determine whether the changes occur peripherally at the effector level or more centrally in the regulatory system.

Materials and methods

Winter flounder, *Pseudopleuronectes americanus* Walbaum, were caught locally by SCUBA divers using hand nets and laboratory-acclimated in stock tanks under seasonal temperatures (range −1.75 to +13°C) and illumination periodicity. Subsequently, flounders were background-adapted in individual black or white Plexiglas aquaria (400 mm × 225 mm × 203 mm) continuously illuminated (60 W, 1 m above) and supplied with running sea water at seasonal temperatures. Aquaria were covered with wide-mesh nylon net mounted on close-fitting wooden frames. Black and white background reversals were achieved by gently transferring flounder by hand between aquaria. Melanophore responses were estimated microscopically at appropriate times by plucking scales, each with a small (<0.7 mm × 1.5 mm × 0.3 mm) slip of skin (Burton, 1978), from the mid region of the integumentary pattern, i.e. from the extensive general background component (Osborn, 1939; Burton, 1981). Scale slips were frozen at −78°C immediately after plucking with fine forceps, then fixed in 37% formaldehyde and temporary glycerine mounts were prepared. The chromatic effect of stress related with these procedures has been discussed (Burton, 1979) and it has no significant influence on the general background component of the pattern. Dermal and epidermal melanophore index (DMI and EMI) scales, adapted from Hogben and Slome (1931), were used to quantify responses, 1.0 representing complete melanosome aggregation and 5.0 complete dispersion.

Melanophore responses to L-noradrenaline bitartrate (Sigma) occurred after subcutaneous injection (0.1 ml per 100 g), the vehicle being a balanced salt solution (BSS) with the following composition in $mmol\,l^{-1}$: NaCl, 175.0; KCl, 2.7; $MgCl_2.6H_2O$, 0.64; $CaCl_2$, 1.53; $NaHCO_3$, 5.0; glucose, 5.6. Data from dose–response experiments indicate that noradrenaline at 5×10^{-7} mol kg^{-1} was suitable for this study.

For *in vitro* experiments, scale slips were incubated initially in melanosome-dispersing fluid (DF) after removal from black-adapted flounder. The composition of DF (in $mmol\,l^{-1}$) was NaCl, 180.0; $NaHCO_3$, 5.0; glucose, 5.6. A thermal microscope stage was used to incubate the preparations (Burton and Everard, 1990) at the ambient seawater temperature. After initial equilibration to DF, the DMI and EMI were recorded. The incubation medium was then replaced, without temperature change, by melanosome aggregating fluid (AF), the composition of

which (in mmol l^{-1}) was, KCl, 180; $KHCO_3$, 5.0; glucose, 5.6. The DMI and EMI were recorded at suitable intervals.

All estimates of DMI and EMI in each *in vivo* and *in vitro* experiment were made using two scale slips from each fish. Statistical comparisons were made by means of the Mann–Whitney *U*-test using extended tables (Rohlf and Sokal, 1981).

Results

Six flounders which had DMI and EMI values of 5.0 after 7 days on a black background were subjected to three successive black–white–black cycles of background change (Figs 1 and 2). Forty minutes after the first change from black to white background, dermal and epidermal melanophores of only 33 % of scale slips sampled displayed partial melanosome aggregation (Fig. 1A,B). After 4 h all scale slips displayed intermediate degrees of melanosome aggregation, but universal melanosome aggregation took more than 3 days (Fig. 1A,B). Forty minutes after the flounders had been returned to the black background, all but 1 of 12 scale slips displayed partial melanosome dispersion in the dermal melanophores, but only 33 % of epidermal melanophores displayed such a change

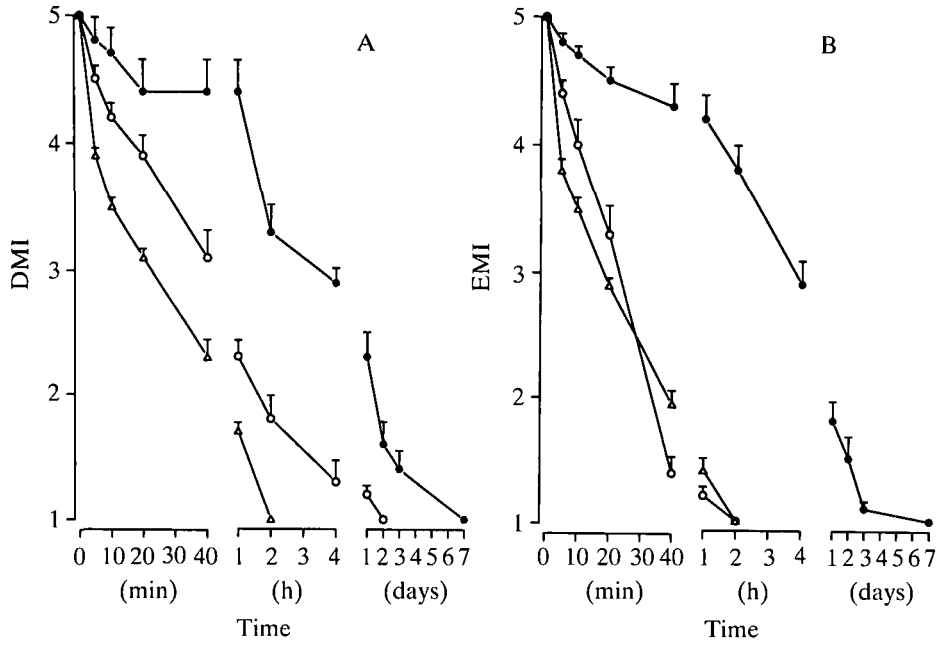

Fig. 1. Melanophore responses of a single group of flounders on transfer to a white background during three successive black–white–black background cycles. (A) Dermal melanophores; (B) epidermal melanophores. First (●——●), second (○——○) and third (△——△) cycles. DMI and EMI values are means for six flounders (two replicates per flounder). Water temperature 6–10°C. Bars represent standard errors.

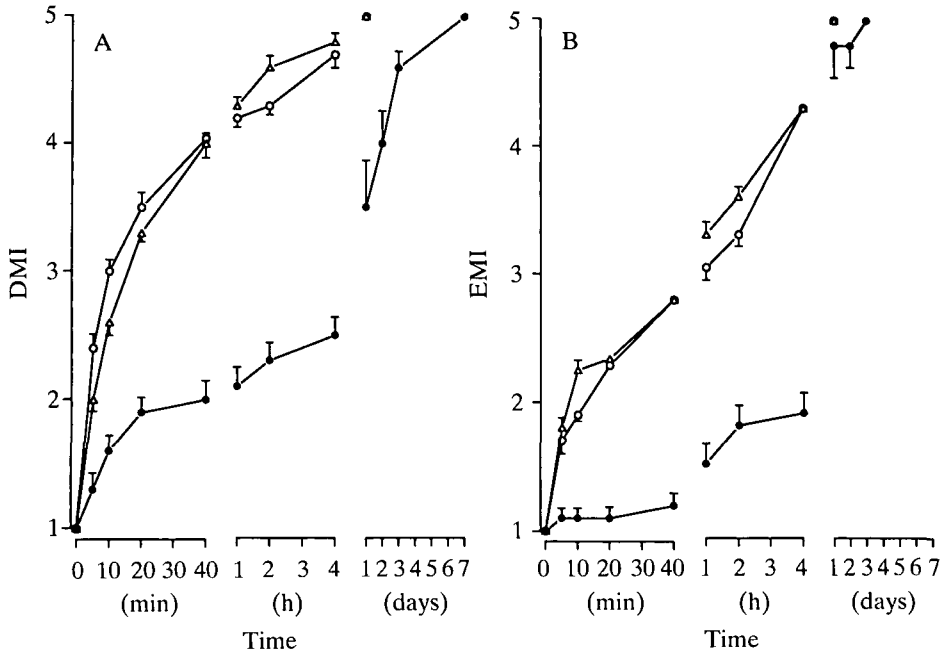

Fig. 2. Melanophore responses of the group of six flounders (two replicates per flounder) in Fig. 1 on transfer to a black background during three successive black–white–black background cycles. (A) Dermal melanophores; (B) epidermal melanophores. Water temperature 7–10.5°C. The key is as in Fig. 1. Bars represent standard errors.

(Fig. 2A,B). Complete melanosome dispersion in all scale slips during this first cycle of background change required 3 days or more (Fig. 2A,B). However, the final stages of melanosome dispersion were attained more readily in the epidermal melanophores than in the dermal melanophores.

During the two further black–white–black cycles of background change there was considerable enhancement of the rates of response during both melanosome aggregation and dispersion (Figs 1 and 2). Compared with the first cycle, all DMI values after 5 min in the second cycle were significantly ($P \leq 0.025$) lower and melanosome aggregation was completed by 2 days in the dermal melanophores. There was further enhancement in the third cycle (Fig. 1A), with significantly ($P \leq 0.025$) lower DMI values compared with those at equivalent times in the second cycle, and completion of melanosome aggregation within 2 h. The epidermal melanophores also displayed considerable enhancement of melanosome aggregation during the second cycle (Fig. 1B), with significantly ($P \leq 0.025$) lower EMI readings after 5 min and completion of aggregation within 2 h. Differences between the second and third cycle rates of EMI decrease (Fig. 1A,B) were not as pronounced or as clearly defined as the DMI changes, and melanosome aggregation in the epidermal melanophores was completed within the same time in both cycles. In the case of melanosome dispersion (Fig. 2A,B),

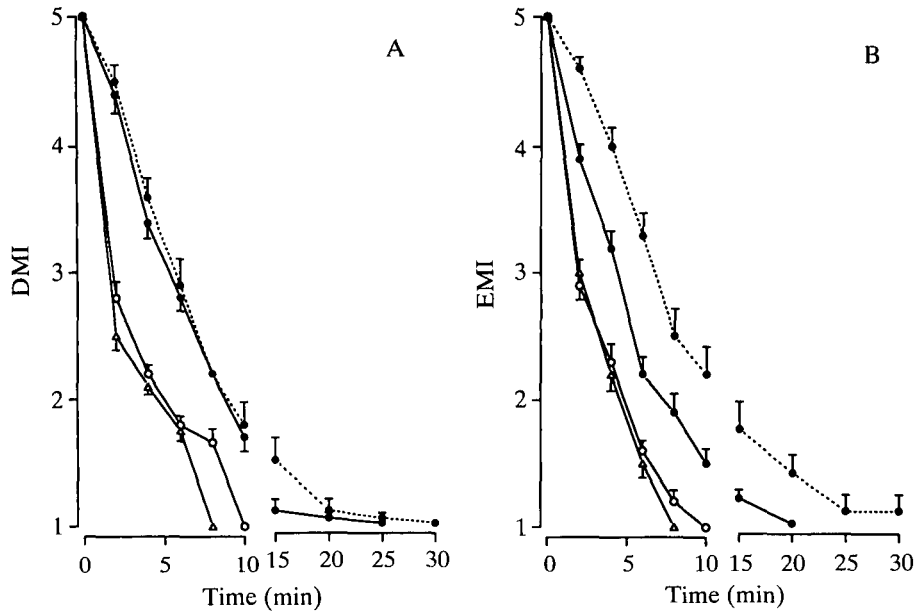

Fig. 3. Melanosome aggregation induced *in vitro* by K^+ in scale-slip preparations from the six flounders in Figs 1 and 2 adapted to a black background during the three successive black–white–black background cycles. (A) Dermal melanophores; (B) epidermal melanophores. Preparations from the initial, precyclic, period of adaptation to black (●---●) and from the first (●——●), second (○——○) and third (△——△) background reversal cycles. DMI and EMI values are means±S.E.M. for 12 scale slips. Temperature 6–10.5°C.

the DMI and EMI readings 5 min after the flounders had been transferred to the black background were significantly ($P<0.005$) higher for the second and third cycles than for the first, and melanosome dispersion was completed within 1 day compared with 3–7 days for the first cycle. Mean DMI and EMI values at the sampling times for the second and third cycles were similar or identical (Fig. 2A,B).

After 5 days of the initial, i.e. precyclic, period of adaptation to the black background, and at the equivalent times during the black-background phase of each of the three subsequent cycles, scale slips from each dark-adapted flounder were incubated in AF after equilibration in DF (Fig. 3A,B). Universal completion of melanosome aggregation *in vitro* in the dermal melanophores in response to K^+ in the precyclic period and in the first cyclic adaptation of the flounder to a black background (Fig. 3A) required up to 30 and 25 min, respectively. There were no statistically significant differences ($P>0.05$) between these two responses. Compared with these two responses, the rate of *in vitro* K^+-evoked dermal melanophore aggregation in preparations from the second and third cycles for the flounders was considerably enhanced (Fig. 3A), with universal melanosome aggregation in 10 min for the second cycle and 8 min for the third cycle and with

significantly ($P<0.001$) more aggregation during the early stages. However, the dermal melanophore responses to K^+ in these two later preparations had similar time courses, the only significant ($P<0.001$) difference being at completion of the final *in vitro* response (Fig. 3A). In the case of the epidermal melanophores, a minimum mean EMI of 1.1 ± 0.03 was attained after 25 min of incubation in AF in the precyclic preparations. Scale slips plucked during the three subsequent background reversal cycles progressively took 20, 10 and 8 min to attain a universal EMI of 1.0 (Fig. 3B). After incubations of 2 min and longer, EMI values were significantly ($P<0.001$) lower during the first cycle compared with precyclic preparations (Fig. 3B); that is, the *in vitro* epidermal melanophores were more sensitive than the dermal melanophores to a single albedo change for the flounder, which is consistent with their more rapid facilitation *in vivo* (Fig. 1). The further differences in EMI at all times *in vitro* (Fig. 3B) between the second cycle and precyclic preparations were also statistically significant, but differences between the second and third cycles were not significant ($P>0.05$).

When six black-adapted flounders were injected at 2-day intervals with noradrenaline (5×10^{-7} mol kg^{-1}), there was also enhancement of melanosome aggregation (Fig. 4). With this noradrenaline dose, maximal mean responses of both dermal and epidermal melanophores to the first injection were attained within 20 min. Subsequently, there was partial melanosome dispersion after

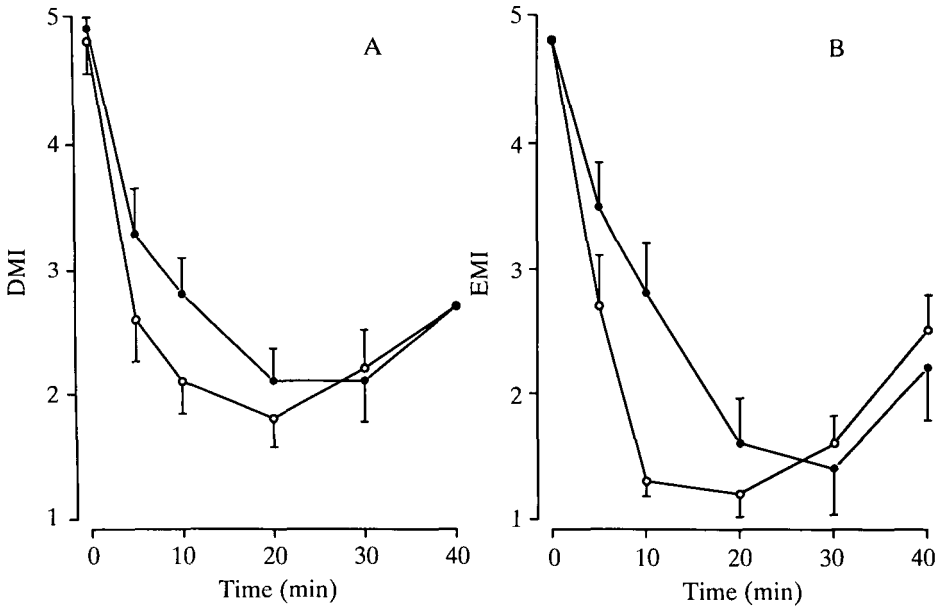

Fig. 4. Melanophore responses of a single group of black-adapted flounders to two injections of noradrenaline (5×10^{-7} mol kg^{-1}) given 2 days apart. (A) Dermal melanophores; (B) epidermal melanophores. First injection (●——●), second injection (○——○). DMI and EMI values are means±S.E.M. for six flounders (two replicates per flounder). Water temperature 12°C.

30 min, associated with clearance of the plasma-borne neurotransmitter. The melanosome-aggregating response to the second injection was faster than to the first, the DMI and EMI after 10 min being significantly ($P<0.025$) less for the second injection than for the first (Fig. 4). However, the response characteristics of a third injection were similar to those of the second, with no significant ($P>0.05$) differences between them. Interestingly, the epidermal melanophores were more sensitive than the dermal melanophores to the second and third injections. The minimum mean EMI attained was 1.2 compared with 1.8 for the DMI (Fig. 4); for the second injection, individual EMI values of 1.0 were obtained for five of the six flounders whereas only one flounder had a DMI of 1.0. In response to BSS control injections, melanosomes of both dermal and epidermal melanophores remained in a highly dispersed condition after 10 min. Pre-injection mean DMI and EMI were 4.9±0.1 and 5.0, respectively, while 10 min after BSS injection corresponding values were 4.7±0.2 and 4.8±0.2, neither difference being statistically significant ($P>0.05$).

Discussion

The current microscopic description of the enhancement of melanophore responses by repetition of albedo changes is consistent with earlier (Sumner, 1911; Mast, 1916; Osborn, 1939) macroscopic accounts in flatfish. However, it is now becoming apparent that this phenomenon is not limited to the effect of repeated cycles of background changes.

The degree of enhancement during repeated background change cycles is remarkable in flounders. A clearly identifiable physiological 'priming' is associated with an initial chromatic response to sharply contrasted backgrounds under experimental conditions and with variable degrees of enhancement during subsequent melanosome translocation. Sumner (1911) suggested that such enhancement represents a form of habituation. However, in current usage this term describes a simple form of learning with a gradual waning of response to repeated stimulation. This does not describe the present phenomenon as initial extensive melanosome translocation makes it easier for subsequent responses to occur and 'facilitation' appears to be a more appropriate description. The present use of an *in vitro* protocol in conjunction with repeated transfers of flounders between contrasting backgrounds has demonstrated clearly that such facilitation of chromatic responses is essentially a peripheral phenomenon, rather than one involving central regulatory change. Classical neuromuscular facilitation involves summation and increasing amplitude of contraction in response to repeated stimulation. However, repeated stimulation of the melanophores increased the rate of the responses, which were much longer in duration than muscle contraction.

The sympathetic nerve endings and melanophores on scale slips represent miniature neuroeffector preparations in which the antagonistic actions of K^+ and Na^+ can be interpreted (Burton and Everard, 1990) solely in terms of neuronal

depolarization and repolarization, as in action potentials, regulating noradrenaline release. Abbott (1968) has suggested that if melanophores are regulated only by melanosome-aggregating nerves, dispersion may result from any interruption to the activity of these nerves by any means.

In goldfish scale-slip incubations, the epidermis has been described as a barrier to the diffusion of adrenaline at low concentrations, but not at high concentrations, and EDTA has been employed to remove this epithelium (Stone and Chavin, 1974). In flounder, the use of 'complete' scale slips enables data to be collected for the epidermal as well as for the dermal melanophore neurally mediated responses to the high ionic concentrations in the incubation media. Also, the scale-slip *stratum spongiosum* tissue severed at plucking provides an alternative diffusion pathway above the scale through which K^+ and Na^+ can reach the dermal neurones associated with the melanophore area observed, which seldom extends more than 300 μm from the severance plane. The rapidity of flounder melanosome aggregation in response to K^+, compared with *in vivo* aggregation, reflects the universality of the neuronal depolarization in these preparations. Thus, the melanosome-aggregating effect of K^+ is not dependent on diffusion gradients to individual melanophores, but on the rapid spread of depolarization as these ions come into contact with nerve endings close to the severance plane. Also, the pronounced facilitation of the *in vitro* K^+-evoked melanosome aggregation in the current work demonstrates that these preparations are sensitive to the enhancement of the holistic chromatic responses of flounder to repeated changes of background. Interestingly, melanophores from flounders exposed to a black background for a week or longer also display facilitated melanosome aggregation and dispersion, respectively, when scale slips are subjected to repeated cycles of K^+ and Na^+ incubation *in vitro* (Burton and Everard, 1991).

Plasma-borne injected neurotransmitters can stimulate a multiplicity of potential regulatory levels, making interpretation of results difficult. However, it is clear that, compared with the response to black–white background change, melanophores from black-adapted flounders have the capacity to respond relatively rapidly, and with facilitation, to the dose of noradrenaline injected. Also, the relative rapidity of *in vitro* melanosome aggregation in response to K^+-evoked universal depolarization of the scale-slip nerve endings before black-adapted flounders experienced albedo change is indicative of the adequacy of the neuronal noradrenaline reserves and also of the capacity of the melanophores for melanosome aggregation before the flounder displayed facilitation of the chromatic responses. Thus, before initial transfer between backgrounds, the flounder neuromelanophore system has the potential for a more rapid melanosome aggregation in response to a relatively high concentration of neurotransmitter than is observed during the first or subsequent cycles of albedo change. However, *in vivo*, flounder melanophores are unable to respond rapidly to physiological noradrenaline levels released by depolarization at nerve endings as a result of photic stimulation without preliminary priming. The enhancement of melanosome aggregation during repeated albedo changes could be explained by increases in the physiologi-

cal levels of released noradrenaline and in adrenoceptor frequency. However, the increase in melanosome dispersion rate during repeated exposures of flounders to a black background indicates that it is the melanosome translocation process that is involved in this type of facilitation. Although the cellular mechanism driving pigment movement is not understood (Schliwa, 1986), available evidence (Luby and Porter, 1980; Porter and Tucker, 1981; Schliwa, 1984, 1986; Obika, 1986) implicates a relatively primitive system based on the cytoskeleton, with microtubules defining the channels in which melanosomes migrate. It is a matter of dispute whether microtubules undergo cycles of disassembly and reassembly during pigmentary responses (Obika, 1986). The current data from experiments with noradrenaline *in vivo* and K^+ *in vitro* suggest that, in flounders, the intracellular channels for melanosome translocation can function effectively in response to high noradrenaline concentrations before any physiological priming and that any subsequent facilitation involves a modulation of existing microtubular channels, possibly involving a smoothing process. However, ultrastructural studies on flounder skin have so far not revealed (D. Burton and M. Hatcher, unpublished observation) a microtubular system that is as clearly organized as that described for some teleosts (Obika, 1986; Schliwa, 1986). This may account for the generally slow responses of flounder melanophores.

Epidermal melanophores are not universally found in teleosts and are not considered important in vertebrate physiological colour change (Bagnara and Hadley, 1973). In contrast to their role in morphological colour change in flounders, which is distinct from that of dermal melanophores (D. Burton, unpublished data), the current results demonstrate that their physiological responses are similar to those of dermal melanophores. The spatial relationship between epidermal melanophores and the integumentary innervation remains to be established. Based on the relatively high sensitivity of the epidermal melanophores to plasma-borne injected noradrenaline, their normal neural regulation could involve relatively low neurotransmitter levels associated with a field effect. Ultrastructural and fluorescent histochemical studies suggest (Fujii and Oshima, 1986) that within the dermis many axons pass close to melanophores, releasing neurotransmitter along their length to control a number of melanophores. This suggests that catecholaminergic regulation may have paracrine neurohormonal characteristics in this system with associated local field effects, which may be important in epidermal melanophore control.

The effect of repeated chromatic stimulation in teleosts has been neglected in recent years. The current work demonstrates that enhancement of melanophore physiological activity can be induced by different experimental protocols and it is suggested that the phenomenon represents a form of facilitation occurring peripherally at the melanophore level. The possibility of such facilitation is clearly a factor that must be considered in designing comparative experiments on teleost melanophores. The phenomenon also gives rise to interesting questions about the relationship between local habitat variability and the capacity for rapid chromatic adaptation under environmental conditions.

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