AN IN VITRO STUDY OF α AND β ADRENOCEPTORS IN MELANOSOME DIFFERENTIAL AGGREGATION AND DISPERSION ASSOCIATED WITH CRYPTIC PATTERNING IN WINTER FLOUNDER (PLEURONECTES AMERICANUS)

DENNIS JOSEPH MAYO

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AN *IN VITRO* STUDY OF α AND β ADRENOCEPTORS IN MELANOSOME DIFFERENTIAL AGGREGATION AND DISPERSION ASSOCIATED WITH CRYPTIC PATTERNING IN WINTER FLOUNDER (*PLEURONECTES AMERICANUS*)

BY

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ABSTRACT

The remarkable cryptic patterning capability of flatfish gives rise to important questions about the regulatory processes which must be involved in the differential control of melanophores. Winter flounder, *Pleuronectes americanus*, possess three distinct pattern components on its dark upper (ocular) surface that are important for cryptic patterning. These are the white spot, general background and dark band components that display differential chromatic responsiveness which has been studied using electrophysiological and pharmacological methods in the current work.

In vitro experiments with K^+ rich aggregating fluid (AF) or noradrenaline and phentolamine demonstrate that α-adrenoceptors mediate melanosome aggregation. Studies examining the effects of increasing concentrations of prazosin and yohimbine on melanosome aggregation evoked by electrical stimulation indicate that both α_1- and α_2-adrenoceptors play a role in melanosome aggregation, but there is a predominance of the latter. Experiments with atenolol suggest that there may be some β-adrenoceptor synergism involved in pigment aggregation.

Topical application of low concentrations of isoproterenol and noradrenaline enhance rates of *in vitro* melanosome dispersion in balanced salt solution which could be depressed with propranolol and suggests β-adrenoceptor mediation. The subtype of this adrenoceptor appears to be most likely the β_2 conformation based on studies with terbutaline. Denervation of scale slips in 6-OH dopamine followed by incubation in a

high concentration of isoproterenol and propranolol results in complete melanosome aggregation. Incubation of denervated scale slips in the same concentration of isoproterenol and phentolamine results in the blockade of pigment aggregation.

This study shows that α- and β_2-adrenoceptors play roles in pigment aggregation and dispersion respectively and that the melanophore response that is initiated is dependent upon neurotransmitter concentration. High concentrations of noradrenaline or isoproterenol evoke melanosome aggregation via α-adrenoceptors while low concentrations induce melanosome dispersion via β_2-adrenoceptors.

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1. INTRODUCTION

Many fish and other vertebrates are able to alter the shade or color of their skin. This is accomplished by an intracellular pigment redistribution within chromatophores which has been reviewed in general terms for lower vertebrates by Bagnara and Hadley (1973). It is commonly known that this redistribution is considered to be a physiological change and it is in contrast to a morphological change which is due to differences in the amount of pigment stored in the chromatophores or in the number of chromatophores (Abbott, 1973; Fujii and Oshima, 1986). Flatfish chromatophores are commonly classified into various categories depending on the colors they display. These include the black melanophores, red erythrophores, yellow xanthophores, and reflecting iridophores (Cunningham and McMunn, 1893)

Histological studies conducted by Kuntz (1917) on the flounder, *Paralichthys*, determined that melanophore cell shape is not altered during paling or darkening of the skin. Instead he found that the color change resulted from pigment movement either toward or away from the center of the cell. The conventional terminology used to describe this pigment movement is aggregation of melanosomes (pigment containing organelles) toward the center of the cell while dispersion refers to the opposite process.

There is great diversity among fish with regards to the basic mechanisms

underlying melanophore control which have been summarized by Khokhar (1971). It is thought that the endocrine system might predominant in some fish , e.g. *Anguilla anguilla* (Neill, 1940), while in others like *Macropodus opercularis*, the nervous system seems to be the most important controlling factor (Umrath and Walcher, 1951). There also appears to be varying levels of coordination between the hormonal and neural regulatory mechanisms in some other fish (Khokhar, 1971).

Pouchet (1876) and von Frisch (1911) established that the coordination of teleost color changes is under neural control. The central controlling mechanism is poorly understood in comparison to what is known about the peripheral mechanism. However, location of a paling center in the medulla is a landmark study by von Frisch (1911) on *Phoxinus*. Nerve fibres from this centre run down the spinal cord and into the sympathetic chains at localized spinal outflow levels and flow by spinal nerves to the skin.

Pigment aggregation occurs when the sympathetic fibers that innervate the melanophores are stimulated electrically. It has been shown that noradrenaline acts as the neurotransmitter, being released from the presynaptic nerve endings and exerting its postsynaptic effects on the melanophores (Fujii and Oshima, 1986; Kumazawa and Fujii, 1984).

Parker (1934) and Parker and Rosenblueth (1941) suggested that teleost melanophores are doubly innervated based on work with a catfish, *Ameiurus*. The

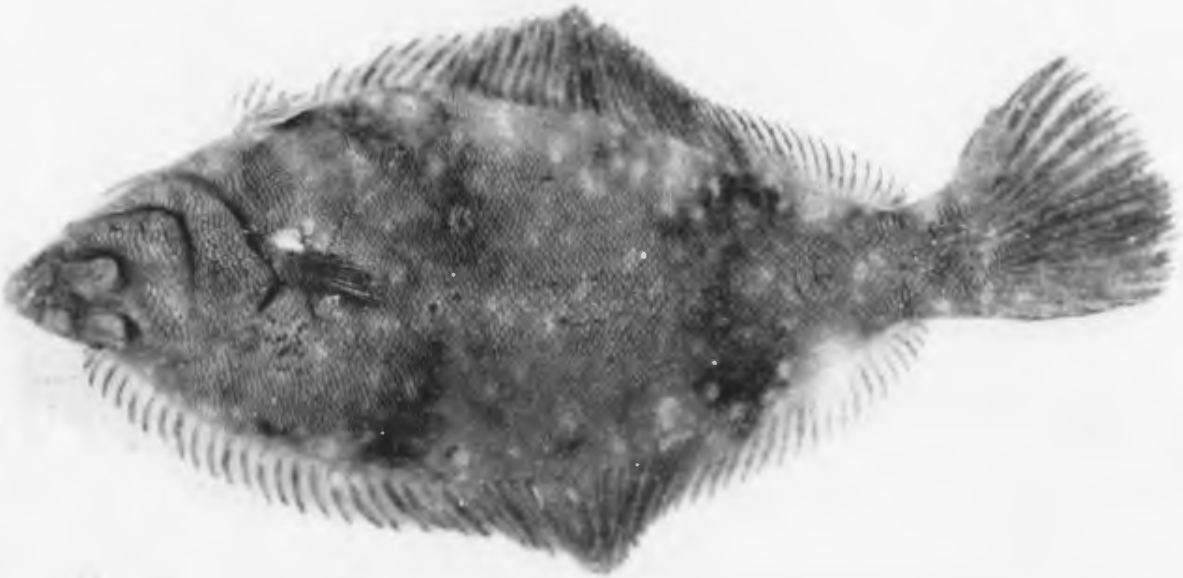
authors proposed that in addition to the sympathetic adrenergic transmission, there is also a second cholinergic transmission route which is melanosome dispersing. This regulatory mechanism has been the subject of controversy over the years and does not receive much support currently (Fujii and Oshima, 1986).

Winter flounder, *Pleuronectes americanus* (formerly *Pseudopleuronectes americanus*), is a flatfish that has a dark upper surface including three distinct pattern components (Figure 1). These are the white spots, general background and dark band pattern components which are morphologically distinct in that each of these components contains varying proportions of dermal and epidermal melanophores. The dark bands have the most epidermal melanophores, the white spots have few or none, while the general background component has an intermediate number of epidermal melanophores (Burton, 1980).

In addition to the morphological differences, it has been observed macroscopically (Osborn, 1939) and microscopically (Burton, 1975, 1980) that the pattern components are physiologically different as well. Previous studies have indicated that the physiological responses of the melanophores in all three pattern components are under neural control in the winter flounder (Burton, 1981).

Burton (1975,1980) established at the cellular level that the dark band melanosomes dispersed rapidly when transferred from a white to a black background and aggregated slowly when transferred from a black to a white background. General

Figure 1. Winter flounder (*Pleuronectes americanus*) after one hour on a white background

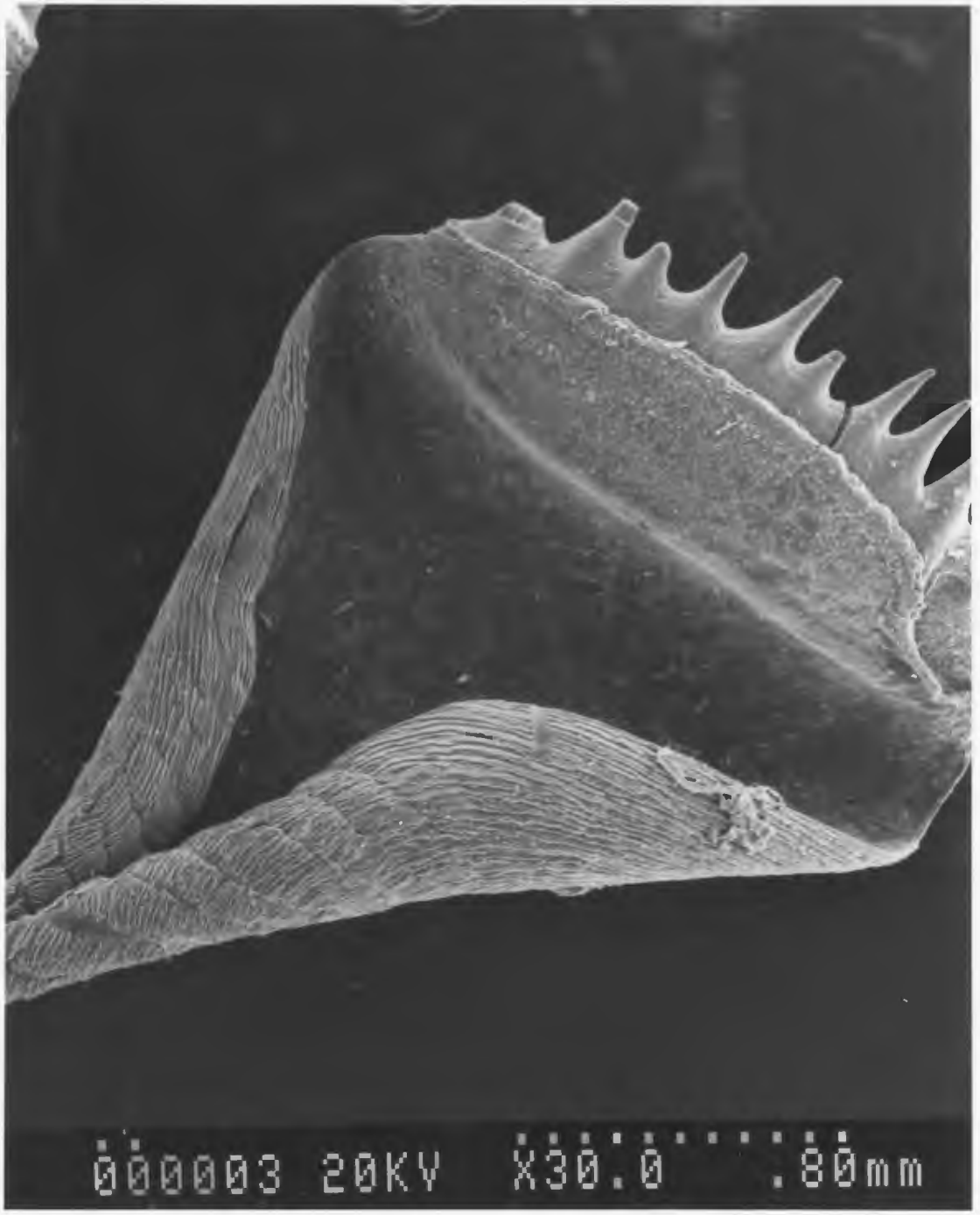


background melanosomes initially exhibited rapid aggregation when transferred to a white background, but complete aggregation was not seen until 1-6 days later, whilst on black background initial melanosome dispersion was faster in the dark bands, although maximum dispersion was not observed until 3-6 days later. The white spots were quite different as compared to the other two pattern components and can be extremely pale in otherwise dark fish. The shade of the white spots can be influenced by factors other than background, such as stress and the same is true for the dark bands in paled flounder (Osborn, 1939; Burton, 1980).

Teleost scale slips (Figure 2) have been used *in vitro* to study the physiological and pharmacological properties of melanophores since Spaeth's (1913) classical work on *Fundulus*. Previous studies with flounder scale slips from each pattern component have demonstrated that the melanophores display pattern-related variability with respect to rates of aggregation and dispersion (Burton and Snow, 1993). It was found that the white spot melanosomes displayed the fastest responsiveness to K^+-rich melanosome aggregating fluid and the dark band melanosomes were the slowest, with the general background melanosomes being intermediate. With respect to melanosome dispersion induced by Na^+ ions, white spots responded the fastest followed by the dark band and general background melanosomes.

More recently, Burton et al. (1995) investigated the role of adrenoceptors in

Figure 2. Scanning electron micrograph of a winter flounder scale slip (including 0.8 mm reference point)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cryptic patterning of winter flounder. *In vitro* it was demonstrated that α-adrenoceptors mediate melanosome aggregation in all pattern components, with the relative potencies of the catecholamines being in accordance with Ahlquist (1948) criteria (i.e. noradrenaline > adrenaline > isoproterenol) and on the effect of the α-adrenoceptor antagonist phentolamine.The fact that isoproterenol (β-adrenoceptor agonist) can evoke melanosome aggregation which can be reversed by propranolol (β-adrenoceptor antagonist) (Burton et al., 1995) suggests that β-adrenoceptors may have a modulatory role in pigment movements in this species.

A drawback with the pharmacological approach is that the epidermis on the scale slips acts as a partial barrier for the diffusion of topically applied drugs (Spaeth, 1913; Stone and Chavin, 1974). To utilize the endogenous neurotransmitter, a method of electrical stimulation of isolated scale slips has been developed which stimulates its release (Andersson et al., 1984; Fujii and Oshima, 1986). The primary objective of the present study was to investigate the physiological differences between the general background, dark band and white spot pattern components using electrophysiological and pharmacological methods. A secondary objective was to study the cellular features affecting melanosome aggregation and dispersion in all three pattern components (general background, dark band and white spots) in order to better understand the nature of the melanosome control mechanisms at the cellular level.

2. MATERIALS AND METHODS

2.1 General

Winter flounder were collected by SCUBA divers using handnets near St. John's, Newfoundland. The fish were maintained in stock tanks supplied with running seawater at ambient temperature and under seasonal photoperiod conditions. The winter flounder that were to be used for experiments were transferred to "Plexiglass" black aquaria (400mm x 225mm x 203mm) supplied with running seawater at ambient temperature. These fish were maintained under seasonal photoperiod using 60 watt incandescent illumination 1 meter above the standardized tank conditions, illumination conditions which result in complete melanosome dispersion on black background without observably stressing the fish. The fish were chromatically adapted to these tanks for at least 2 days before experiments were conducted.

Scale slips required for each experiment were obtained by plucking scales with fine forceps from each pattern component located on the dark upper surface of the winter flounder. The scale slips were incubated in a balanced salt solution (BSS) composed of the following in mM: NaCl, 175.0; KCl, 2.7; $NaHCO_3$, 5.0; $MgCl_2 6H_2O$, 0.64; $CaCl_2$, 1.53; glucose, 5.6. BSS being relatively Na^+ rich can serve as a melanosome dispersing fluid. Another incubation solution used was melanosome aggregating fluid (AF) composed of the following in mM: NaCl, 127.9; KCl ,49.8; $KHCO_3$, 5.0; $MgCl_2.6H_2O$, 0.64; $CaCl_2$, 1.53; glucose, 5.6. Thin glass culture microslides containing 0.33-0.4 ml of

incubation medium, mounted on a thermal stage (Bailey instruments, Model TS-2), were used to conduct experiments. All experiments were performed at 10°C which corresponds to the ambient seawater temperature during the time most experiments were conducted. Experimental results are based on 10 scale slips from each pattern component

2.2 Scale slip and melanophore morphological measurements

Measurements of the diameter of the intracellular pigment mass were made in order to estimate the relative sizes of melanophores from the different pattern components. The melanophore measurements were made using a calibrated ocular grid while viewing scale slips at 100x magnification. The diameter of the pigment mass in a fully aggregated melanophore was measured. However, measuring fully dispersed melanosome mass was more difficult given its highly irregular shape associated with the melanophore processes. As a result of this the maximum diameter was recorded for each pigment mass. Mean (± SEM) values were calculated based on 50 size estimates for each pattern component.

Skin region measurements were taken using a calibrated ocular grid while viewing scale slips at 40x magnification. When measuring this region, measurements were taken along axes which corresponded to the anterior-posterior axis and transverse

axis of the fish. Due to the highly irregular shape of the slips of skin on the scales, the maximum value for each dimension was recorded. Mean (± SEM) values were calculated based on measurements taken from 10 scale slips for each pattern component.

2.3 Neurotransmitter agonists and antagonists

Solutions 10^{-3}M in BSS of phentolamine mesylate (α-adrenoceptor antagonist) (Ciba-Geigy, Montreal, Quebec, Canada), L-propranolol hydrochloride (β-adrenoceptor antagonist) (Sigma, St. Louis, Missouri, U.S.A.), prazosin hydrochloride (α_1-adrenoceptor antagonist) (Sigma), yohimbine hydrochloride (α_2-adrenoceptor antagonist) (Sigma), terbutaline hemisulfate salt (β_2-adrenoceptor agonist) (Sigma), and atenolol (β_1-adrenoceptor antagonist) (Sigma) were prepared. The adrenoceptor activity of each of these compounds is based on mammals and is described in the source book by Bevan (1983). Serial dilutions in BSS were used for concentration-response experiments with scale slips being equilibrated to equal logarithmic concentration increments for 10 min following a brief wash with each new solution. For electrical stimulation experiments, the appropriate electrical stimulation parameters as determined were applied following the equilibration period.

Mixtures in BSS of phentolamine (10^{-3}M) with L-noradrenaline bitartrate (3.15 x 10^{-6}M) (Sigma), terbutaline (10^{-3}M) with L-noradrenaline (3.15 x 10^{-6}M and 10^{-6}),

atenolol (10^{-3}M) with L-isoproterenol hydrochloride (β-adrenoceptor agonist in mammals (Bevan, 1983)) (10^{-4}M and 3.15 x10^{-5}M) (Sigma) were also prepared. The first reagent in each mixture listed was serially diluted with a solution of BSS and the second reagent, whose concentration remained constant. Concentration-response experiments were conducted as indicated above.

Solutions in BSS of L-isoproterenol (3.15 x 10^{-4}M, 10^{-7}M, 10^{-8}M, and 10^{-10}M) and noradrenaline (10^{-8}M, 10^{-9}M, and 10^{-10}M) were prepared for time-response experiments. Mixtures in BSS of L-propranolol (10^{-4}M) with noradrenaline (10^{-10}M), L-propranolol (10^{-4}M) with isoproterenol (10^{-8}M and 3.15 x 10^{-5}M), L-isoproterenol (3.15 x 10^{-4}M) with L-propranolol (10^{-4}M), and phentolamine (10^{-4}M) with isoproterenol (3.15 x 10^{-4}M) were prepared for time-response experiments as well.

Solutions of phentolamine mesylate (10^{-3}M) in AF were prepared and serially diluted with AF for use in concentration response experiments in a manner similar to that described previously.

All solutions were prepared fresh before the start of each experiment and kept on ice while the experiment was being conducted. 1 ml pipettes were used to transfer the required volume of the solution to the culture microslide.

2.4 Comparison of melanosome aggregation and dispersion by K^+ and Na^+

Before conducting pharmacological experiments, all scale slips were immersed in BSS for 15 min. Where specific experiments dictated, this was followed by a 15 min incubation in K^+ enriched aggregating fluid (AF) to evoke complete melanosome aggregation.

2.5 Electrical stimulation

Two silver wires (1 mm diameter) were mounted 10 mm apart on a culture microslide containing 0.3 ml of solution. This setup permitted the observation of a single winter flounder scale slip during electrical stimulation. The silver wires were coated with AgCl by immersing the wires in a 2% KCl solution, applying 15 volts DC for 30 sec, then reversing the polarity and repeating to coat the other electrode. A Grass S88 stimulator was used to deliver the appropriate electrical stimulation as determined for each pattern component. An oscilloscope (Topward, model 7025) was used to monitor the voltage being applied to the scale slips. Scale slips were placed on the microslide such that the transverse axis of the skin region was placed across the two electrodes.

All experiments were conducted using 10 scales slips from each pattern component that had been incubated in BSS for at least 15 min. For the time course for melanophore responses to electrical stimulation, individual scale slips were transferred to

the microslide containing 0.3 ml of BSS. Following this, electrical stimulation with parameters determined as appropriate for each particular pattern component was applied with melanophore responses being recorded at 5 sec intervals.

The effect of repeated electrical stimulation on the capacity of dermal melanophores to respond was determined by subjecting scale slips incubated in BSS to trains of electrical stimulation once every 10 min for 80 min.

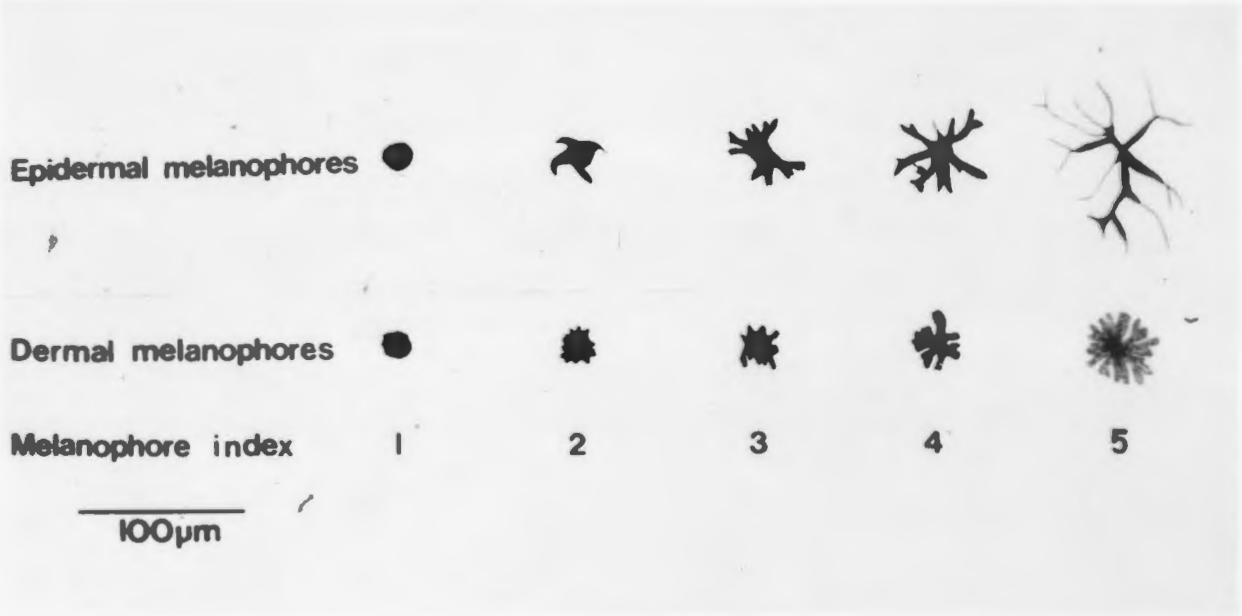
2.6 Denervation of scale slips

Scale slips from the general background pattern component were denervated by incubation in various concentrations of 6-OH dopamine hydrochloride (Sigma) for varying time periods. Following incubation the scale slips received 2 x 20 min washes with BSS. After this the scale slips were subjected to electrical stimulation to check the success of the denervation process.

2.7 Estimation of melanosome aggregation and dispersion and statistical analysis

The dermal and epidermal melanophore indices (DMI and EMI) were used to estimate the degree of overall melanosome aggregation or dispersion (Figure 3) (Hogben and Slome, 1931). All DMI and EMI values are means ± SEM for 10 scale slips. In concentration-response experiments, logarithmic transformation of drug concentrations

Figure 3. Dermal and epidermal melanophore indices


Epidermal melanophores
Dermal melanophores
Melanophore index
1
2
3
4
5
100μm

were used to provide data for obtaining the geometric mean EC_{50} with 95% confidence interval based on individual curves (Fleming et al., 1972). ANOVA and LSD or T-tests were used for statistical comparisons of the geometric mean EC_{50} where appropriate. For statistical comparisons of mean melanophore index values the non-parametric Kruskal-Wallis test or Mann-Whitney U test was used since the indices represent a ranking procedure. The Kruskal-Wallis test was used for overall comparisons between three or more sets of data and the Mann-Whitney U test for comparisons between pairs of data.

3. RESULTS

3.1 Scale slip and melanophore morphological measurements

Table 1 gives the mean diameters of the intracellular pigment areas in dermal melanophores with both fully aggregated and fully dispersed melanosomes. In both of these conditions values for white spot melanophores are the smallest, those for general background melanophores are intermediate in size while those for the dark band melanophores are the largest. These differences are statistically significant for both the fully dispersed (Anova: $F=19.02$, $df=2,47$, $p<0.001$) and fully aggregated conditions (Anova: $F=48.91$, $df=2,47$, $p<0.001$). For the fully dispersed state, it was found that the difference between the general background and dark band melanophores was significant at the 0.05 level while the other comparisons were significant at the 0.001 level. However for the fully aggregated state it was found that all comparisons were equally statistically significant at the 0.001 level.

The dimensions of the skin region of scale slips from each pattern component are summarized in Table 2. The dimensions of the skin region are of particular interest since this is where the melanophores are located. The differences in the dimensions of the anterior-posterior axis and transverse axis are statistically significant (Anova, $F=11.44$, $df=2,27$, $p<0.001$ for each dimension). Least significant differences were calculated for each dimension. It was found by L.S.D. that all comparisons were equally statistically

Table 1. Mean diameters of the intracellular pigment mass in individual dermal melanophores of *Pleuronectes americanus* when fully aggregated and fully dispersed in each pattern component

	White Spots	General Background	Dark Bands
Fully dispersed state	47.7μm (± 1.61)	62.4μm (± 1.84)	71.8μm (± 1.40)
Fully aggregated state	15.0μm (± 0.36)	22.1μm (± 0.53)	27.0μm (± 0.55)

Note: Average values (for 50 size estimates) are followed by the SEM.

Table 2. Dimensions of the skin region of scale slips from each pattern component of *Pleuronectes americanus*

Pattern Component	Anterior-posterior axis (mm)	Transverse axis (mm)
White Spots	0.73 ± 0.03	2.06 ± 0.08
General Background	0.94 ± 0.04	2.63 ± 0.07
Dark Bands	0.77 ± 0.02	2.58 ± 0.08

Note: measurements are based on ten scale slips from each pattern component
values = mean ± SEM

significant ($p<0.001$) for the anterior-posterior axis measurements but for the transverse axis measurements the general background and dark band comparison was significant at the 0.05 level while the other comparisons were significant at the 0.001 level.

3.2 Comparison of melanosome aggregation and dispersion by K^+ and Na^+

Incubation of melanophores from each pattern component in BSS for 15 min resulted in complete melanosome dispersion. Subsequent transfer to AF gave complete dermal melanophore pigment aggregation for each pattern component (Figure 4A). This aggregation occurring after 4 min, 8 min, and 11 min for white spot, general background and dark band dermal melanosomes respectively. These pattern related differences were statistically significant after 4 min (Kruskal-Wallis: $H=21.79$, $df=2$, $p<0.001$). Melanosome aggregation in epidermal melanophores followed a similar trend (Figure 5A) which was statistically significant after 7 min (Mann-Whitney: $n_1=n_2=10$, $U=93$, $p<0.001$).

Dermal melanophores from each pattern component exhibited complete melanosome aggregation after a 15 min incubation in aggregating fluid (AF). After transfer back to balanced salt solution (BSS) (i.e. without K^+ stimulation), within 3 min for the white spots and 7 min for the other components, melanosomes in dermal melanophores were completely dispersed (Figure 4B). Pattern related differences after 3

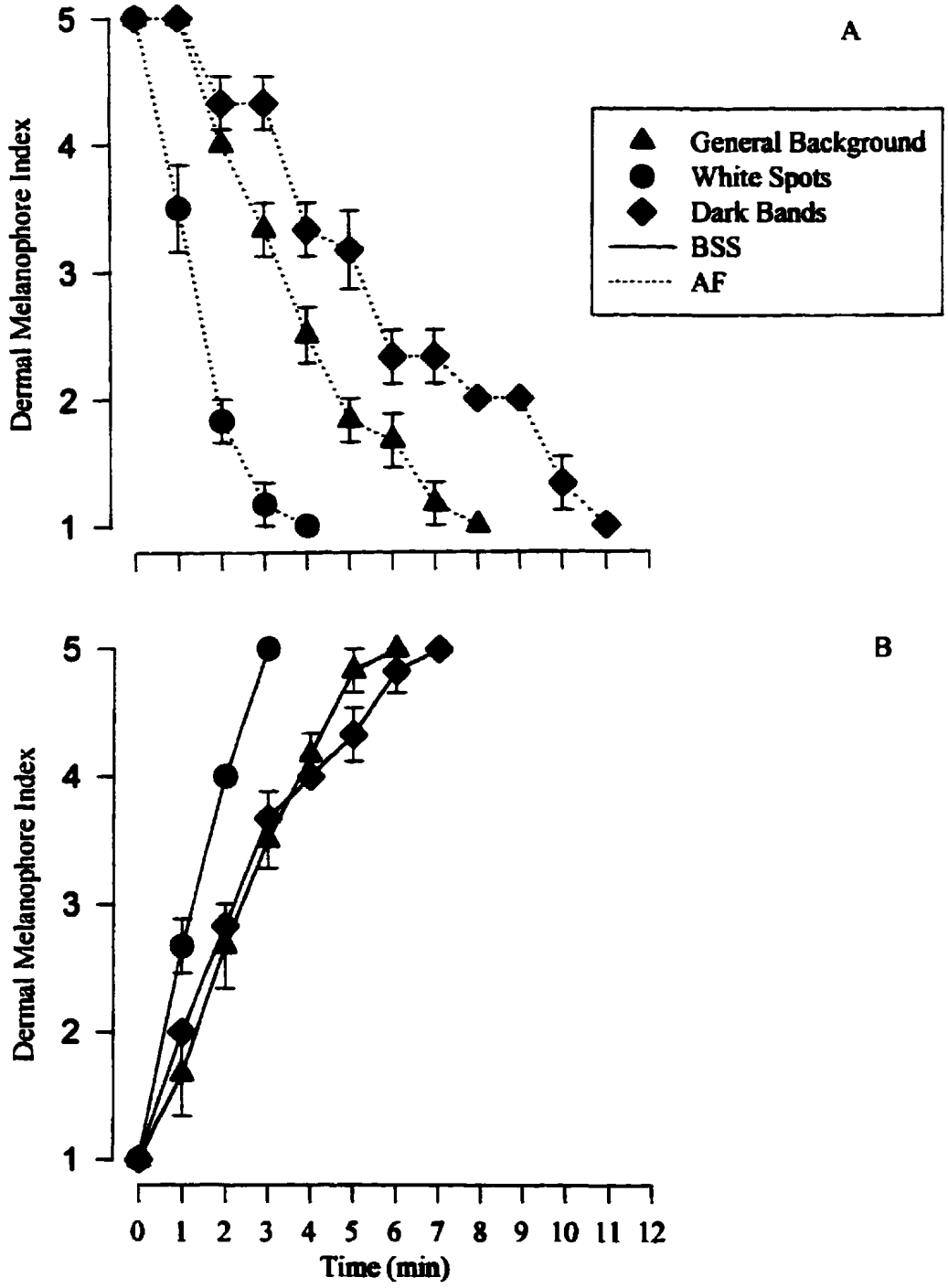


Figure 4. *In Vitro* rates of pigment aggregation and dispersion in dermal melanophores
A) Melanosome aggregation induced by K^+ enriched AF
B) Melanosome dispersion induced by BSS

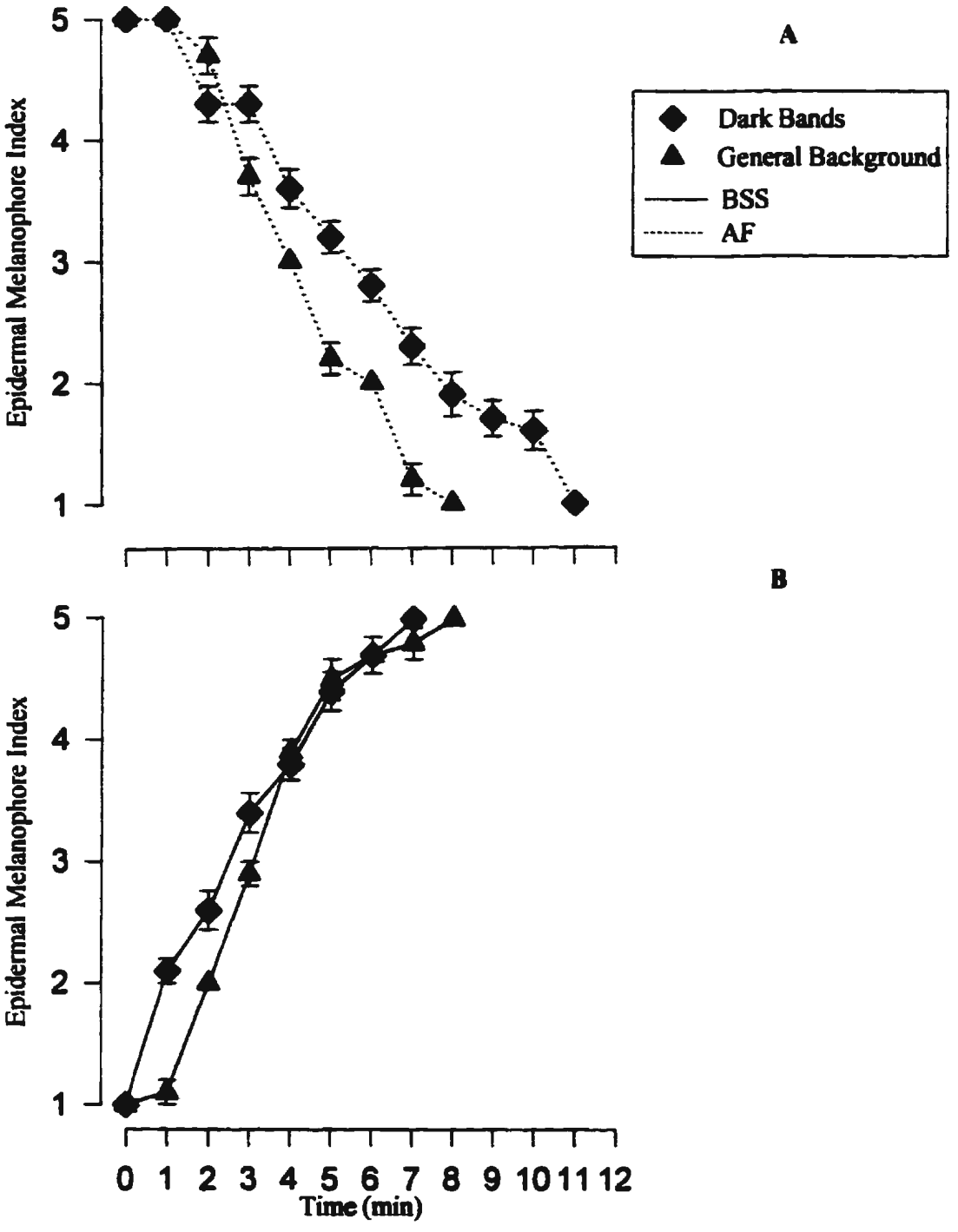


Figure 5. *In Vitro* rates of pigment aggregation and dispersion in epidermal melanophores
A) Melanosome aggregation induced by K^+ enriched AF
B) Melanosome dispersion induced by BSS

min were statistically significant (Kruskal-Wallis: $H=17.47$, $df=2$, $p<0.001$). Similarly, pigment dispersion in epidermal melanophores occurred within 7 min for the dark bands and 8 min for the general background (Figure 5B). These pattern related differences were statistically significant after 1 min (Mann-Whitney: $n_1=n_2=10$, $U=95.5$, $p<0.001$).

3.3 Comparison of phentolamine induced dispersion in K^+ and noradrenaline

Concentration-response experiments with the α-adrenoceptor antagonist, phentolamine in AF and phentolamine in noradrenaline (3.15×10^{-6}M) result in pattern related differences in melanosome dispersion in dermal melanophores (Figure 6). 3.15×10^{-6}M noradrenaline was used as this is the lowest concentration that will consistently induce complete melanosome aggregation. Melanosomes from all pattern components incubated in solutions of progressively stronger phentolamine in AF dispersed at lower concentrations of phentolamine than those incubated in solutions of progressively stronger phentolamine in noradrenaline (3.15×10^{-6}M). This is indicated by the EC_{50} values for each pattern component (Table 3). Comparisons between the pattern components were statistically significant for each experiment (Anova: $F=228.05$, $df=2,15$, $p<0.001$) and comparisons between drug treatments were statistically significant for each pattern component (T-test, $p<0.001$). The epidermal melanophores followed the same general trends as the dermal melanophores.

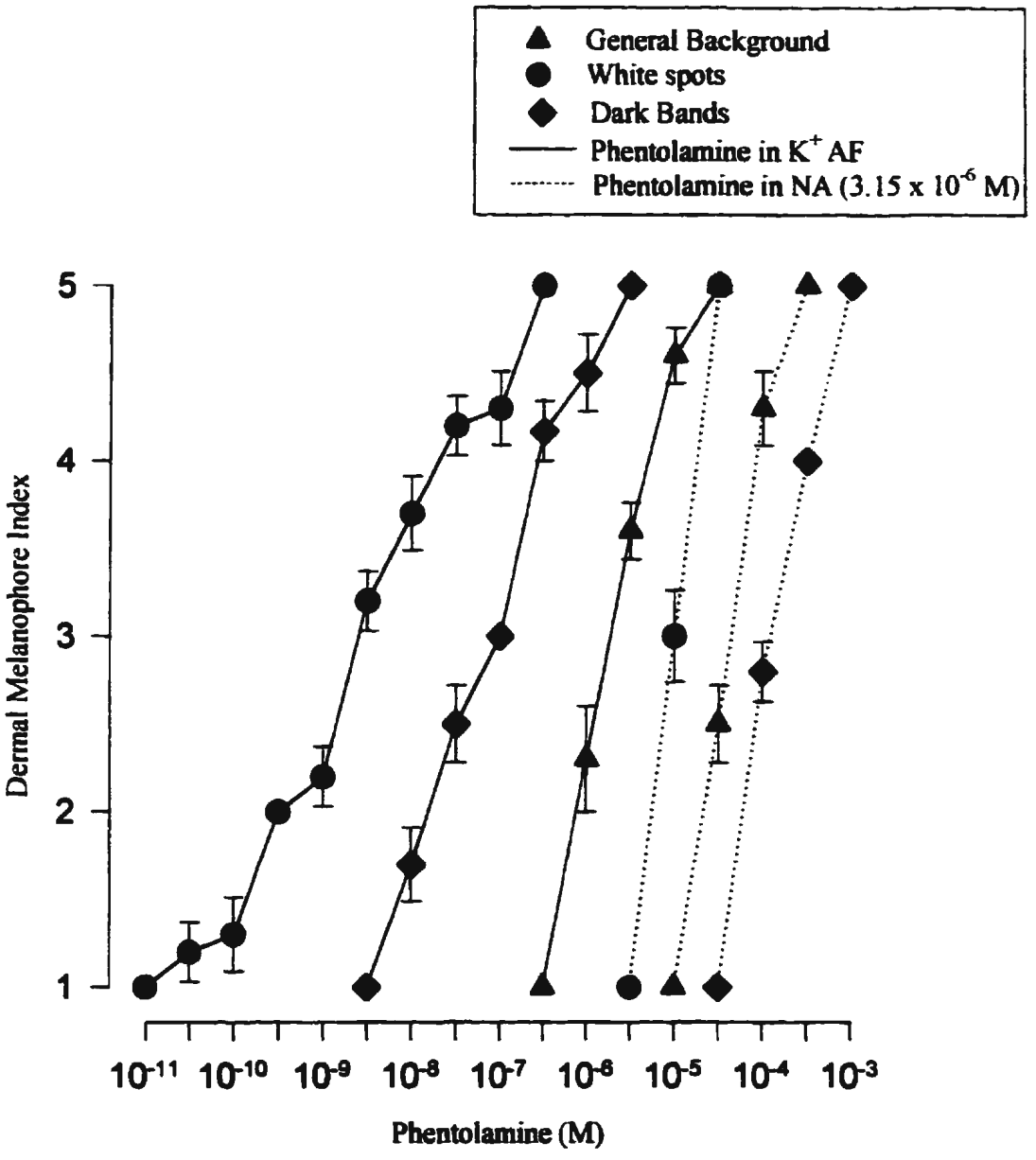


Figure 6. Concentration response curves for phentolamine in K^+ enriched aggregating fluid (AF) and in noradrenaline (3.15×10^{-6}M)

Table 3. Ec_{50} values with 95% confidence limits for melanosomes incubated in solutions of phentolamine in aggregating fluid (AF) and in solutions of phentolamine in noradrenaline (3.15×10^{-5}M) (n=6 scale slips for each pattern component)

	White Spots	General Background	Dark Bands	ANOVA
Phentolamine in aggregating fluid	**2.85×10^{-9}M (1.37×10^{-9}M - 5.93×10^{-9}M)**	**2.47×10^{-6}M (1.95×10^{-6}M - 3.12×10^{-6}M)**	**7.03×10^{-8}M (5.22×10^{-8}M - 9.46×10^{-8}M)**	**F=228.35 df= 2,15 p<0.001**
Phentolamine in noradrenaline solution	**1.00×10^{-5}M (7.78×10^{-6}M - 1.30×10^{-5}M)**	**4.47×10^{-5}M (3.32×10^{-5}M - 6.01×10^{-5}M)**	**1.50×10^{-4}M (1.37×10^{-4}M - 1.64×10^{-4}M)**	**F=227.05 df=2,15 p<0.001**
T-tests	**t=27.285, df=10 p<0.001**	**t=8.072, df=10 p<0.001**	**t=63.588, df=10 p<0.001**	

3.4 Electrical stimulation

The specific electrical stimulation parameters required to induce complete melanosome aggregation in dermal melanophores in each pattern component are shown in Table 4. The voltages given are the lowest which consistently induce complete melanosome aggregation for each pattern component. Noteworthy is the fact that no electrical stimulation parameters could be established which would induce melanosome aggregation in epidermal melanophores.

An experiment utilizing the electrical stimulation parameters in Table 4 investigated the time course for melanosome aggregation in dermal melanophores for each pattern component followed by melanosome dispersion (Figure 7). It was found that the white spots and general background melanosomes in dermal melanophores aggregated the fastest followed by those of the dark band. At 20 sec this pattern-related difference was statistically significant (Kruskal-Wallis: $H=14.31$, $df=2$, $p<0.001$). Overall, the melanosome aggregation induced by electrical stimulation was much faster than that observed with AF (Figure 4A).

After termination of the electrical stimulation, complete melanosome dispersion occurs in dermal melanophores. This dispersion is fastest for the white spots where complete melanosome dispersion occurs within 25 sec after cessation of electrical field

Table 4. Electrical stimulation parameters for complete melanosome aggregation in dermal melanophores from each pattern component of *Pleuronectes americanus*

Pattern Component	Frequency (Hz)	Pulse Duration (ms)	Train (s)	Volts (output)	Volts (delivered)
General Background	10	2	30	45	26
White Spots	10	2	30	62	36
Dark Bands	2	2	35	89	52

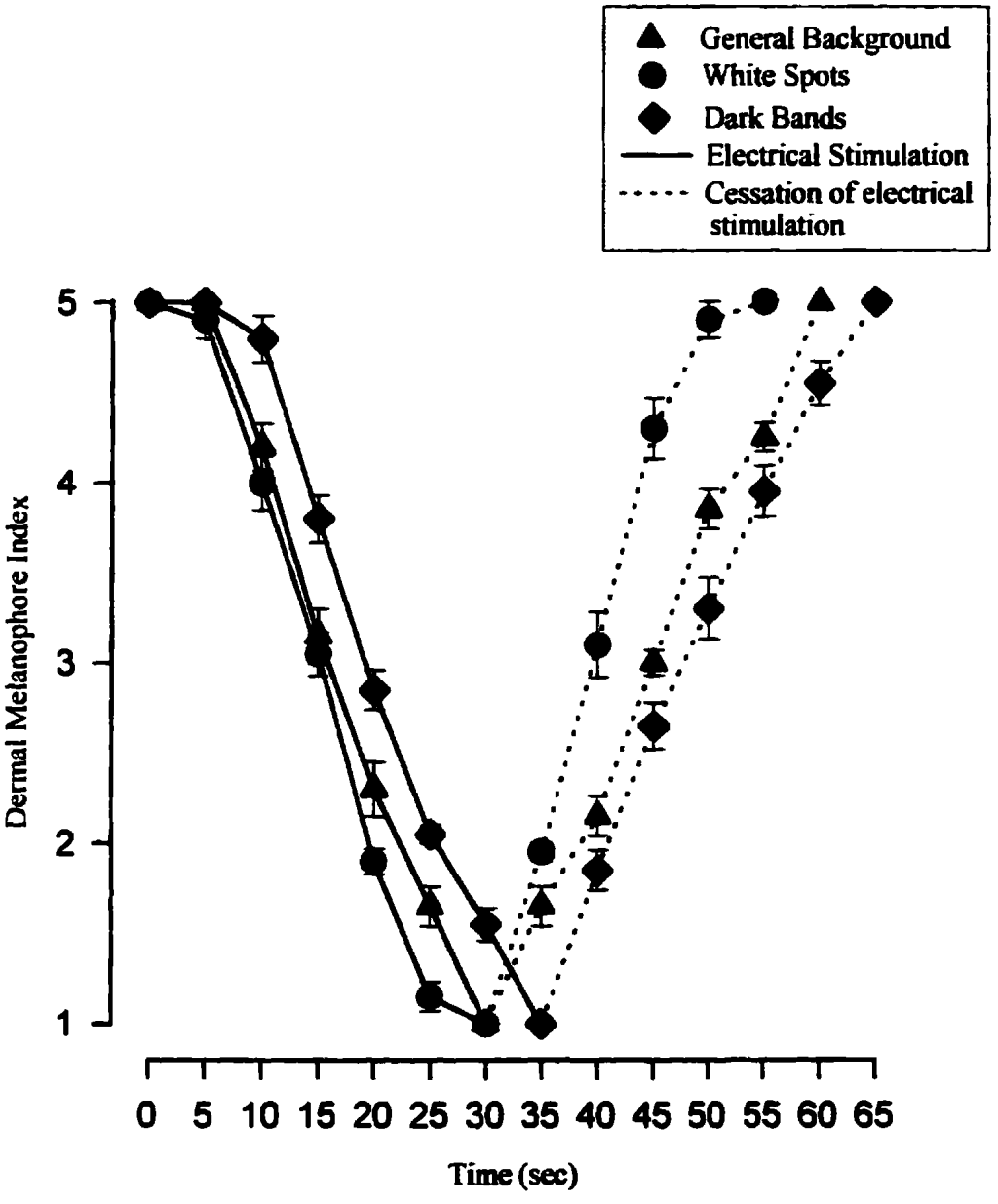


Figure 7. Time course for melanophore responses to electrical stimulation

stimulation. In contrast, the general background and dark band melanosomes require 30 sec to achieve complete dispersion. The differences are statistically significant 15 sec after cessation of electrical stimulation (Kruskal -Wallis: $H=16.22$, $df=2$, $p<0.001$).

The effect of repeating electrical stimulation using the parameters in Table 4 on dermal melanophore responsiveness is shown in Figure 8. These data indicate that 7 such trains of electrical stimuli can be applied to melanosomes before there is a degradation in the responsiveness of any of the dermal melanophores. The results from this experiment act as a control for all other experiments combining the electrical stimulation protocol with increasing drug concentrations.

The electrical stimulation protocol was combined with progressively increasing concentrations of the α-adrenoceptor antagonist, phentolamine (Figure 9). The results indicate that complete blockade of melanosome aggregation within the dermal melanophores occurs at a concentration of 10^{-6}M for white spots and 3.15×10^{-6}M for the general background and dark bands. The pattern related difference between the white spots and general background with 3.15×10^{-7}M phentolamine is statistically significant (Mann-Whitney: $n_1=n_2=10$, $U= 89$, $p<0.005$) and the difference between the white spots and dark bands is statistically significant at this concentration as well (Mann-Whitney: $n_1=n_2=10$, $U=78$, $p<0.025$).

Using the β-adrenoceptor antagonist, propranolol combined with electrical stimulation results in the inhibition of melanosome aggregation at much higher

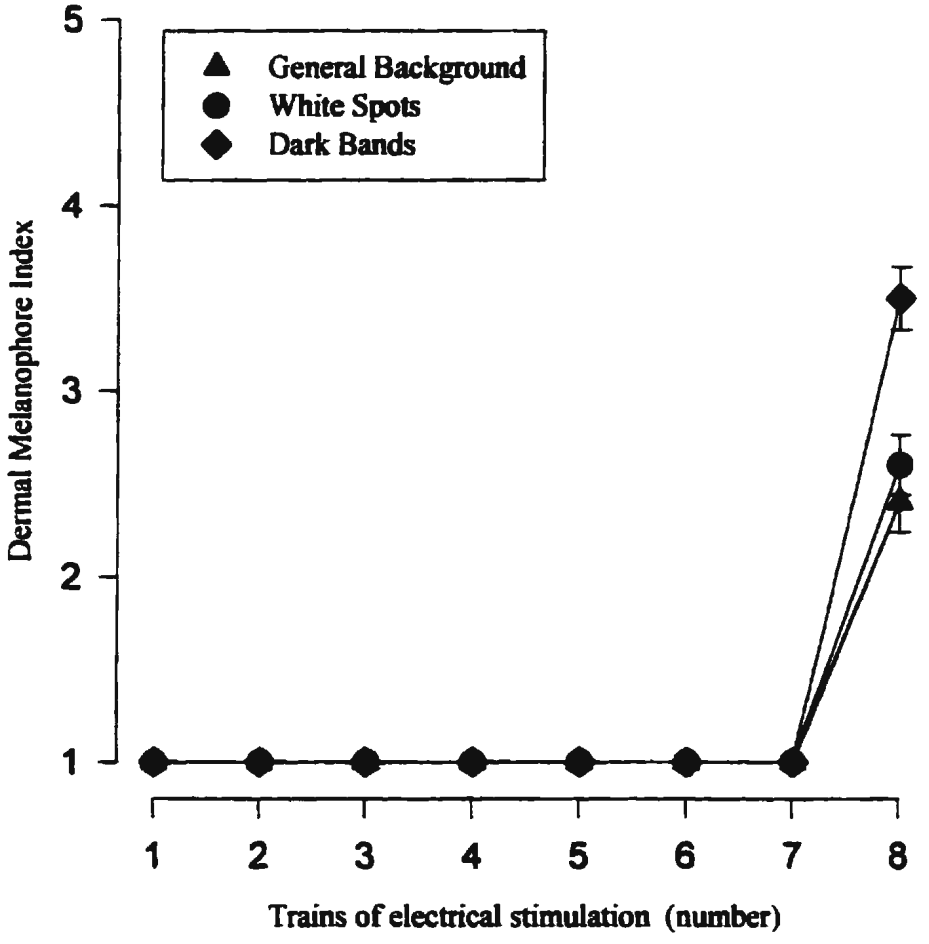


Figure 8. The effect of repeated trains of electrical stimulation on dermal melanophore responsiveness

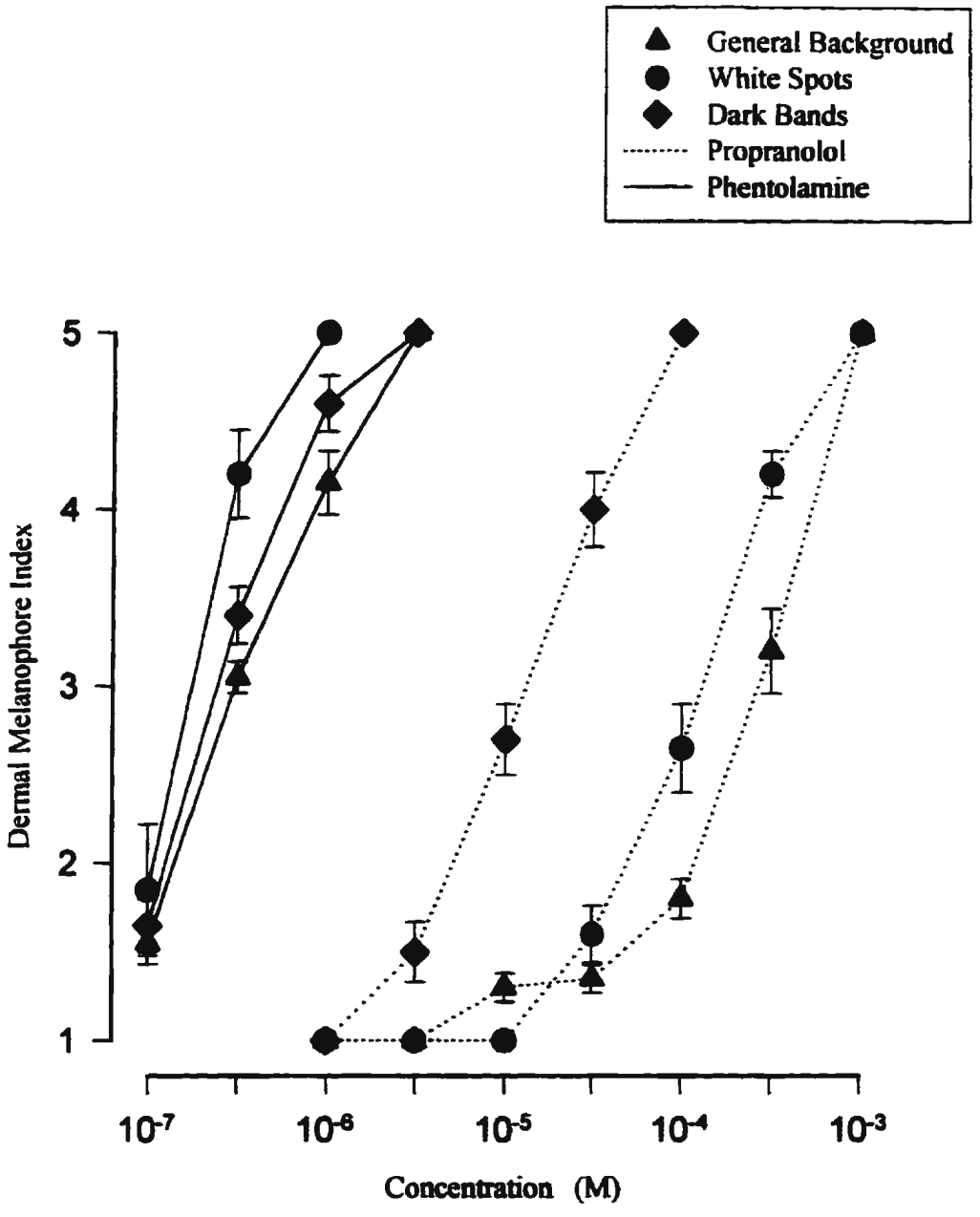


Figure 9. Effect of increasing concentrations of phentolamine and propranolol on melanosome aggregation induced by electrical stimulation

concentrations than with phentolamine (Figure 9). Complete inhibition occurs at a concentration of 3.15×10^{-5}M for the dark bands and 3.15×10^{-4}M for the white spots and general background pattern components. These pattern related differences are statistically significant at 10^{-4}M (Kruskal-Wallis: $H=22.79$, $df=2$, $p<0.001$).

Separate experiments were conducted to determine if phentolamine and propranolol were toxic to the melanophores rather than exerting their blocking effects at the nerve endings. Scale slips from each pattern component were transferred to either phentolamine (10^{-4}M) or propranolol (10^{-4}M) for 90 min followed by a 20 min incubation in BSS. Following this, electrical stimulation was applied to each scale slip using the appropriate parameters (Table 4). All melanosomes were able to achieve DMI = 1 which indicates that the melanosome dispersing effect of the adrenoceptor antagonists at 10^{-4}M was fully reversible.

Concentration response curves were also obtained with the α_1-adrenoceptor antagonist, prazosin and the α_2-adrenoceptor antagonist, yohimbine during electrical stimulation (Figure 10). Both of these drugs are able to completely block dermal melanosome aggregation. Prazosin at 10^{-4}M completely blocks this response for all three pattern components. Yohimbine completely blocks the aggregation response at 3.15×10^{-6}M for the white spots and dark bands and at 10^{-5}M for the general background pattern component. For melanosomes incubated in the prazosin and yohimbine solutions the

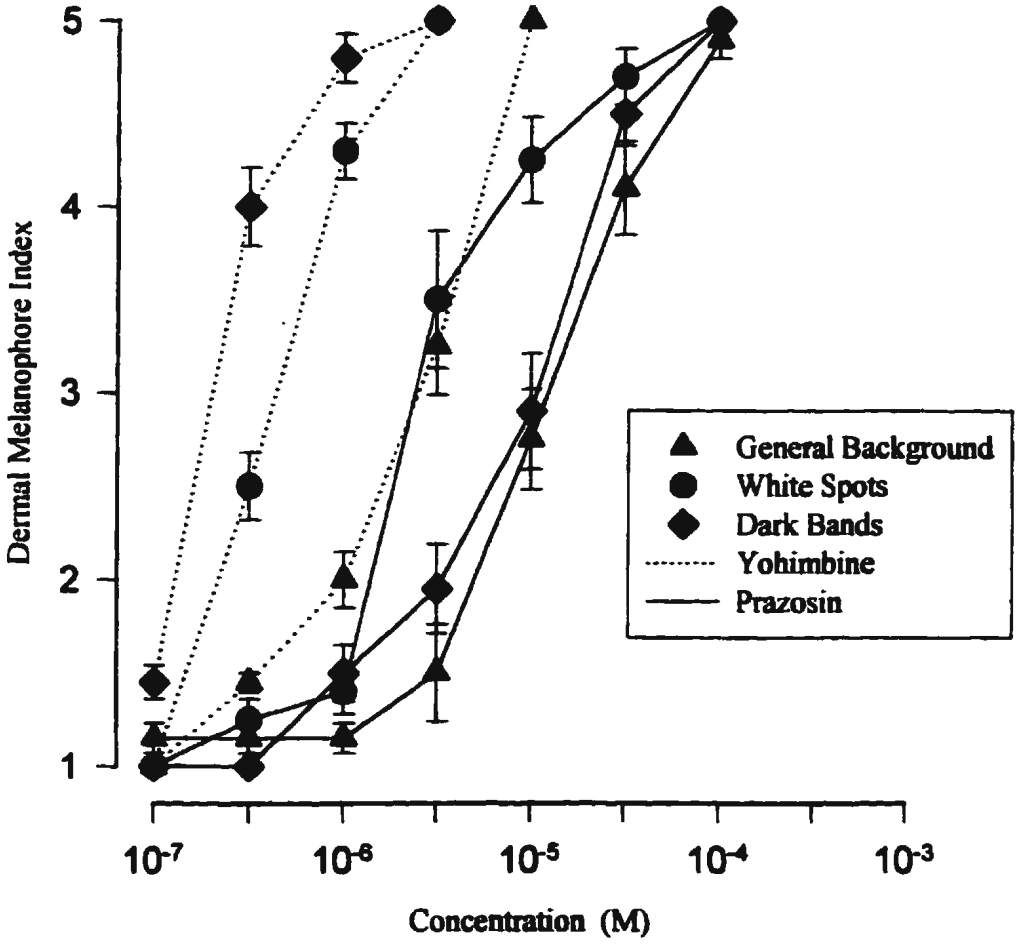


Figure 10. Concentration response curves for prazosin and yohimbine during electrical stimulation

EC_{50} values with the 95% confidence limits are given in Table 5. The differences between EC_{50} values for these two drugs for each pattern component were statistically significant (T-test, $p<0.001$).

Toxicity tests for prazosin (10^{-4}M) and yohimbine (3.15×10^{-6}M) were conducted in a manner similar to that previously described for phentolamine and propranolol. Results indicated that the effects of yohimbine and prazosin were fully reversible at the indicated concentrations.

3.5 Melanosome dispersion - involvement of β-adrenoceptors

A series of experiments was conducted on dermal melanophores using low concentrations of the β-adrenoceptor agonist, isoproterenol (10^{-7}M, 10^{-8}M, and 10^{-10}M) (Figures 11, 12 and 13 respectively). The results from these three experiments indicate that following melanosome aggregation in aggregating fluid (AF), these low concentrations of isoproterenol enhance the rate of pigment dispersion in dermal melanophores in BSS for all pattern components as compared to the controls (BSS alone). Similar results were obtained for the epidermal melanophores.

Using isoproterenol (10^{-7}M and 10^{-8}M), melanosomes from white spot melanophores disperse faster than the general background and dark bands which show similar rates of dispersion (Figures 11 and 12). With 10^{-7}M isoproterenol the white spot

Table 5. Ec_{50} values with 95% confidence limits for dermal melanophores incubated in solutions of prazosin and yohimbine

	White Spots	General Background	Dark Bands
Prazosin	3.08×10^{-6}M (2.06×10^{-6}M - 4.62×10^{-6}M)	1.24×10^{-5}M (1.17×10^{-5}M - 1.31×10^{-5}M)	9.02×10^{-6}M (5.45×10^{-6}M - 1.49×10^{-5}M)
Yohimbine	4.68×10^{-7}M (3.92×10^{-7}M - 5.58×10^{-7}M)	2.26×10^{-6}M (1.58×10^{-6}M - 3.24×10^{-6}M)	2.24×10^{-7}M (1.73×10^{-7}M - 2.91×10^{-7}M)
T-tests	t=9.751, df=18 p<0.001	t=6.106, df=18 p<0.001	t=14.689, df=18 p<0.001

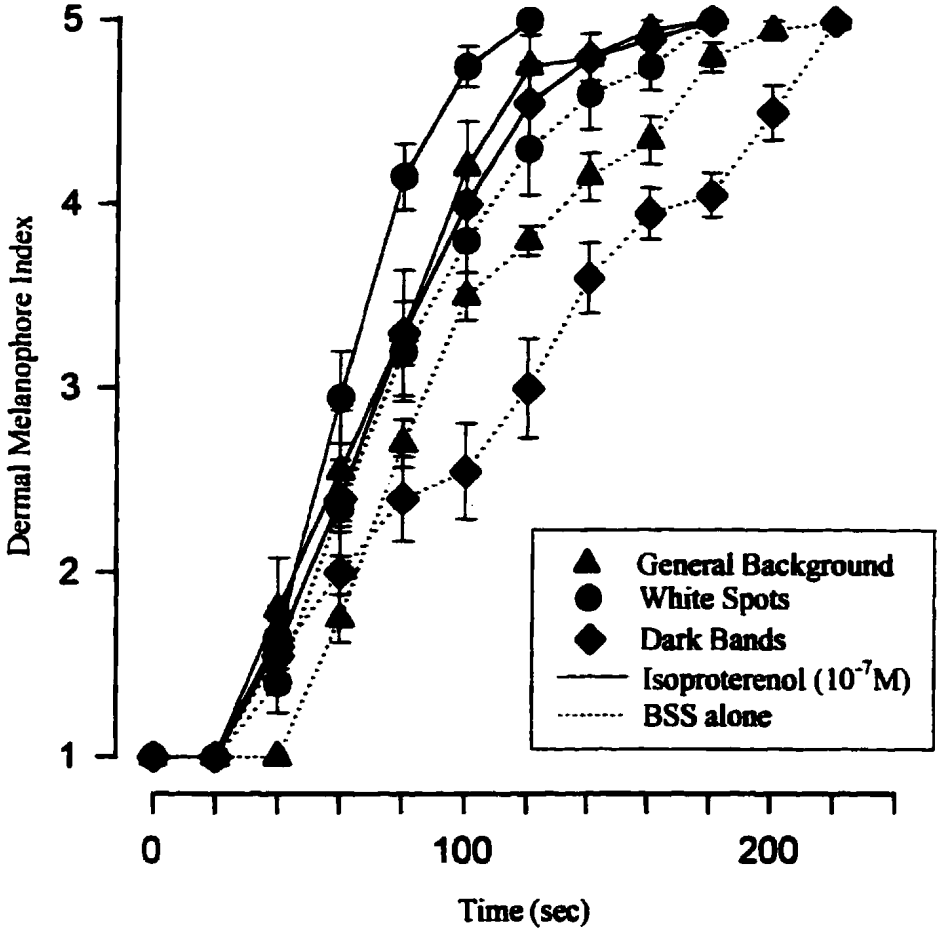


Figure 11. The effect of 10^{-7}M isoproterenol on the rate of melanosome dispersion in BSS

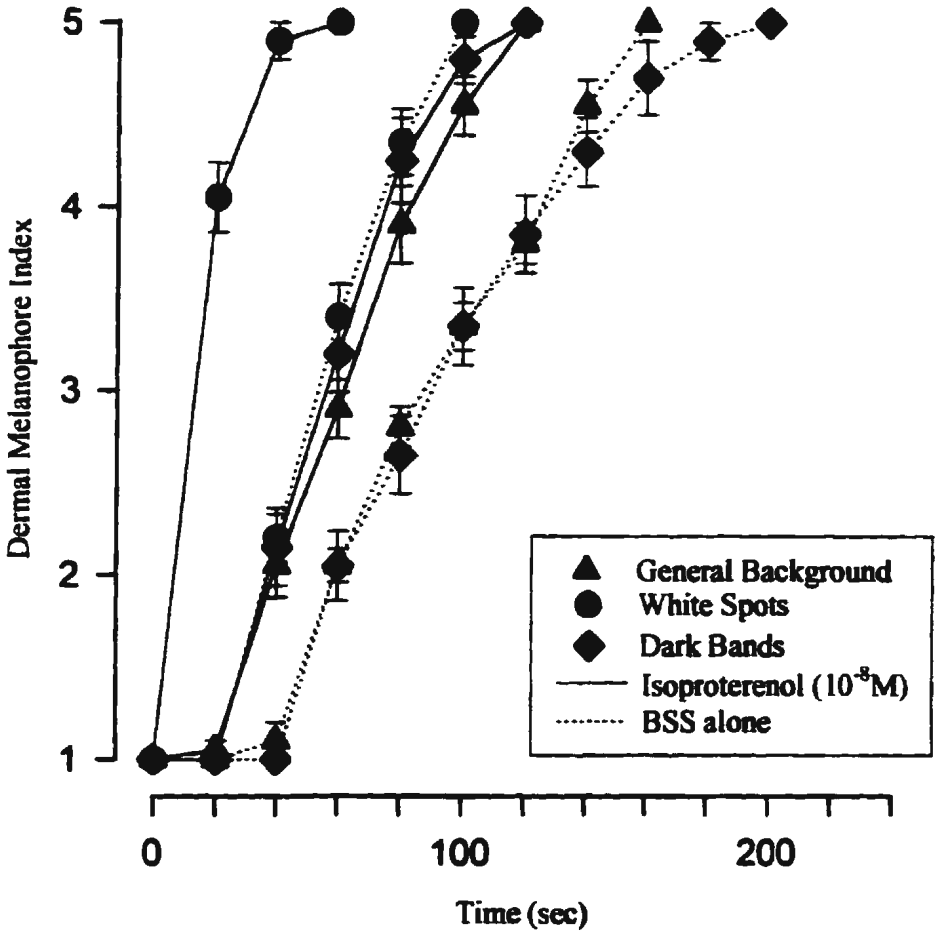


Figure 12. The effect of 10^{-8}M isoproterenol on the rate of melanosome dispersion in BSS

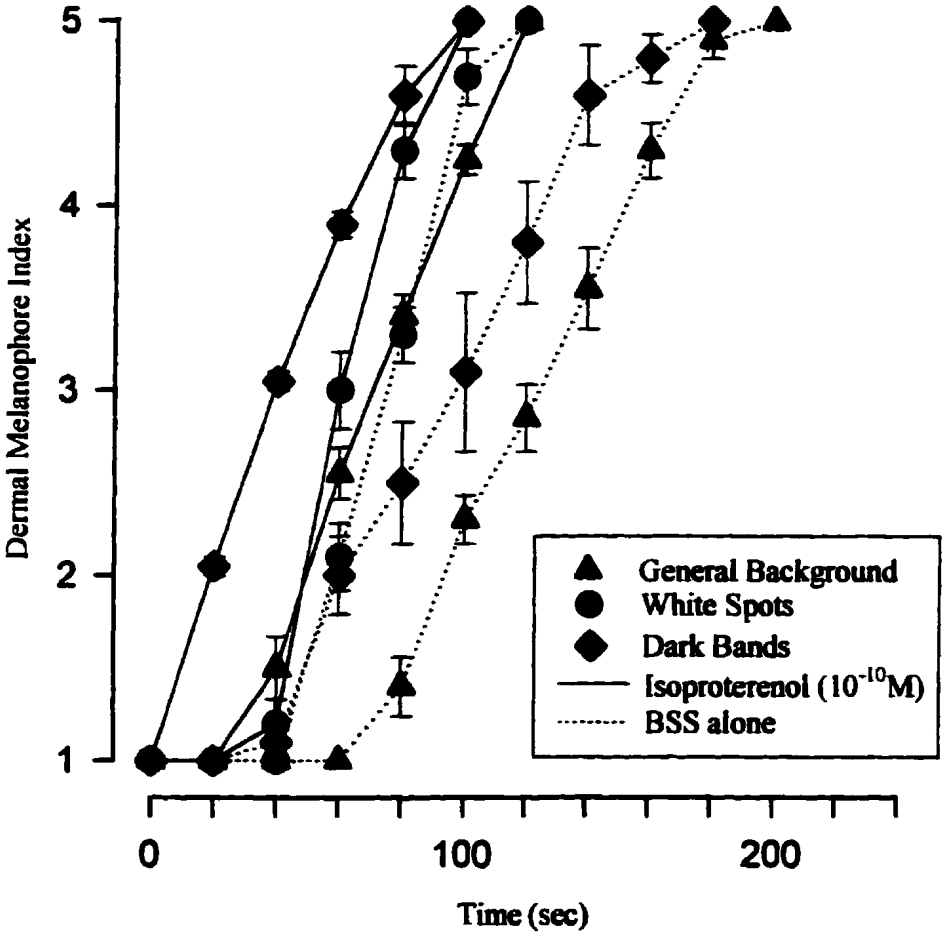


Figure 13. The effect of 10^{-10}M isoproterenol on the rate of melanosome dispersion in BSS

mean DMI was statistically significantly different compared to its control after 100 sec (Mann-Whitney: $n_1=n_2=10$, U=84, p=0.005). Similarly, the dark band and general background values were statistically significantly different from controls after 100 sec (Mann-Whitney: $n_1=n_2=10$, U=89, $p<0.005$) and 200 sec (Mann-Whitney: $n_1=n_2=10$, U=86, $p<0.005$) respectively. With 10^{-8}M isoproterenol all pattern component DMI values were statistically significantly different as compared with their controls (Mann-Whitney: $n_1=n_2=10$, U=100 and 92.5 for white spots and general background respectively, $p<0.001$ and U=89 for dark bands, $p<0.005$) after 60 sec.

The dark band and white spot melanosomes achieve complete dispersion by 100 sec with 10^{-10}M isoproterenol while the general background melanosomes require 120 sec to become totally dispersed (Figure 13). After 80 sec the white spot DMI was statistically significantly different compared to its control (Mann-Whitney: $n_1=n_2=10$, U=89.5 $p<0.005$). The general background and dark band DMI values are statistically significantly different compared to their controls at this time as well (Mann-Whitney: $n_1=n_2=10$, U=100 and 96 respectively, $p<0.001$).

The effect of these low concentrations of the β-adrenoceptor agonist, isoproterenol, in enhancing melanosome dispersion provided the rationale for the next two experiments. Figure 14 summarizes the effect of various low concentrations of noradrenaline on melanosome dispersion in BSS in scale slips from the general

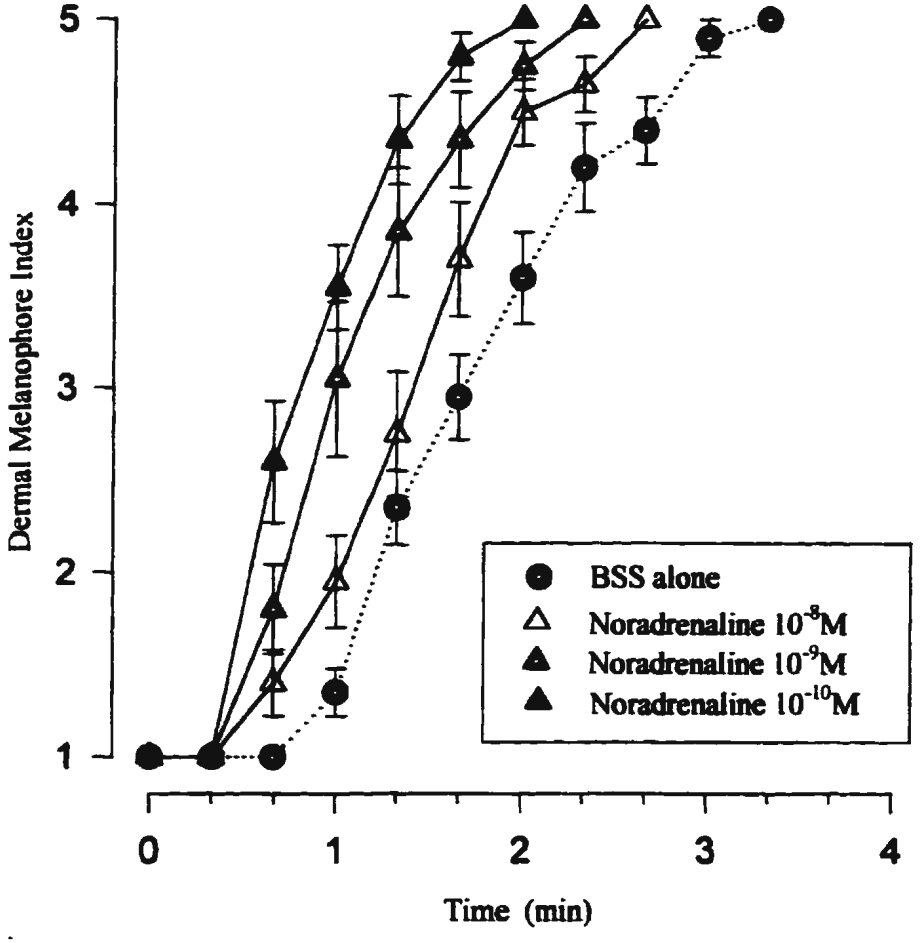


Figure 14. The effect of low concentrations of noradrenaline on the rate of melanosome dispersion in BSS in scale slips from the general background pattern component

background pattern component. The results clearly indicate that low concentrations of noradrenaline increased the rate of melanosome dispersion in BSS in the general background pattern component as compared to its control (BSS alone). Noradrenaline (10^{-10}M) gave faster melanosome dispersion than either 10^{-9}M or 10^{-8}M solutions. The overall differences in DMI for the 3 concentrations of noradrenaline after 1 min was statistically significant (Kruskal-Wallis: $H=20.88$, $df=2$, $p<0.001$).

The next experiment expands on the previous one which was primarily exploratory. This experiment examines the effect of noradrenaline (10^{-10}M) on the rates of melanosome dispersion in BSS for all three pattern components (Figure 15). The results indicate that noradrenaline at this concentration enhances the rate of melanosome dispersion in each pattern component following pallor induced by melanosome aggregating fluid (AF). The white spot and dark band melanosomes are fully dispersed by 80 sec with the general background melanosomes achieving full dispersion at 140 sec. The degree of this dispersion was statistically significant for each pattern component as compared to their controls after 60 sec (Mann-Whitney: $n_1=n_2=10$, $U= 97$, 96 and 94.5 for white spots, general background and dark bands respectively, $p<0.001$). This rate of dispersion is faster than that observed with BSS alone (control). For the control, complete melanosome dispersion was achieved at 140, 160 and 180 sec for the white spots, dark

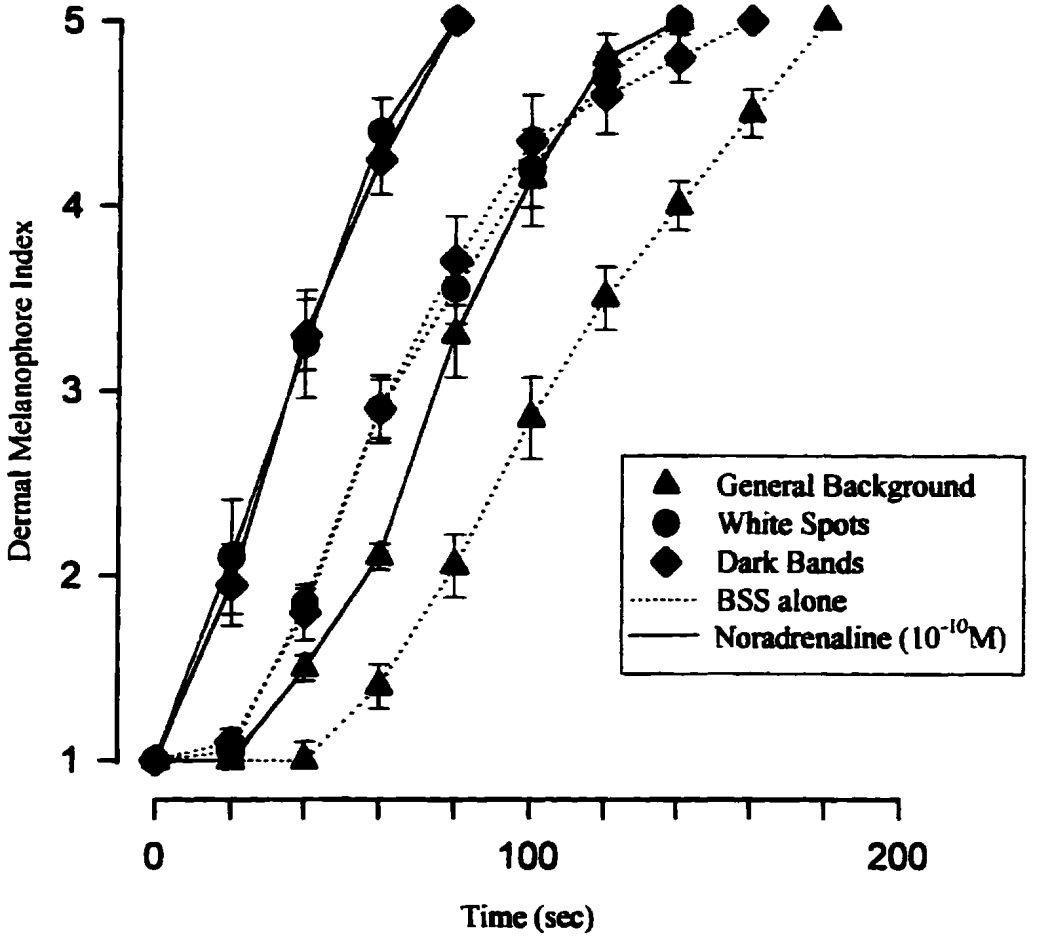


Figure 15. The effect of a low concentration of noradrenaline (10^{-10}M) on the rate of pattern related melanosome dispersion in BSS

bands and general background respectively. Similar trends were observed for the epidermal melanophores.

Propranolol (10^{-4}M) has a pronounced effect on noradrenaline (10^{-10}M) and isoproterenol (10^{-8}M) enhanced rates of melanosome dispersion (Figure 16). Initial incubation of scale slips from the general background pattern component in 10^{-4}M propranolol results in the elimination of any enhanced rates of pigment dispersion. Complete pigment dispersion was not attained until 10 min for scale slips incubated in a noradrenaline and propranolol mixture while only incomplete dispersion was attained after a 10 min incubation in an isoproterenol and propranolol mixture. These rates of melanosome dispersion are much slower than that observed without initial incubation in propranolol or even in BSS alone (Figures 12 and 15).

An initial investigation on the effect of different concentrations of the β_2-adrenoceptor agonist, terbutaline, on noradrenaline (3.15×10^{-6}M) did not result in any melanosome dispersion for all pattern components. However, when the concentration of noradrenaline was reduced to 10^{-6}M and the same experiment was repeated, pattern related differences with respect to melanosome dispersion were evident (Figure 17). The white spot melanosomes achieved complete dispersion at a terbutaline concentration of 10^{-4}M while the dark bands and general background exhibited incomplete pigment dispersion in dermal melanophores at a concentration of 10^{-3}M. The pattern related

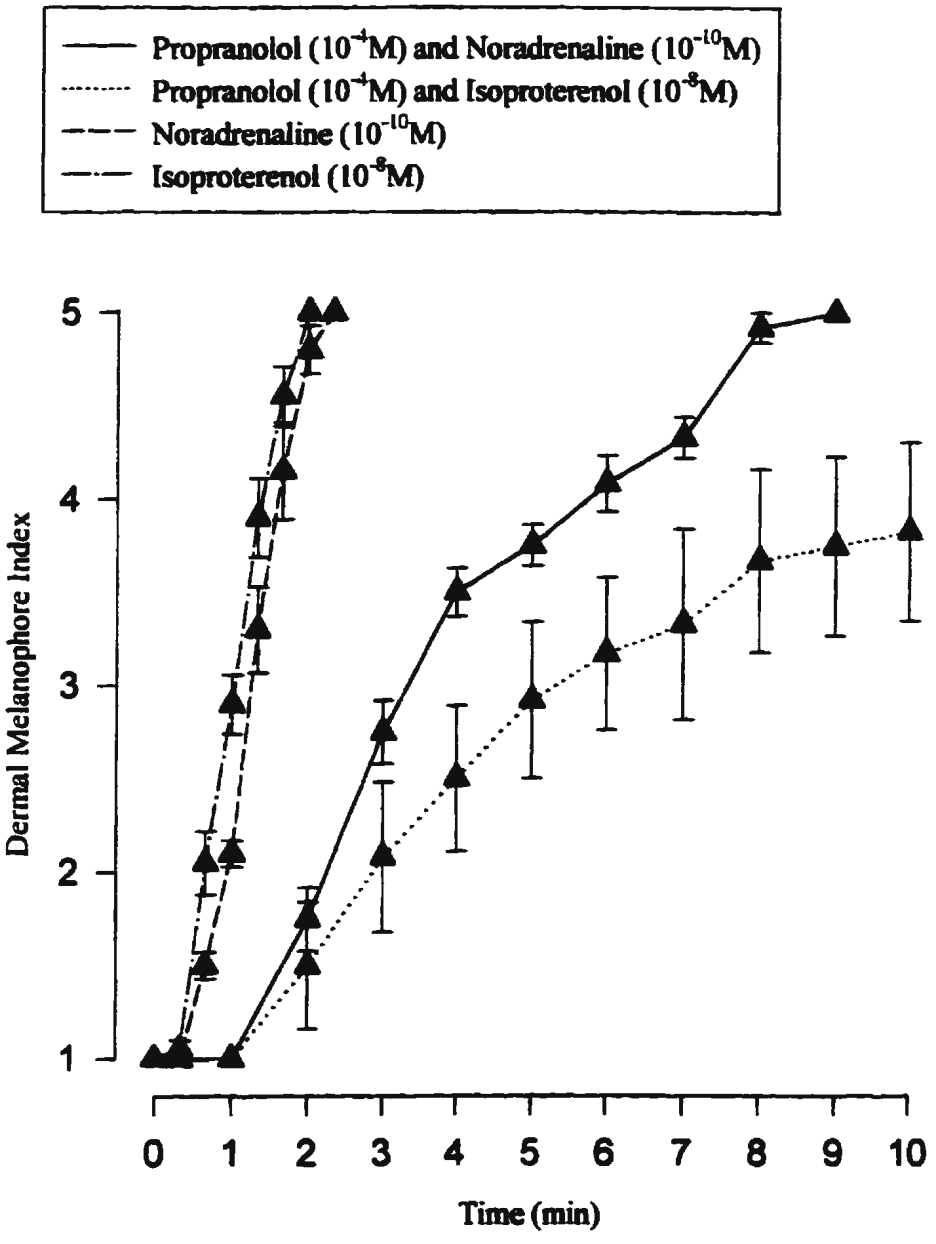


Figure 16 . The effect of propranolol (10^{-4}M) on noradrenaline (10^{-10}M) and isoproterenol (10^{-8}M) enhanced rates of melanosome dispersion in scale slips from the general background pattern component

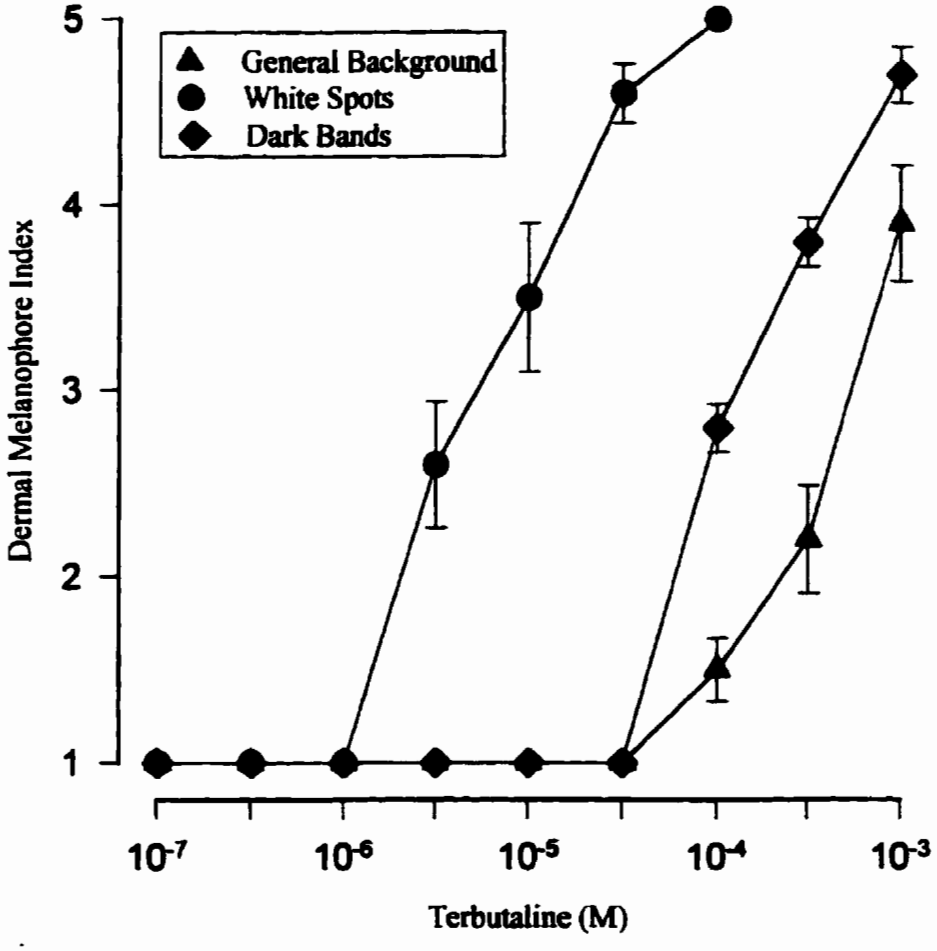


Figure 17. The effect of increasing concentrations of terbutaline on noradrenaline (10^{-6}M) evoked melanosome aggregation

differences are statistically significant with 10^{-4}M terbutaline (Kruskal-Wallis: H=24.58. df=2, $p<0.001$).

Increasing concentrations of terbutaline while stimulating the melanophores electrically gave incomplete melanosome dispersion for all three pattern components with the highest concentration (10^{-4}M) employed (Figure 18). At 10^{-5}M terbutaline the differences in the degree of melanosome dispersion is statistically significant (Kruskal-Wallis: H=11.33, df=2, $p<0.005$).

3.6 Further investigation of β-adrenoceptors

The effect of isoproterenol (3.15×10^{-4}M) on dispersed melanosomes of dermal melanophores in BSS was investigated (Figure 19). It was found that this concentration of isoproterenol induces complete aggregation within 3, 4, and 6 min respectively for the white spot, general background and dark band melanosomes. These pattern related differences were statistically significant after 3 min (Kruskal-Wallis: H=17.05, df=2, $p<0.001$). The rate of melanosome aggregation was faster than that evoked by AF (Figure 4A).

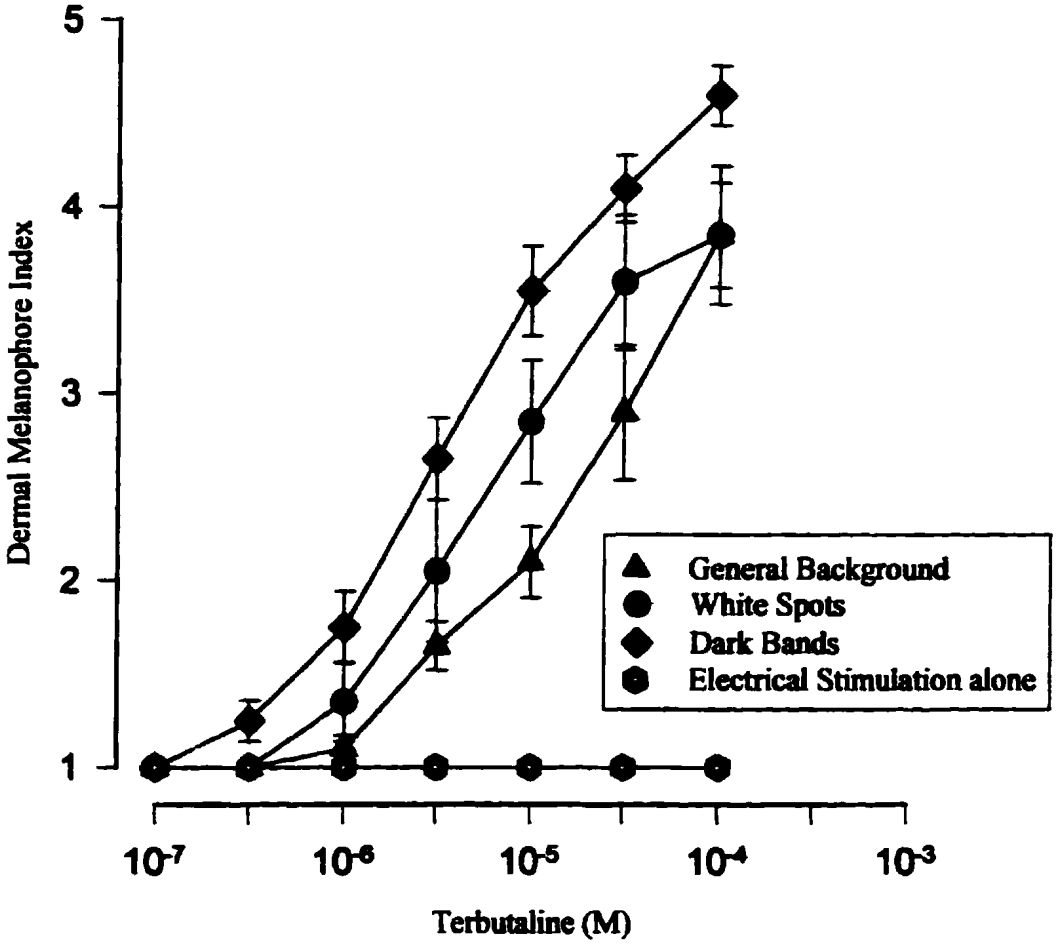


Figure 18. The effect of increasing concentrations of terbutaline on melanosome aggregation evoked by electrical stimulation

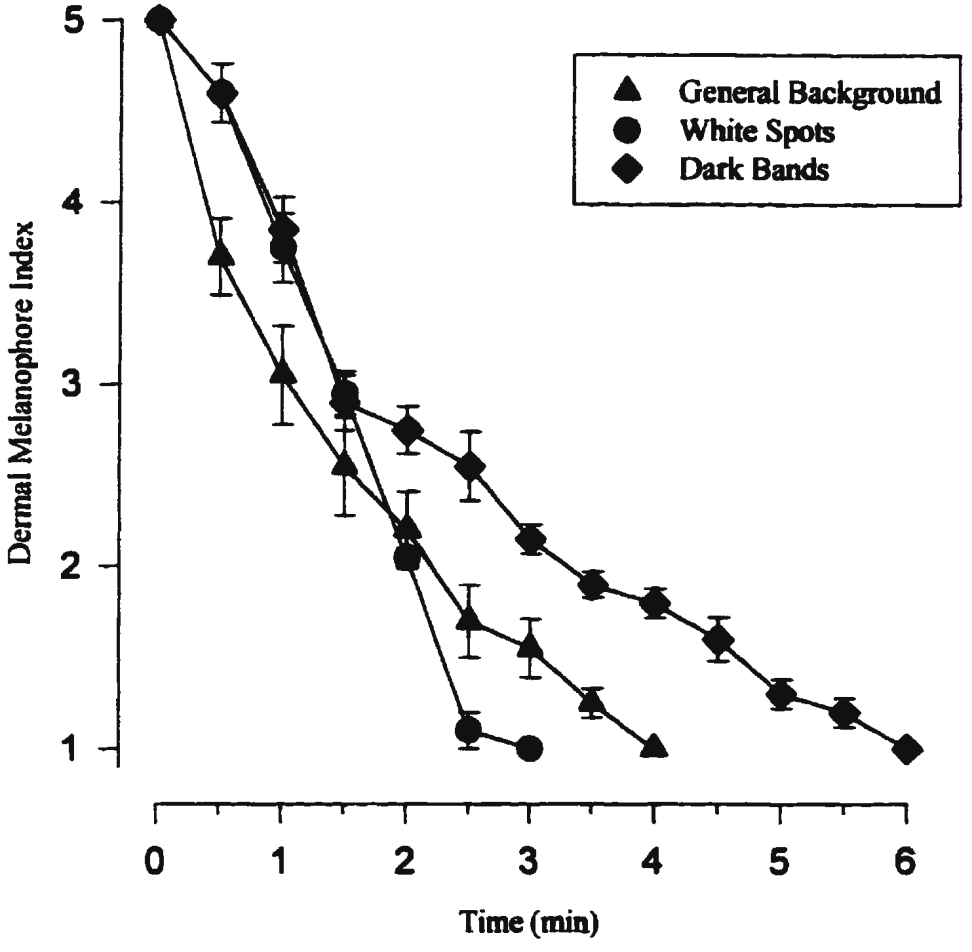


Figure 19. The effect of isoproterenol (3.15×10^{-4}M) on melanosome dispersion in BSS

The effect of different concentrations of 6-OH dopamine and incubation times on sympathetic nerve endings in scale slips is summarized in Table 6. Incubation times of 5 h or less had no measurable effect on the sympathetic nerve endings regardless of the concentration of 6-OH dopamine. This is indicated by the fact that complete melanosome aggregation could still be evoked by electrical stimulation. However, using 10^{-3}M 6-OH dopamine with an incubation time of 28 h resulted in some degree of nerve ending destruction since electrical stimulation could only evoke a mean DMI of 3.5. Finally, using 10^{-3}M 6-OH dopamine with a 44 h incubation time, a chemical sympathectomy is achieved since electrical stimulation could evoke no degree of melanosome aggregation. At the same time the fundamental functionality of the melanophores was not compromised since subsequent incubation of the scale slips in 10^{-5}M noradrenaline induced complete melanosome aggregation.

A time response experiment investigating the effect of isoproterenol (3.15 x 10^{-4}M) on melanosomes after incubation in 6-OH dopamine for 44 h was conducted (Figure 20). Utilizing isoproterenol at this concentration results in complete melanosome aggregation in dermal melanophores for each pattern component at a faster rate than with isoproterenol with the nerve endings intact (Figure 19), although pattern differences in

Table 6. The effect of different concentrations of 6-OH dopamine on sympathetic nerve endings in scale slips from the general background pattern component following various incubation times as determined by electrical stimulation

concentration (M)	sample size	incubation time (h)	mean DMI after incubation	mean DMI after 2 x 20 min BSS washes	mean DMI at end of electrical stimulation
10^{-7}	2	1	5	5	1
10^{-6}	2	1	5	5	1
10^{-5}	2	1	5	5	1
10^{-4}	2	1	1.25	5	1
10^{-3}	2	1	1	5	1
3.15×10^{-4}	2	2	1	5	1
10^{-3}	2	2	1	5	1
3.15×10^{-4}	3	5	1	5	1
10^{-3}	3	5	1	5	1
10^{-3}	4	28	1	5	3.5
10^{-3}	4	44	1	5	5 *

* Following electrical simulation these scale slips were transferred to 10^{-5}M noradrenaline and all 4 scale slips exhibited complete melanosome aggregation (DMI=1)

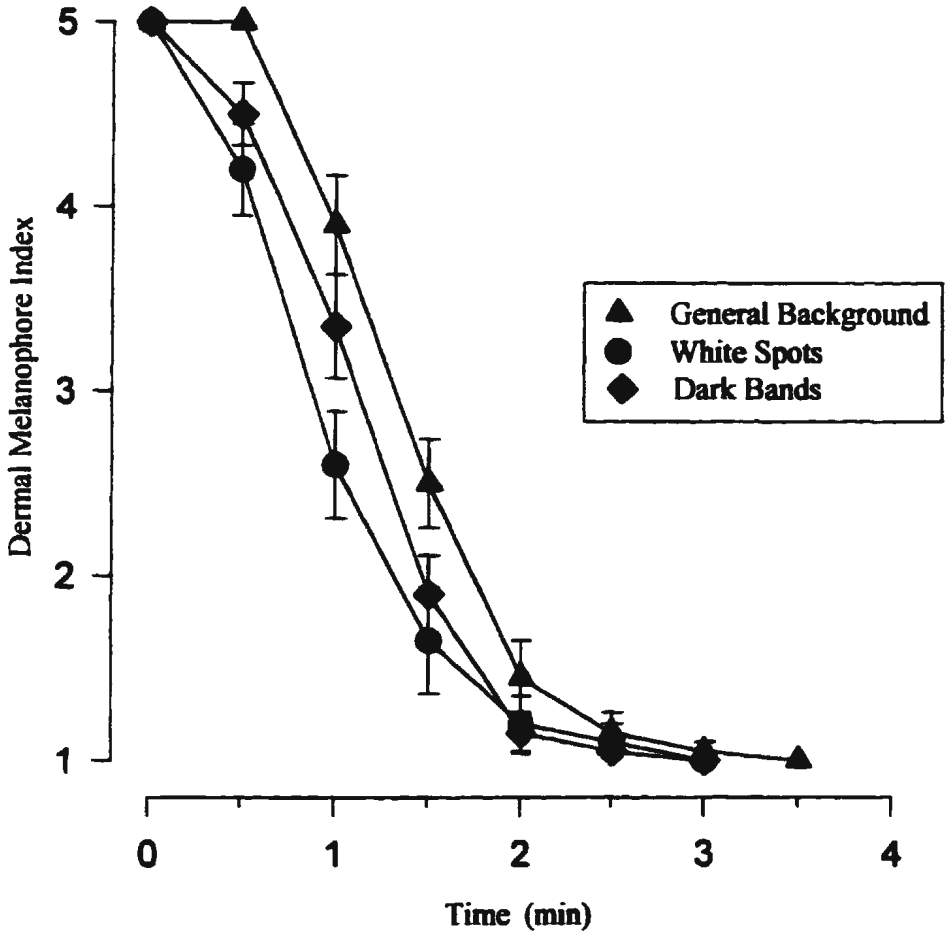


Figure 20. Melanophore responses to isoproterenol (3.15×10^{-4}M) after incubation in 6-OH dopamine for 44 h

DMI after 1 min were not statistically significant (Kruskal-Wallis: H=12.87, df=2 p>0.01).

Repeating the above experimental protocol with a mixture of isoproterenol (3.15×10^{-4}M) and propranolol (10^{-4}M) yields similar results (Figure 21). However, with a mixture of isoproterenol (3.15×10^{-4}M) and the α-adrenoceptor antagonist, phentolamine (10^{-4}M) no pigment aggregation in dermal melanophores occurred, the DMI remaining at 5.0 for all pattern components.

The melanosome aggregation induced in control scales by a lower concentration (3.15×10^{-5}M) of isoproterenol can be inhibited by 10^{-4}M propranolol after longer incubation with a mixture of the two. This is particularly pronounced in the white spot melanophores (Figure 22). This difference is statistically significant after 15 min (Kruskal-Wallis: H=22.61, df=2, p<0.001).

The preceding experiment was repeated with scale slips that had been previously incubated in 6-OH dopamine (Figure 23) to produce a chemical sympathectomy. This results in more rapid and more extensive melanosome dispersion for the white spots and general background than that observed previously (Figure 22) and no dispersion for the dark band pattern component. These pattern related differences after 8 min are statistically significant (Kruskal-Wallis: H=18.05, df=2, p<0.001).

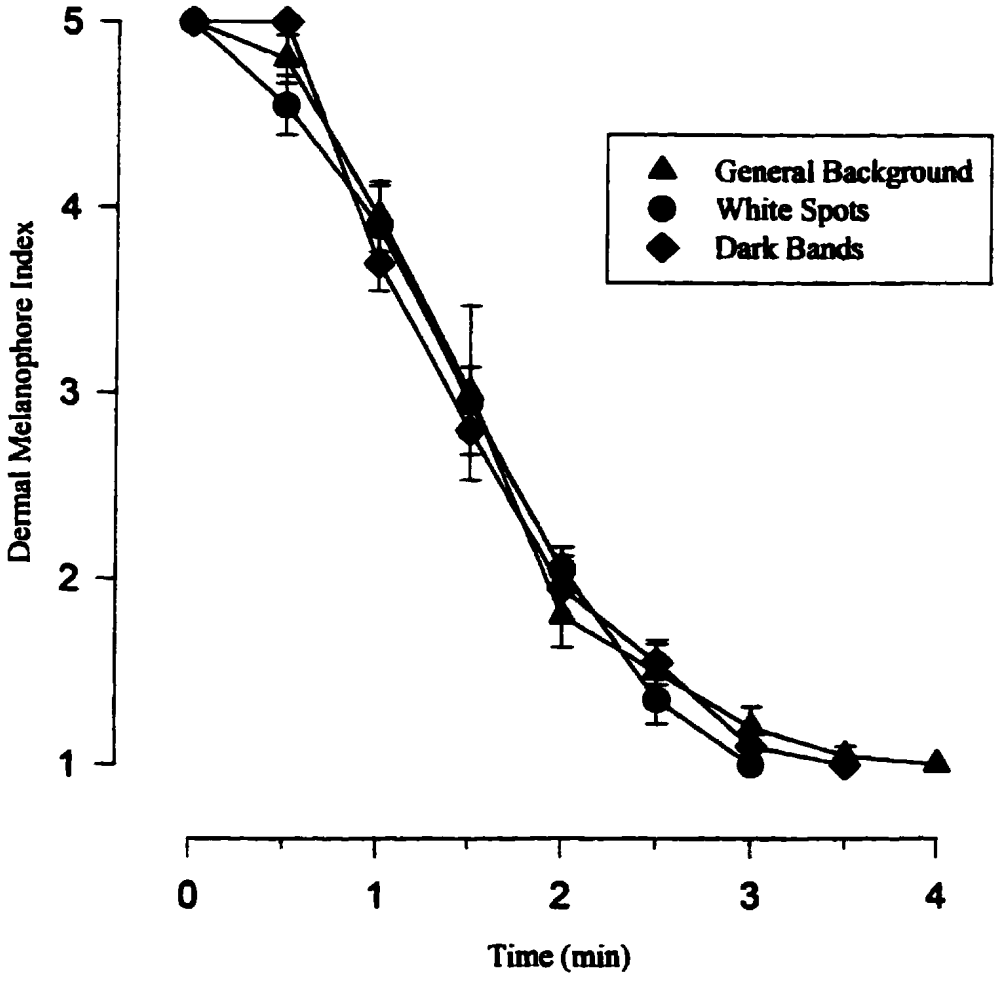


Figure 21. Melanophore responses to isoproterenol (3.15×10^{-4}M) with propranolol (10^{-4}M) after incubation in 6-OH dopamine for 44 h

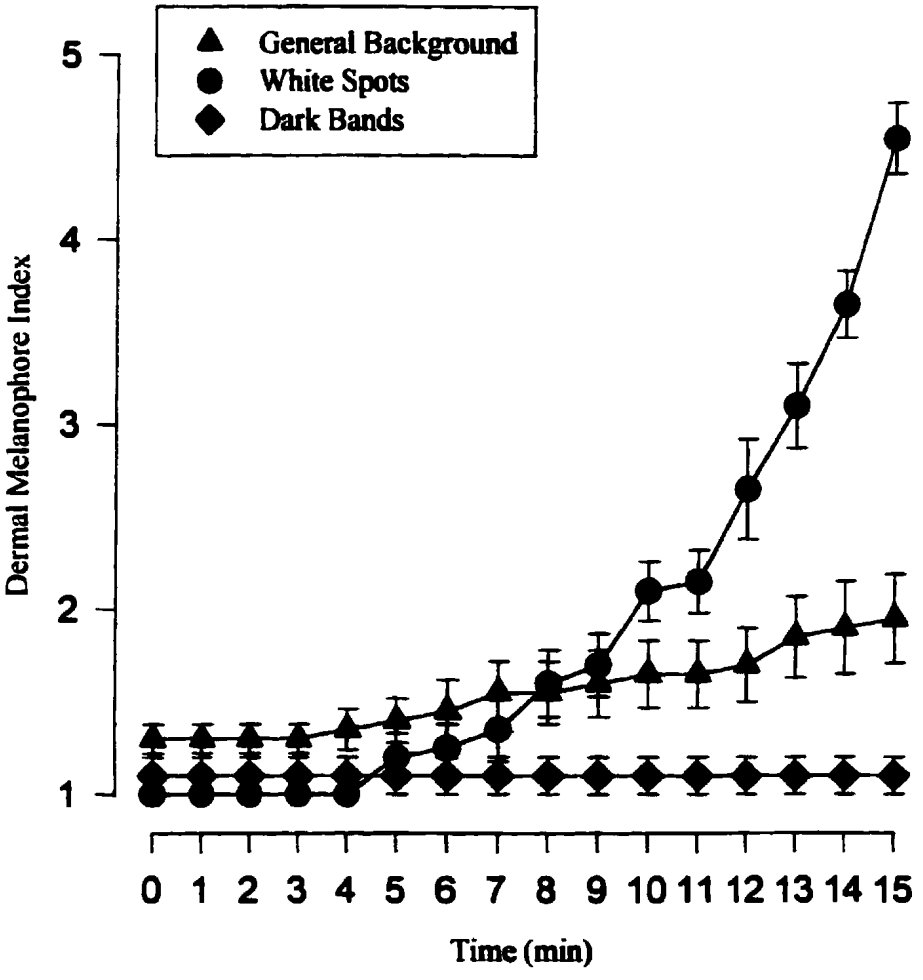


Figure 22. *In vitro* effects of propranolol (10^{-4}M) on melanosome aggregation induced by isoproterenol (3.15×10^{-5}M)

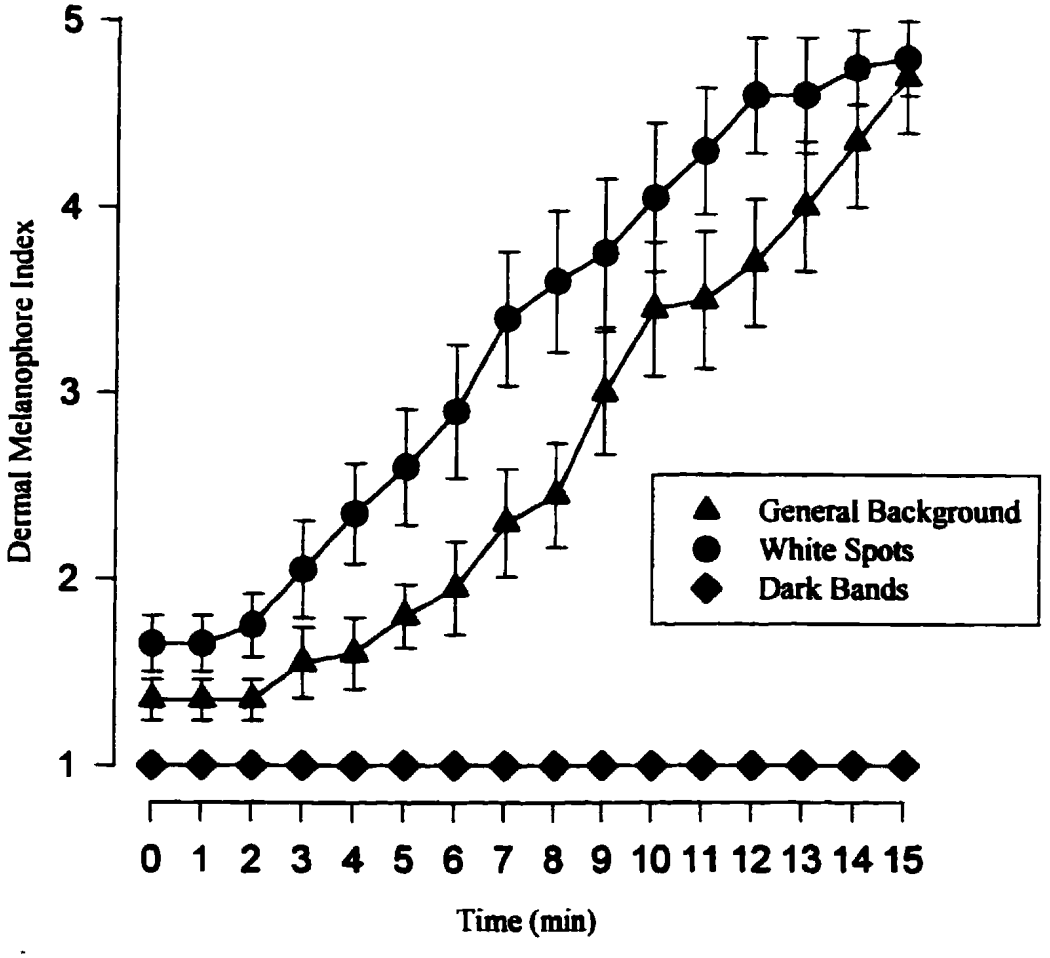


Figure 23. The effect of propranolol (10^{-4}M) on isoproterenol (3.15×10^{-5}M) induced melanosome aggregation in scale slips that have been incubated in 6-OH dopamine for 44 h

10^{-4}M isoproterenol evokes complete melanosome aggregation. However, increasing the concentration of the β_1-adrenoceptor antagonist, atenolol, inhibits isoproterenol evoked melanosome aggregation. This results in incomplete melanosome dispersion in dermal melanophores for the white spots with 10^{-4}M isoproterenol and no dispersion for the corresponding general background and dark band melanosomes (Figure 24). However, when the isoproterenol concentration is reduced to 3.15×10^{-5}M, the white spot melanosomes completely disperse while incomplete dispersion occurs in the other two pattern components (Figure 25). These pattern related differences are statistically significant with 10^{-3}M atenolol (Kruskal-Wallis: $H=21.03$, $df=2$, $p<0.001$).

Increasing concentrations of atenolol during electrical stimulation results in incomplete dispersion for each pattern component (Figure 26). The white spot melanosomes disperse the most followed by the dark band melanosomes while the general background melanosomes disperse the least with 10^{-3}M atenolol. These differences in the degree of dispersion for each pattern component are statistically significant with 10^{-3}M atenolol (Kruskal-Wallis: $H=14.72$, $df=2$, $p<0.001$). With electrical stimulation the dark band and general background pattern components begin to exhibit some initial melanosome dispersion at a lower concentration of atenolol than that observed with atenolol and isoproterenol. In contrast, the white spots begin to exhibit melanosome dispersion at a higher concentration of atenolol than seen with atenolol and isoproterenol (Figure 25).

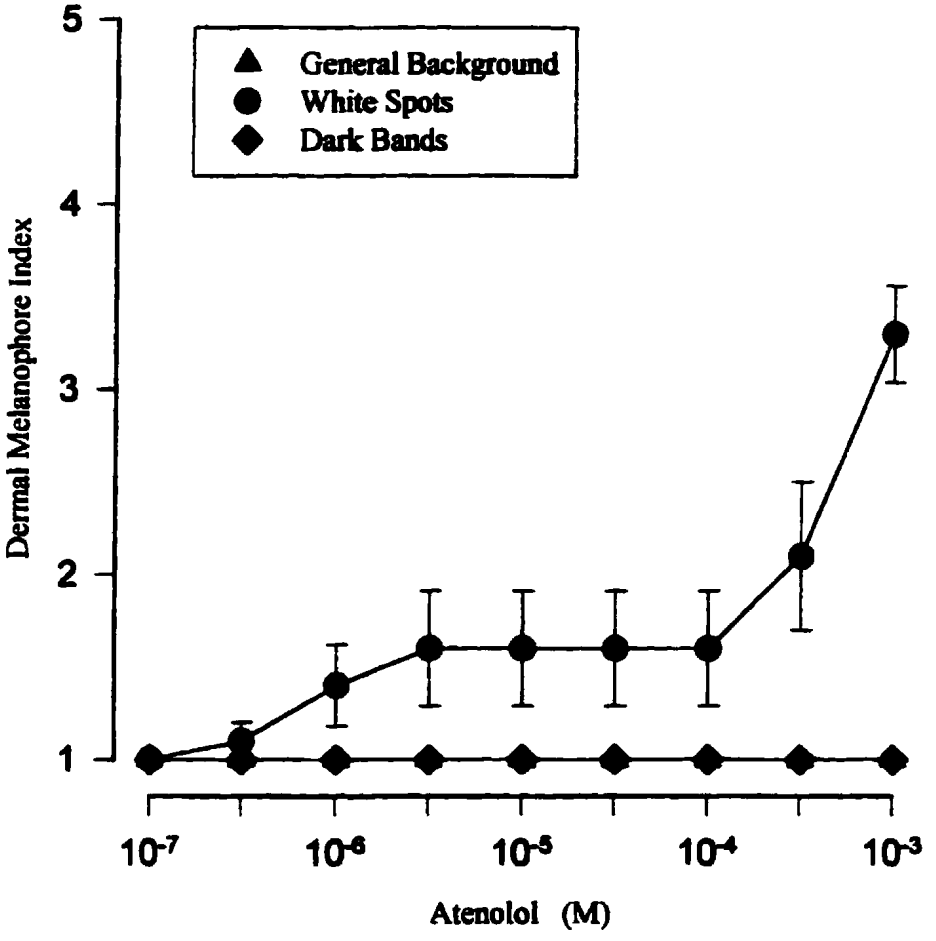


Figure 24. The effect of increasing concentrations of atenolol on isoproterenol (10^{-4}M) evoked melanosome aggregation

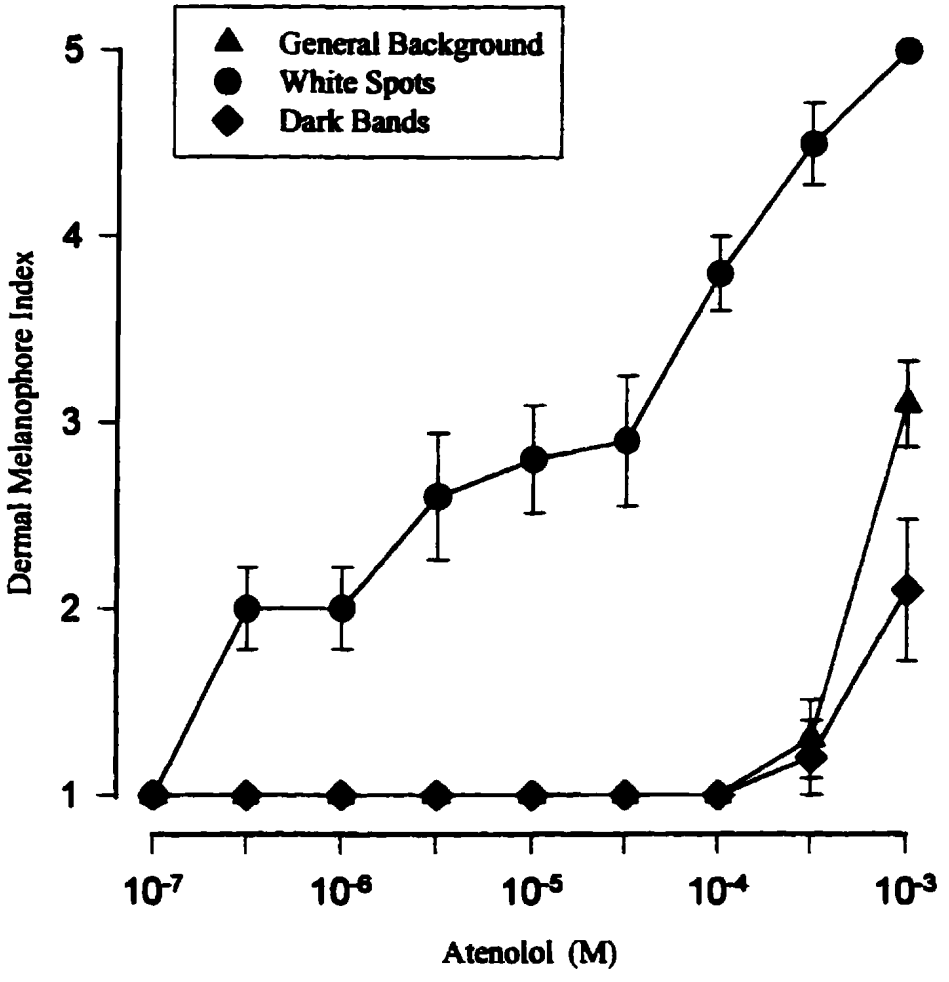


Figure 25. The effect of increasing concentrations of atenolol on isoproterenol (3.15×10^{-5}M) evoked melanosome aggregation

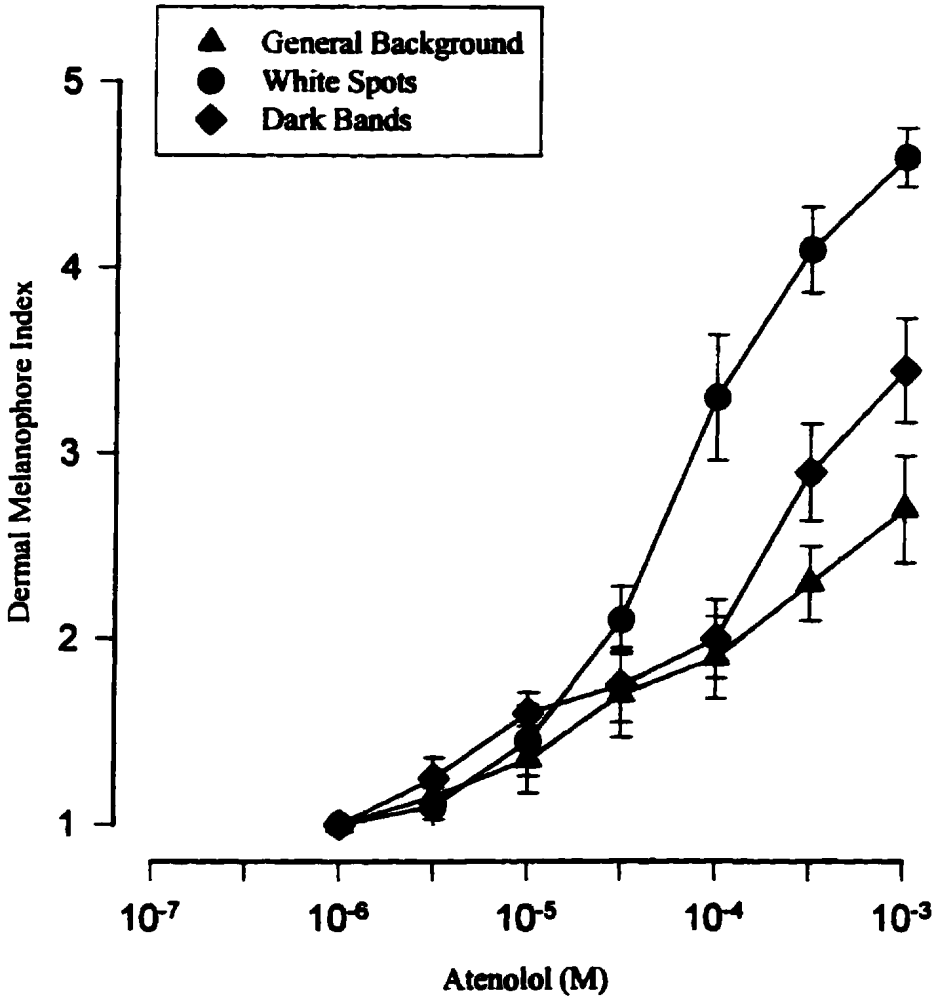


Figure 26. The effect of increasing concentrations of atenolol during electrical stimulation

4. DISCUSSION

The central finding of this study was that adrenergic innervation alone controls neurally mediated melanosome aggregation and dispersion in winter flounder. High concentrations of noradrenaline mediate pigment aggregation through α-and possibly β_1-adrenoceptors and pigment dispersion is mediated by noradrenaline at lower concentrations via β_2-adrenoceptors. This finding contrasts with the melanosome dispersive mechanism proposed by Parker (1934) and Parker and Rosenblueth (1941) for the catfish, *Ameiurus nebulosus*, or the mechanism suggested by Kumazawa and Fujii (1984) for tilapia, *Sarotherodon niloticus*.

The literature on the physiology and pharmacology of teleost melanophores has a paucity of information on cryptic patterning processes. Even the melanophores associated with the remarkable chromatic patterning capability of flatfish have so far been investigated only in a general manner, without any detailed study of their differential cellular responsiveness, except for the series of studies in progress on winter flounder.

The *in vitro* differential dispersion of melanosomes in response to Na^+ reflects that observed *in vivo* except that the *in vitro* rates are much faster (Burton, 1980; Burton and Snow, 1993). *In vivo*, when winter flounder are transferred from a white to a black background, the initial rate of melanosome dispersion that occurs in the dark bands is more rapid than that observed in the general background pattern component (Burton, 1980; Burton and Snow, 1993). Complete dispersion is not attained until several hours

after transfer for the dark bands and after a day or several days for the general background, but may not be attained even after 6 days for the white spots in individual fish. The authors also observed that melanosome aggregation is a slower process than dispersion. Burton (1980) transferred winter flounder from black to white background and found that dark band melanosomes showed no initial rapid aggregation while the general background melanosomes exhibited rapid aggregation initially. However, complete pigment aggregation was not observed until 1-6 days after transfer. The author also found that white spot melanosomes do not show the same range of background related activity as exhibited by the dark band and general background melanophores. A similar relationship was observed in the present *in vitro* work except that the melanophore responses were much faster as compared to those observed *in vivo* by Burton (1980) and Burton and Snow (1993).

The fact that there are differences in the rates of *in vitro* melanophore responsiveness suggests that there are varying degrees of neuromodulation for the pattern components at the peripheral level including the balance between α- and β-adrenoceptors on melanophores. The differences in the overall melanophore responsiveness *in vivo* (hours) as compared to *in vitro* (minutes) reflects the central processing of the various input signals from the environment as compared with more direct topical stimulation of the effectors.

Phentolamine blocks K^+ and noradrenaline-induced melanosome aggregation indicating that α-adrenoceptors play a role in melanosome aggregation. It is known that

K^+ acts on adrenergic nerves by causing the release of the neurotransmitter (Burton, 1989; Burton and Everard, 1989; Fujii and Oshima, 1986; Karlsson et al., 1988). The effect of this endogenous neurotransmitter is blocked by a much lower concentration of phentolamine than that required to block exogenously applied noradrenaline induced melanosome aggregation. Also, the differences between the pattern components are much greater than those seen with the blockade of exogenous noradrenaline. In addition to this, there is a difference in the order in which the pattern components exhibit melanosome dispersion in terms of decreasing sensitivity to phentolamine. The order observed with blockade of K^+ was white spots followed by dark band then the general background melanosomes. By comparison, the order observed with blockade of exogenous noradrenaline was white spots followed by general background then dark band melanosomes. The order and responsiveness of pigment aggregation observed with the release of neurotransmitter by K^+ is most likely more representative of the neurotransmitter-effector relationship *in vivo* than that observed with direct topical application of noradrenaline. The responsiveness of melanophores to an application of a specific concentration of noradrenaline will be predominantly a direct relationship. In contrast, the K^+ application does allow for the possibility of differing degrees of peripheral neuromodulation of neurotransmitter released from the nerve endings.

The white spots have the smallest dermal melanophores while the dark bands have the largest. Burton (1975) measured dermal melanophore size in the dark bands and general background in *Pleuronectes americanus*. It was found that the dark band

melanophores were larger than the general background melanophores but no reference was made to the white spot melanophores. The physical size of the pigment mass may play a role in the rates of melanophore responsiveness *in vitro*. This possibility is discussed by Burton and Snow (1993) who also state that size does not explain the capacity of the dark band melanosomes to display rapid initial dispersion as is consistent with the present observation.

It does not appear as if melanophore size or scale slip dimensions have any effect on the electrical stimulation parameters required to evoke complete melanosome aggregation. The pigment mass in the white spot dermal melanophores is smaller than that in the other two pattern components yet they require a voltage that is intermediate to that required by the general background and dark band melanophores. Similarly, the dimensions of the skin region of the white spots are smaller than those of the dark bands, with general background scale slips having the largest skin region dimensions. There seems to be no obvious relationship between the morphological measurements and electrical stimulation parameters for each pattern component. This suggests that the electrical stimulation parameters are indicative of physiological neuroeffector differences between the pattern components. It is noteworthy that electrical stimulation parameters could not be established which would evoke pigment aggregation in epidermal melanophores. This may indicate that epidermal melanophores are not innervated but simply rely on a diffusion of neurotransmitter from the nerves associated with the dermal melanophores. However, dermal melanophore responsiveness to electrical stimulation is

very rapid, the time period being too short to permit diffusion of endogenous neurotransmitter to epidermal melanophores. In contrast, dermal melanophore responsiveness to AF is much slower with sufficient time to permit endogenous neurotransmitter diffusion to the epidermal melanophores.

It has been suggested that the epidermis is a diffusion barrier to drugs that are topically applied to scale slip preparations (Fernando and Grove, 1974 b; Spaeth, 1913; Stone and Chavin, 1974). As a result of this, melanophores are not permitted completely free access of exogenous chemicals (Burton et al., 1995). Stone and Chavin (1974) demonstrated that there is a tenfold increase in sensitivity to noradrenaline concentration upon removal of the epidermis from goldfish scale slips. Burton et al. (1995) found that removal of the epidermis from winter founder scale slips results in a faster melanosome aggregation rate and thus provides additional information on the extent to which the epidermis is a diffusion barrier. These authors also pointed out that the effect of the diffusion barrier is not pattern-related and more recent observations (Vokey and Burton, unpublished data) demonstrate that there are no pattern-related significant differences in epidermal thickness.

Electrical stimulation of melanophores offers the advantage of circumventing the epidermal diffusion barrier since such stimulation causes release of neurotransmitter from nerve endings (Andersson et al., 1984; Fujii and Oshima, 1986; Gray, 1956; Karlsson et al., 1988). By stimulating the release of endogenous neurotransmitter, application of exogenous noradrenaline is not necessary and the findings of such

experiments should be a function of the capacity for neurotransmitter release and its effects on melanophores at the different electrical stimulation parameters employed.

In vivo, differential melanosome aggregation and dispersion occurs for each pattern component (Burton, 1980). *In vitro*, differential melanophore responsiveness was observed as well, but it was more rapid than that seen *in vivo*. Burton (1980) made these observations when transferring the winter flounder from black to white aquaria and vice versa. Thus central processing as well as possible peripheral neuromodulation probably played a major role in coordinating cues from the external environment and translating them into actual melanophore responses which would account for the differing time scales in the two types of experiments. The different parameter values used in electrically stimulating the melanophores *in vitro* to attain full melanosome aggregation illustrate the degrees of peripheral neuromodulation between each pattern component, but it does not address the role of central processing in the pattern-related differences.

Topical application of either phentolamine or propranolol results in blockade of melanosome aggregation during electrical stimulation thus indicating the involvement of both α and β adrenoceptors in the response. It is apparent that α-adrenoceptors predominate but there also appears to be some pattern related β-adrenoceptor synergism as suggested by Burton et al. (1995).

Application of either prazosin or yohimbine during electrical stimulation blocks melanosome aggregation with yohimbine being more potent than prazosin suggesting that

both α_1 and α_2 adrenoceptors play a role in the aggregation but with a predominance of the latter. Andersson et al. (1984) have suggested that post-ganglionic α_2-adrenoceptors regulate melanosome aggregation in *Labrus ossifagus* and that α_1-adrenoceptors have no role in melanosome aggregation. No reference was made as to whether or not *Labrus* possess any cryptic patterning. Fujii and Oshima (1986) state that the conclusion reached by Andersson et al. (1984) requires more studies to confirm the findings. Unpublished data (Burton and Vokey) indicate that there is differential mediation involving both α_1- and α_2-adrenoceptors in flounder pattern related melanophore responsiveness and that it is likely that both are postsynaptic.

It is known that α-adrenoceptors are mediators of pigment aggregation in teleost melanophores (Burton and Everard, 1989; Fernando and Grove, 1974 a, b; Fujii and Miyashita, 1975). Evidence is accumulating which indicates that β-adrenoceptors may be involved in pigment dispersion in different teleost species (Fujii et al., 1985; Kasukawa et al., 1985; Miyashita and Fujii, 1975). The current study demonstrates that β-adrenoceptors mediate this pigment dispersion in *Pleuronectes americanus* and that they are probably of the β_2 conformation based on experiments conducted with terbutaline. These results provide evidence for a mechanism by which the melanosome aggregating and dispersion responses in flounder are controlled via adrenergic innervation alone and not by adrenergic and cholinergic innervation as suggested by Parker (1934) and Parker and Rosenblueth (1941) for the catfish, *Ameiurus nebulosus*. Thus in winter flounder, it is suggested that pigment aggregation is mediated by higher concentrations of

noradrenaline through α-adrenoceptors and pigment dispersion is mediated by lower concentrations of noradrenaline through β_2-adrenoceptors. Functionally, this would mean that in this species melanosome aggregation will occur in response to catecholamines released from nerve endings, whilst the falling concentration after cessation of neural activity will enhance pigment dispersion.

β-adrenoceptors are implicated as having a role in melanosome dispersion since the β-adrenoceptor agonist, isoproterenol, enhances this rate as compared to BSS alone. The extent of this enhancement is pattern-related and is apparent for several relatively low concentrations of isoproterenol. This is in contrast to its effect at higher concentrations at which it evokes melanosome aggregation. Thus it appears that isoproterenol can evoke different melanophore responses depending on its concentration. It is argued from its effects on the teleost vascular system that isoproterenol might operate via α-adrenoceptors at high concentrations (Wahlqvist and Nilsson,1981), which is consistent with its antagonism by phentolamine in the present work, and via β-adrenoceptors at low concentrations, which is consistent with the effect of propranolol in the present work.

Wahlqvist and Nilsson (1981) investigated the sympathetic nervous control of the vasculature in the tail of *Gadus morhua*. They found that isoprenaline (isoproterenol) at relatively high concentrations would constrict the tail vasculature in a manner similar to adrenaline and a lower concentration of isoprenaline would produce vasculature dilation

in some preparations. From this they suggested that α-adrenoceptors mediate vasoconstriction and β-adrenoceptors mediate vasodilation. Fujii and Miyashita (1975) demonstrated that some β-adrenoceptor agonists induce melanosome aggregation in *Lebistes reticulatus* and that this effect was antagonized by α-adrenoceptor blocking agents. Further, β-adrenoceptor blocking agents were ineffective in blocking the pigment aggregation induced by the β-adrenoceptor agonists. This accumulating evidence supports the idea that isoproterenol may operate via α-adrenoceptors at high concentrations.

Isoproterenol is a synthetic compound and thus it is not present *in vivo*. Because of this the enhanced dispersion observed with isoproterenol is not biologically significant unless it can be observed with a neurotransmitter that is present naturally. The fact that it was found that flounder melanosome dispersion can also be enhanced by relatively low concentrations of noradrenaline and that this can be depressed by propranolol in the present work represents an important development in understanding melanophore control in this species. Again this effect is opposite to that observed at higher concentrations at which it induces melanosome aggregation. This dispersive role of noradrenaline can be explained if one considers the possibility that this neurotransmitter can act via β-adrenoceptors at low concentrations.

Experiments conducted with terbutaline indicate that noradrenaline could be enhancing dispersion by acting via β_2-adrenoceptors. Morishita et al. (1985) found that terbutaline reduced the noradrenaline evoked melanosome aggregating response of

melanophores in *Oryzias latipes*, suggesting that terbutaline competitively suppresses the aggregating response, which is confirmed for flounder in the present work. It is suggested by Morishita et al. (1985) that pigment aggregation is antagonized by the pigment dispersing action of β_2-adrenoceptors.

It would appear that scale slips in *Pleuronectes americanus* only require adrenergic innervation to regulate melanophore responses. It has been proposed that *Ameiurus* melanophores may be doubly innervated with one set of nerves controlling pigment aggregation and the other dispersion (Parker, 1934; Parker and Rosenblueth, 1941). It has further been suggested that the pigment aggregating nerves are adrenergic while the dispersing nerves are cholinergic (Parker et al., 1945). Pye (1964) was unable to prove the existence of such double innervation in *Phoxinus*. Currently the existence of double innervation in melanophores is not widely accepted (Fujii and Oshima, 1986).

Kumazawa and Fujii (1984) have suggested a purinergic means of melansome dispersion based on accumulating evidence in various fish species which include tilapia, (*Sarotherodon niloticus*) (Kumazawa and Fujii, 1984); guppy, (*Lebistes reticulatus*) (Fujii and Miyashita,1976); and a catfish, (*Parasilurus asotus)* (Miyashita et al.,1984). Kumazawa and Fujii (1984) propose that ATP is released from nerve endings as a co-transmitter with noradrenaline. Following melanosome aggregation induced by noradrenaline, ATP is dephosphorylated by exoenzymes to produce adenosine. Adenosine then acts on it specific receptors on the melanophore plasma membrane to mediate melanosome dispersion.

It is apparent that there is support for the purinergic mechanism of melanosome dispersion in the fish species previously mentioned (Fujii and Miyashita,1976; Kumazawa and Fujii, 1984; Miyashita et al.,1984). However, the current work on winter flounder suggests that melanosome dispersion in this fish occurs via a β-adrenergic mechanism rather than by a purinergic means. Previous work conducted on winter flounder using ATP and related compounds could find no evidence for these compounds having a melanosome dispersive role in this species (Burton, unpublished data).

It has been shown here, that in flounder, α- and β_2-adrenoceptors play roles in pigment aggregation and dispersion, respectively, and that the type of response initiated by the melanophore is dependent upon the concentration of neurotransmitter employed. At high concentrations of noradrenaline pigment aggregation is mediated via α-adrenoceptors while at low concentrations pigment dispersion is mediated via β_2-adrenoceptors. Subsequently, melanophores only require adrenergic innervation and this study does not support the idea of doubly innervated melanophores as suggested by Parker (1934) and Parker and Rosenblueth (1941). Also, the mechanism in winter flounder requires only a catecholamine and not the presence of ATP as a co-transmitter (Kumazawa and Fujii,1984).

Indirect support for β-adrenoceptors mediating pigment dispersion has been demonstrated in a catfish, (*Parasilurus asotus*) (Fujii et al., 1985); blue damselfish, (*Chrysiptera cyanea*) (Kasukawa et al., 1985); guppy, (*Lebistes reticulatus*) (Miyashita and Fujii, 1975); and medaka,(*Oryzias latipes*) (Morishita et al., 1985). Miyashita and

Fujii (1975) found that low concentrations of adrenaline or isoproterenol dispersed melanosomes while high concentrations of these drugs aggregated them. These findings lend support to the idea that noradrenaline can exhibit α or β adrenergic activity depending on its concentration. However, these earlier experiments on other species utilized more than one drug which may have other unknown effects on melanophores which may affect the interpretation of the findings. Fujii et al. (1985) and Kasukawa et al. (1985) used a melatonin solution to aggregate melanosomes before application of possible pigment dispersing compounds. Miyashita and Fujii (1975) subjected melanophores to reserpine treatment followed by a brief incubation in noradrenaline to bring about a prolonged pigment aggregation. Following this, various drugs could be examine for pigment dispersing effects. Morishita et al. (1985) utilized denervated melanophores that were kept in physiological saline at room temperature for greater than 15 h before conducting experiments. By using more than one drug, these authors have introduced other unknown variables into their experiments which makes interpretation of the findings difficult. After incubation of scale slips in melanosome aggregating fluid, the present work is the first study to demonstrate an enhanced melanosome dispersion rate with noradrenaline alone in all pattern components of winter flounder without the complications of using other drugs.

The drug 6-OH dopamine is known to cause noradrenaline depletion from sympathetic nerve endings and selective degeneration of adrenergic fibers (Furness et al., 1970; Holmgren and Nilsson, 1976; Tranzer and Thoenen, 1968). It is possible to induce

a chemical sympathectomy of the nerve endings in scale slips which isolates the adrenergic receptors from the effects of nervous innervation. After such destruction, electrical stimulation of melanophores does not bring about pigment aggregation, which is consistent with the absence of a response to K^+ ion incubation following treatment with phentolamine, and confirms that the plasma membrane of the melanophore is a non-excitable membrane.

A high concentration of isoproterenol can evoke pigment aggregation in chemically sympathectomized scale slips. Isoproterenol and propranolol combined gives a similar finding. This is in agreement with the earlier suggestion that high concentrations of isoproterenol may operate via melanophore α-adrenoceptors to evoke melanosome aggregation in all pattern components. This suggestion is reinforced by the finding that phentolamine blocks this response in chemically sympathectomized scale slips.

An isoproterenol concentration that is only 10% of the above can have its melanosome aggregating effects reversed by propranolol, particularly in the white spots. This suggests some α- and β-adrenoceptor synergism in pigment aggregation in white spots and that its sensitivity is increased by denervation. This would mean that β_1-adrenoceptors are implicated as having a modulatory role in melanosome aggregation since atenolol blocks isoproterenol and electrically stimulated pigment aggregation. This is an extension of the findings of Burton et al. (1995) who suggest that there might be some β-adrenoceptor synergism. Atenolol appears to be much more potent in the white

spots as compared to the dark bands and general background pattern component. The results with atenolol suggest the β-adrenoceptor mediation in aggregation is of the β_1 conformation and that these receptors are differentially distributed.The β_1-adrenoceptors possibly mediate the distinctive paling responses of the white spots by being sensitive to plasma borne adrenaline from chromaffin tissue during stress which would explain their paling in otherwise dark flounder.

Winter flounder melanophores display pattern related differential responsiveness that probably involves coordination between neural aggregating and dispersing elements. (Burton, 1985). β-adrenoceptors have been implicated in mediating melanosome dispersion in other teleosts (Fujii et al., 1985; Kasukawa et al., 1985; Miyashita and Fujii, 1975). This study on winter flounder has implicated β-adrenoceptors of the β_2-subtype as controlling this process. The present work is an extension of previous work (Burton et al., 1995) and represents a significant step towards characterizing the mechanisms involved in controlling melanosome aggregation and dispersion at the peripheral neural level. It can now be stated that neurally regulated pigment aggregation in all pattern components of winter flounder is mediated through α-adrenoceptors and that β_2-adrenoceptors are involved in pigment dispersion. The differential character of the pattern-related responsiveness of the melanophores involves neuromodulation beyond this basic aggregation-dispersion mechanism. Indications of such additional neuromodulation are implicated in the current work but it has yet to be investigated in detail. This includes the

possibility of the involvement of neuropeptides, the role of which is virtually unknown in melanophore work (Grove, 1994).

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