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Signaling for color change in melanophores

and a biosensor application

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Theories come and theories go.
The frog remains.
JEAN ROSTAND (1960)

Papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

- I Karlsson AM, Lerner MR, Unett D, Lundström I, Svensson SPS. 2000. Melatonin-induced organelle movement in melanophores is coupled to tyrosine phosphorylation of a high molecular weight protein. *Cellular Signalling* 12:469-474.
- II Nilsson HM, Karlsson AM, Loitto V-M, Svensson SPS, Sundqvist T. 2000, Nitric oxide modulates intracellular translocation of pigment organelles in *Xenopus laevis* melanophores. *Cell Motility and the Cytoskeleton* 47:209-218.
- III Karlsson AM, Wiman ÅK, Svensson SPS. Potential tyrosine phosphorylation of spectrin and talin in the regulation of pigment organelle movement.
- IV Karlsson AM, Bjuhr K, Testorf M, Öberg PÅ, Lerner E, Lundström I, Svensson SPS. Biosensing of opioids using frog melanophores.

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Abbreviations

α -MSH	α -melanocyte stimulating hormone
A	absorbance
AC	adenylyl cyclase
ADP	adenosine diphosphate
A _f	final absorbance
A _i	initial absorbance
ATP	adenosine triphosphate
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
DAF-2 DA	4,5-diaminofluorescein diacetate
ES	electrospray
FAK	focal adhesion kinase
GC	guanylyl cyclase
G _{i/o} proteins	inhibitory G proteins
GPCR	linking G protein coupled receptor
GST	glutathion-S-transferase
HMW	high molecular weight
IPTG	isopropylthio-beta-D-galactoside
L-NAME	N ω -nitro-L-arginine methyl ester
MALDI	matrix assisted laser desorption ionization
MAPK	mitogen-activated protein kinase
MCH	melanin concentrating hormone
MEK	mitogen-activated protein kinase kinase, or MAP-ERK kinase; mitogen-activated protein-extracellular regulated kinase kinase
NAD	nicotinamide adenine dinucleotide
NOS	NO synthase
ODQ	1H-(1,2,4) oxadiazolo(4,3-a)quinoxalin-1-one
OP3	opioid receptor 3
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
SDS	sodium dodecylsulphate
T	transmittance
T _f	final transmittance
T _i	initial transmittance



Introduction

Chromatophores are adaptable pigment cells

(Excellently reviewed by (deOliveira, Castrucci et al. 1996), (Filadelfi and Castrucci 1996), (Nery and Castrucci 1997), (Fujii 2000)). Some vertebrates such as fish, amphibia, and reptiles, and many invertebrates have adaptable color patterned integument. This pattern can for instance be used for camouflage, sun protection, thermoregulation, and social interactions. The coloration can change quickly in response to changes in the environment, such as photic or thermal variations. The color change is mediated by specialized pigment cells in the integument, called chromatophores that synthesize and store pigments.

Chromatophores in fish are about 100 μm in diameter, located mostly in the dermal layer of the integument but can also be found in the epidermal layer. These pigmented cells are derived from the neural crest and migrate dorsolaterally beneath the ectoderm around the periphery of the developing embryo (Gilbert 1994). The morphology of the chromatophores varies from highly dendritic to discoid shape, depending on the location in the animal and on animal species (Obika 1986).

The color of the integument is a result of the absorption of light rays of certain wavelengths, and also due to scattering and reflection of light. There are six kinds of chromatophores, each recognized by its color. The light absorbing chromatophores are the melanophores (brown), erythrophores (red), cyanophores (blue), and xanthophores (yellow). Melanophores are full of melanin-filled granules, melanosomes, which gives the cells their characteristic dark brown color. Leucophores (white) and iridophores (metallic) lack color and their particles reflect light.

Color change by melanophores

All classes of chromatophores can regulate the integument color, although this thesis is focused on melanophores. The dark melanophores can either hide colorful cells so that the animal appears dark, or expose colors from underneath, see Figure 1. Variations in the environment are detected and the animal regulates its colors and patterns via communicating nerve cells and hormones in the blood stream, which affect the melanophores. In response to hormonal or neural stimulation, pigment granules migrate toward or away from the cell center in an ordered manner. Hormones as well as neurotransmitters act on transmembrane receptors located on the melanophore cell surface.





Figure 1. Micrographs show a *Xenopus laevis* melanophore. The melanosomes are fully dispersed in the cell at the picture to the left. Aggregation of melanosomes to the cell center in the other pictures is a result of melatonin stimulation. The pictures show the same cell 0, 15, 30, and 45 minutes after stimulation with 10 nM melatonin. Martin Testorf kindly provided the micrographs.

Several substances are documented in the regulation of melanosome movement and some of them are discussed here. Color changes in fish and amphibians can be induced by α -melanocyte stimulating hormone (α -MSH), a tridecapeptide secreted from the intermediate lobe of the pituitary. The melanosomes disperse throughout the cytoplasm when α -MSH is applied to melanophores. α -MSH is secreted when fishes are on a dark background and the scales accordingly appear dark. The α -MSH secretion is inhibited when fishes have a light surrounding and thereby the scales lighten (Ganong 1993). Melanin concentrating hormone (MCH) is a heptadecapeptide that can bring about a lightening in the scales of neopterygian fish. MCH thereby antagonizes the darkening caused by α -MSH. The MCH peptide is secreted from the neurohypophysis and it acts by concentrating pigment granules to the center of melanophores.

Noradrenaline from the sympathetic division of the autonomic nervous system and adrenaline from the adrenal medulla regulate the distribution of melanosomes differently, depending on the type of adrenergic receptor activated in melanophores. Activated α -adrenoceptors mediate aggregation of melanosomes, and activated β -adrenoceptors mediate dispersion. Melatonin-stimulation of melanophores results in aggregation of melanosomes in amphibians, although its effect is not always apparent in fish. Melatonin synthesis in the pineal gland follows a 24-hour rhythm, reaching maximum in the dark period of the day. Visible light leads to dispersion of melanosomes in *Xenopus laevis* melanophores (Daniolos, Lerner et al. 1990) and the signal is conveyed by a seven transmembrane photoreceptor, melanopsin (Provencio 1998).

Intracellular signaling

Pigment movement in melanophores is regulated by the intracellular concentration of adenosine 3',5'-cyclic monophosphate (cAMP). High



levels of cAMP lead to dispersion and low levels cause aggregation of melanosomes. This has been shown for many fish and amphibians (reviewed by (Tuma and Gelfand 1999)). α -MSH-induced dispersion of *X. laevis* melanosomes is mediated by increased concentration of cAMP (van de Veerdonk and Konjin 1970), (Daniolos, Lerner et al. 1990), (Potenza and Lerner 1992), and activated protein kinase A (PKA) (Reilein, Tint et al. 1998). Dispersion of melanosomes can also be induced by phorbol esters, mezerin and synthetic diacylglycerol which activate protein kinase C (PKC) (Sugden and Rowe 1992), (Graminski, Jayawickreme et al. 1993). Activation of a recombinant bombesin receptor expressed in melanophores induced dispersion without raising cAMP, demonstrating that dispersion can be mediated by a cAMP-independent pathway (Graminski, Jayawickreme et al. 1993). The authors inferred an alternative pathway via phospholipase C, diacylglycerol and PKC. The endogenous endothelin C receptor is coupled to the phospholipase C pathway, and inhibitors of both PKA and PKC block the endothelin dispersion response (McClintock, Rising et al. 1996). Further studies on phorbol ester induced dispersion showed that PKC can only partially disperse melanosomes in the absence of active PKA (Reilein, Tint et al. 1998), hence a basal level of PKA is probably needed for pigment dispersion.

Aggregation of melanosomes in *X. laevis* melanophores is generated by the hormone melatonin. Pertussis toxin sensitive $G_{i/o}$ proteins (White, Sekura et al. 1987), (Sugden 1991) convey signals from the activated melatonin receptor Mel1c (Ebisawa, Karne et al. 1994), (Reppert 1997). The $G_{i/o}$ proteins probably inhibit adenylyl cyclase and the resulting low cAMP concentration most likely fails to activate PKA. Moreover, active serine and threonine protein phosphatase 2A (PP2A) is necessary for pigment aggregation (Cozzi and Rollag 1992), (Reilein, Tint et al. 1998).

In brief, melanosome dispersion requires the activity of serine threonine protein kinases and aggregation requires serine threonine protein phosphatase activity. It has been shown that α -MSH induces phosphorylation of a 53 kDa protein, which precedes dispersion of melanosomes in *X. laevis* melanophores. Immunoblots suggested that the phosphorylated 53 kDa protein was β -tubulin (de Graan, Oestreicher et al. 1985). Recent experiments confirmed phosphorylation of a 50 kDa band in α -MSH treated cells. The band was detected both in blots probed for phosphotyrosine and phosphothreonine (Reilein, Tint et al. 1998). Reilein *et al* also showed diverse phosphorylation patterns of proteins in the molecular weight region of 87-95 kDa, although the identity of the phosphorylated proteins remains unknown.



Moving melanosomes

The transport system responsible for the ordered movement of melanosomes consists of cytoskeletal tracks and motor proteins that move melanosomes along these tracks (reviewed by (Tuma and Gelfand 1999)). The cytoskeleton is commonly divided into microtubules, actin filaments and intermediate filaments, of which microtubules and actin filaments have been shown important in melanosome movement. Actin filaments and microtubules are polar structures with distinct plus and minus ends, which allow directed movement along the tracks. Microtubules in melanophores radiate from the cell center with their fast growing plus ends directed toward the periphery (Euteneuer and McIntosh 1981), (Rollag and Adelman 1993). Actin filaments in melanophores are arranged in a cortical web, and also as parallel filaments in surface projections (Obika, Menter et al. 1978), (Schliwa, Weber et al. 1981), (Rollag and Adelman 1993). Intermediate filaments are distributed along microtubules by the motor protein kinesin (Gyoeva, Leonova et al. 1987), (Gyoeva and Gelfand 1991).

It is nowadays well established that microtubules are required for melanosome movement, and actin-based movement has also been considered. Two recent reports using actin-disrupting drugs showed that actin filaments are utilized for achievement of uniform distribution of melanosomes in the dispersed state. Disruption of actin filaments in *X. laevis* melanophores caused aggregation of melanosomes. Thus actin filaments was necessary for maintenance of the dispersed state (Rogers and Gelfand 1998). Black tetra melanophores are also dependent on actin filaments for uniform distribution of melanosomes. Disruption of Black tetra actin filaments caused hyperdispersion and melanosomes gathered at the cell margin (Rodionov, Hope et al. 1998). The authors suggested that the reverse polarity of melanosome movement in actin-deprived amphibian and fish cells illustrate different arrangements of actin filaments. A model for melanosome dispersion was proposed: melanosomes initially move to the periphery along radial microtubules and then continue their movement along randomly oriented actin filaments.

Motor proteins are multimeric enzymes that convert energy from adenosine triphosphate (ATP) hydrolysis into directed movement along the cytoskeleton. Generally, kinesins are plus-end directed and cytoplasmic dyneins move towards the minus end of microtubules (reviewed by (Hirokawa 1998)). Melanosome aggregation is mediated by cytoplasmic dynein as shown in *Gadus morhua* (Nilsson, Rutberg et al. 1996), (Nilsson and Wallin 1997), and *X. laevis* (Rogers, Tint et al. 1997). Microtubule-mediated dispersion is probably achieved by kine-



sin II (Tuma, Zill et al. 1998), although conventional kinesin co-localize with aggregated and dispersed melanosomes in *Gadus morhua* (Nilsson, Rutberg et al. 1996). Myosin V is probably the actin-based motor responsible for dispersion in *X. laevis* melanophores (Rogers and Gelfand 1998). Immunoblots have shown that kinesin II, cytoplasmic dynein, (Rogers, Tint et al. 1997) and myosin V (Rogers and Gelfand 1998) are present on purified melanosomes from *X. laevis*. Apparently all three motors are present on melanosomes at the same time. In these two studies, melanosomes were purified from cultured cells that could be assumed to have dispersed melanosomes, as light induces dispersion. When melanosomes were purified from aggregated and dispersed cells, both cytoplasmic dynein and kinesin II remained on melanosomes (Reese and Haimo 2000).

Current models and thoughts

Tuma and Gelfand discussed pigment transport in a recent review (Tuma and Gelfand 1999). My understanding of their suggested model is as follows. Cytoplasmic dynein governs aggregation and kinesin II is responsible for fast dispersing movement, and myosin V is required for short-range spreading of melanosomes. Thus disruption of actin would lead to hyperdispersion due to kinesin II activity, as happens in fish melanophores. In frog cells, myosin V tethers melanosomes to actin filaments, to prevent aggregation by cytoplasmic dynein. Basically, the same model is valid for both fish and frog cells. The differences are explained by their respective equilibrium in motor activity during dispersion. The authors also refer to the abundance of microtubules in fish, compared to amphibian melanophores. The difference may account for the rapid color change in fish.

Rogers and Gelfand comment on another aspect (Rogers and Gelfand 1998). Myosin V seems to have an accessory role in short-range pigment distribution fish cells. However, in frog cells, myosin V seems necessary for long-range dispersion and maintenance of the dispersed state. The authors point out, since frogs require both microtubules and actin for dispersion, this might represent an evolutionary midpoint between a fast microtubule-dominated mechanism in fish and a more actin-based system in mammalian melanocytes. However, recent reports demonstrate that cytoplasmic dynein and kinesin have important roles in microtubule-based melanosome transport in human melanocytes (Vancoillie, Lambert et al. 2000), (Byers, Yaar et al. 2000).

Nilsson and Wallin have previously discussed the possibility that dynein is continuously active during both aggregation and dispersion,



whereas kinesin is the target for regulation (Nilsson and Wallin 1997). Recently, Nilsson proposed that kinesin II, cytoplasmic dynein, and myosin V are all regulated in fish melanosome movement (Nilsson 2000). Nilsson suggests that all three motors are active when dispersing melanosomes reach the cell periphery, both to distribute melanosomes and to balance motor forces. Signaling for aggregation inactivates kinesin II and myosin V, although by different signaling pathways. During aggregation, active cytoplasmic dynein keeps the melanosomes in the cell center. A dispersion signal inactivates cytoplasmic dynein and activates kinesin II. Cytoplasmic dynein and myosin V are reactivated when melanosomes reach the cell periphery.

Reese and Haimo reported that cytoplasmic dynein, dynactin, and kinesin II are cyclically activated and inactivated in *X. laevis* melanosomes (Reese and Haimo 2000). In their model, cytoplasmic dynein and dynactin maintain the aggregated state by binding to microtubules, while kinesin II is inactive. α -MSH-induced active PKA and PKC phosphorylates and inactivates cytoplasmic dynein and dynactin while kinesin II is activated by PKA and melanosomes disperse. Active kinesin II in dispersed cells is inactivated by PP2A following melatonin stimulation. PP2A also activates cytoplasmic dynein to aggregate melanosomes. Protein phosphatase 1 (PP1)-induced activation of dynactin may enhance aggregation or tether melanosomes to the cell center. This proposal is not in agreement with the previously described models, where myosin V and actin filaments clearly contribute to melanosome movement.



Aims of the study

We wanted to investigate signal transduction taking place in melanophores when they were stimulated with melatonin. We also wanted to examine the use of melanophores as biosensors. The aims were to study:

- If tyrosine phosphorylations were implicated in melatonin-induced melanosome aggregation.
- If tyrosine phosphorylation controlled melanosome movement.
- How the phosphorylation signal was induced via melatonin-stimulation.
- The identity of the phosphorylated proteins.
- The function of the phosphorylated proteins.
- If NO synthase was expressed and NO produced in melanophores.
- The role of NO in melanosome distribution.
- If tyrosine phosphorylation and NO signaling was linked.
- Melanophores as biosensors.

The aspiration to truth is more precious than its assured possession.

G. E. LESSING (1778)





Methods

Cells

The cells studied in this thesis were melanophores from the frog *X. laevis*. Cells were isolated from frogs in the early stage of development, when eggs hatch to become tadpoles. Cell lines of melanophores and fibroblasts were established and detailed procedures of isolation and propagation have been described elsewhere (Daniolos, Lerner et al. 1990), (McClintock and Lerner 1997). *X. laevis* fibroblasts were used for production of unknown growth factors needed for melanophores to proliferate. Culture media from fibroblasts were filtered to remove cells and then used to feed melanophores twice a week. For long time storage, stock cells were kept at 18 °C to reduce cell division. Stock cell media was changed once a month. Melanophore and fibroblast cell lines were generous gifts from Michael R. Lerner, Department of Dermatology, University of Texas Southwestern Medical Center, Dallas, TX, USA.

Microtiter plate assays

Analysis of dispersion or aggregation of melanosomes was done with microtiter plate assays, adapted from earlier protocols (Potenza and Lerner 1992), (Martin, Ronde et al. 1999). Melanophores were seeded at 20,000 cells per well in microtiter plates two days before an experiment. Microplates were examined for complete cell coverage of the well bottoms on the day of the assays. For experiments in Paper II, cells were washed with medium (0,7 x Leibovitz L-15 medium) before drug addition. For experiments in Paper I, III, and IV, cells were incubated with medium and exposed to room light for 1 h before drug addition. This allowed light-induced dispersion of melanosomes in the cells. If dispersion of melanosomes was to be assayed, cells were exposed to 1 nM melatonin for 1 h as well, which caused melanosome aggregation. Drugs were stored in aliquots and diluted daily in medium. Control experiments of solvents were made.

Absorbance was measured at 650 nm directly before and 30 minutes after addition of drug using a microtiter plate reader. Fractional change in absorbance, A_f/A_i-1 , was used to quantify melanosome aggregation (Potenza, Graminski et al. 1994). A_i was the initial absorbance immediately before drug addition and A_f was the final absorbance 30 minutes afterward. A_f/A_i-1 was plotted on the y-axis versus drug concentration on the x-axis to illustrate changes in melanosome distribution. When $A_f/A_i-1 = 0$, no change was detected. Aggregation of melanosomes generated a negative value of A_f/A_i-1 and dispersion of melanosomes generated a positive value.



For quantification of melanosome dispersion in Paper I, absorbance readings, A , were transformed to transmittance data, T , using the general equation $T = 10^{-A}$. The formula $1-T_f/T_i$ was used, where T_i was the initial transmission data before drug addition and T_f the final transmission data 30 minutes afterward (Potenza and Lerner 1992). When $1-T_f/T_i = 0$, no change in melanosome distribution was detected. Aggregation of melanosomes generated a negative value of $1-T_f/T_i$ and dispersion of melanosomes generated a positive value.

Immunoblot analysis

In Paper I and III, confluent melanophores growing in 25 cm²-flasks were rinsed with 0.7 x phosphate-buffered saline (0.7 x PBS) before addition of drugs dissolved in medium. Control cells were treated with medium only. Removal of medium and addition of ice-cold 0.7 x PBS stopped the stimulation. In Paper II, growing cells were immediately rinsed with ice-cold 0.7 x PBS.

Cells were lysed in ice-cold lysis buffer and scraped off the flasks using a rubber policeman. Cell suspensions were then left for 30 minutes at 4 °C on an orbital shaker and the resulting lysates were centrifuged at 5,000 x g for 10 minutes at 4 °C. Protein content in the supernatants were determined using a BCA Protein Assay Kit. Supernatants were mixed 1:2 with sample buffer and heated at 97 °C for 5 minutes. Aliquots were separated on 5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The proteins were transferred to nitrocellulose membranes overnight. Electrophoresis and electroblotting were performed using Bio-Rad equipment according to provided manuals. The membranes were blocked for 1 hour in room temperature with 5% (w/v) dry milk in washing buffer to minimize unspecific binding. Then membranes were incubated for 1 hour with respective primary and secondary antibody diluted in washing buffer, and rinsed between incubations. Membranes were analyzed using enhanced chemiluminescence.

Immunoprecipitation

Confluent melanophores growing in 25 cm²-flasks were rinsed with 0.7 x PBS before incubation with 1 nM melatonin or medium for 5 minutes. Removal of medium and addition of ice-cold 0.7 x PBS stopped the stimulation. The cells were lysed as previously described and centrifuged at 10,000 x g for 10 minutes at 4 °C. Pellets were discarded and aliquots of the lysates were removed for subsequent SDS-PAGE. The lysates were incubated with primary antibodies for 1 hour at 4 °C. Twenty μ l of Protein A/G-Agarose was added and the lysates were incubated at 4 °C overnight. Pellets were collected by



centrifugation at 1,000 x *g* for 5 minutes at 4 °C and aliquots of the supernatants were removed for subsequent SDS-PAGE. Pellets were washed 3 times with PBS and resuspended in sample buffer. The samples labeled lysate, supernatant, and pellet, from stimulated and control cells, were separated on 5% SDS-PAGE gels and blotted to membranes as described under “Immunoblot analysis”.

In vitro phosphorylation

The N-terminal 47 kDa domain of chicken talin, corresponding to amino acids 1-433, was expressed as a glutathion-S-transferase (GST) fusion protein in BL21 *E. coli* and purified. The GST talin fusion protein was a gift from David Critchley (University of Leicester, England). Briefly, overnight cultures were diluted 1:10 and grown for an additional 2 hours at 37 °C. Expression was induced by addition of 0.1 mM isopropylthio-beta-D-galactoside (IPTG). After 2 hours, cells were harvested, lysed, sonicated, and Triton X-100 was added to a final concentration of 1% to facilitate solubilization of proteins. The lysate was centrifuged at 10,000 x *g* for 10 minutes at 4 °C. Soluble fusion protein was purified using a GSTrap column. Pigment cells were scraped of the flasks into lysis buffer and centrifuged at 5,000 x *g* for 10 minutes at 4 °C. The supernatants were incubated with 20 or 40 µl fusion protein for 30 minutes at room temperature to allow phosphorylation. Fusion protein was isolated from the mixture using Micro-Spin columns filled with glutathione sepharose 4B. The eluates were mixed with sample buffer, separated on 12% SDS-PAGE gels and proceeded with as described under “Immunoblot analysis”.

Microscopy

Fluorescence microscopy

Melanophores were seeded in chambered coverglasses. Melanophores were incubated with 5 nM 4,5-diaminofluorescein diacetate (DAF-2 DA). Images of melanophores were taken before and after DAF-2 DA addition. Cells were studied in a Zeiss Axiovert 135M microscope equipped for epifluorescence detection with a 100 W HBO mercury arc lamp and a Zeiss 10x objective (0.22 NA). Images of the DAF-2 DA-derived fluorescence were obtained using a 470 ± 20 nm bandpass filter for excitation and a 540 ± 25 nm bandpass filter for emission (i.e. a FITC channel). Filters were from Zeiss. Images were captured using a water-cooled TE4 Astromed 4200 slow-scan CCD-camera controlled by PixCel software. The images were obtained using 100 msecond exposures. Time-lapse sequences, with 5 minutes between successive images, were recorded for up to 30 minutes. All im-



ages were stored on an IBM-compatible computer. The same look-up table was used in all fluorescence images.

Bright-field microscopy in Paper II

Diluted melanophore suspensions were seeded in chambered cover-glasses to obtain single cell cultures. Melanophores were preincubated with L-arginine or N ω -nitro-L-arginine methyl ester (L-NAME) before inducing aggregation with melatonin. Control cells were preincubated with medium only. Images of single cells were taken before and every 10 minutes after melatonin addition, up to 30 minutes. Cells were observed using a Zeiss Axiovert 135M microscope equipped with a Zeiss oil-immersion fluar objective (40x, 1.3 NA). Images were captured using a CCD video camera controlled by a real-time image intensifier attached to an IBM-compatible computer.

Bright-field microscopy in Paper IV

Melanophores were seeded in flat bottom tissue culture plates. Pictures were taken before the addition of 1 μ M DAMGO and 40 minutes afterwards. The micrographs were taken in an inverted microscope (Zeiss Axiovert 100M) using a chilled, black&white CCD camera and stored in an IBM-compatible computer with frame grabber IC-PCI and the software Optimas 6.2.

Electroporation

Electroporation of melanophores were performed using an ECM 399 BTX electroporator, essentially according to a protocol described previously (McClintock and Lerner 1997). The human opioid receptor 3 (OP3) DNA inserted in a pcDNA3 vector was a gift from Jia-Bei Wang (Molecular Neurobiology Branch, Addiction Research Centre, NIDA, Baltimore, MD, USA.). pSVneo was a gift from Susanna Cotecchia (University of Lausanne, Switzerland). The melanophores were co-transfected with the plasmids pcDNA3OP3 and pSVneo. pSVneo contains an antibiotic resistance gene and transfected melanophores were selected by the presence of geneticin in the cell culture medium. Briefly, chilled plasmid DNA, 4 μ g OP3 and 0,1 μ g pSVneo, were mixed with 3 x 10⁶ melanophores on ice. The suspension was transferred to a 4 mm electroporation cuvette and electroporated at 380 V, 150 Ω , and 1050 μ F. The suspension was seeded in a tissue culture plate previously coated with Cell-tak. Cells were propagated in cell culture with 128 μ g/ml geneticin in the medium.



Results and discussion

The study of melatonin-induced tyrosine phosphorylation

Signaling from the G protein coupled melatonin receptor in melanophores was in focus in Paper I, and III. It is nowadays well established that melatonin-stimulation of melanophores results in aggregation of melanosomes to the cell center and that the obvious outcome is more transparent cells (McCord and Allen 1917), (Lerner, Case et al. 1958). As previously described in the Review of the literature section in this thesis, it has been shown that the activity of serine and threonine kinases as well as phosphatases regulates the distribution of melanosomes in the cells. Any role for tyrosine phosphorylations in signaling from the G protein coupled melatonin receptor has not been revealed so far.

In studies on cellular proliferation, a number of authors have described common signaling components linking G protein coupled receptors (GPCR) and receptor tyrosine kinase signaling. GPCR are tyrosine kinase substrates and can also regulate mitogen-activated protein kinase (MAPK) signals (reviewed by (Malbon and Karoor 1998), (Dikic and Blaukat 1999), (Gutkind 2000)). GPCR coupled to inhibitory G proteins have been shown to induce tyrosine phosphorylation (Luttrell, Daaka et al. 1999), hence we wanted to study if these signals were present in the regulation of melanosome aggregation. First, we examined if an inhibitor of tyrosine phosphorylations would interfere with melatonin-induced melanosome aggregation.

Aggregational movement of melanosomes could require tyrosine phosphorylations

We used microplate assays to study the possible effects of the tyrosine phosphorylation inhibitor genistein on melanosome aggregation induced by melatonin. Pre-incubation with genistein inhibited aggregation dose-dependently (Paper I, Figure 1a and b). Daidzein, a negative control compound for genistein, did not inhibit the aggregation response as clearly as genistein. 100 μM daidzein was needed to achieve inhibition comparable to the effect of 10 μM genistein (not shown).

We concluded from these experiments that tyrosine phosphorylation of unknown substrates might be important regulators in the motile machinery. We also proposed the following question: after melatonin stimulation, is tyrosine phosphorylation followed by melanosome aggregation, or is it the other way around?



Melatonin induced tyrosine phosphorylation of a high molecular weight protein

To confirm that tyrosine phosphorylations took place in melanosomes, we made immunoblots. Cells were stimulated with melatonin for 0-30 minutes, and then proteins in cell lysates were blotted onto membranes and labeled with anti-phosphotyrosine antibodies. A clear tyrosine phosphorylated band appeared in cell lysate from cells stimulated for 5 minutes (Paper I, Figure 2a). The intensity of the tyrosine phosphorylated band decreased as stimulation times increased, though the intensity did not completely reach base level after 30 minutes. Cell lysate from unstimulated cells showed a barely detectable degree of tyrosine phosphorylation. A rough estimation of the molecular weight of the tyrosine phosphorylated protein was made by comparison with standard proteins with known molecular weights (not shown). The phosphorylated protein was of high molecular weight (HMW), approximately 270-290 kDa. The kinetics of tyrosine phosphorylation was compared to kinetics of melatonin-induced melanosome aggregation in microplate assays (Paper I, Figure 2b). Both the process of tyrosine phosphorylation and melanosome aggregation seemed to have early onsets. As the tyrosine phosphorylation was most intense shortly after melatonin stimulation, we believe that it was not caused by melanosome migration, as the migration continued further, although at lower speed. Instead, we assumed that phosphorylation preceded melanosome aggregation or that these were simultaneous events.

Another kinetic experiment described in Paper III (Figure 4) revealed that a second melatonin-stimulation produced a second rise in phosphorylation, although the subsequent phosphorylation was somewhat weaker. It is interesting to point out that at the time of the second melatonin-stimulation, the melanosomes are practically completely aggregated in the cell center (not shown). Therefore, we believed that phosphorylation of the HMW protein was not a result of the migration as such. Another interpretation of the results was that the HMW protein was maybe not located on melanosomes, as they might be inaccessible for kinases when they were aggregated in the cell center. The results from the kinetic experiment was also in agreement with the hypothesis that phosphorylation precedes melanosome aggregation, as it could take place in cells with already aggregated melanosomes.

Besides melatonin stimulation, depolymerization of filamentous actin can induce melanosome aggregation. Rogers and Gelfand have shown that actin-perturbing drugs caused aggregation of melanosomes, without addition of melatonin. Actin-bound melanosomes seemed necessary for dispersion and maintenance of the dispersed state. De-



polymerization of actin filaments caused melanosome aggregation along microtubules, as the dispersed state was disturbed (Rogers and Gelfand 1998). To test if this aggregation movement involved tyrosine phosphorylation of the HMW protein, we made immunoblots using latrunculin B as an inhibitor of actin polymerization. Latrunculin B did not induce phosphorylation. In contrast, it seemed to lower basal phosphorylation (Paper III, Figure 3). Thus phosphorylation was not a result of melanosome aggregation, or required for the aggregation movement as such.

When we summarized the experiments described in previous sections, we believed that during melatonin stimulation of melanophores, phosphorylation of the HMW protein preceded melanosome aggregation. Maybe disruption of actin filaments and phosphorylation of the HMW protein shared the same intracellular effect in the cells?

Signaling for tyrosine phosphorylation of the HMW protein

We studied the effect of various substances on melatonin-induced aggregation and tyrosine phosphorylation of the HMW protein in melanophores.

To confirm the results from microplate assays, we made immunoblots of cells pre-incubated with the tyrosine kinase inhibitor genistein and its inactive analogue daidzein, before melatonin-stimulation (Paper I, Figure 3). As expected, genistein clearly inhibited tyrosine phosphorylation of the HMW protein but daidzein only slightly inhibited the phosphorylation.

In another experiment, melanophores were pre-incubated with the $G_{i/o}$ protein inhibitor pertussis toxin before stimulation with melatonin. Immunoblots of these cells showed that tyrosine phosphorylation was clearly reduced by pertussis toxin (Paper I, Figure 4). This indicated that melatonin initiated tyrosine phosphorylation through a $G_{i/o}$ protein coupled receptor, probably Mel1c (White, Sekura et al. 1987), (Sugden 1991), (Ebisawa, Karne et al. 1994), (Reppert 1997).

Knowing so far that melatonin-stimulated melanosome aggregation via a $G_{i/o}$ -coupled receptor involved tyrosine phosphorylation, we used α -MSH to stimulate melanosome dispersion in the cells. α -MSH did not induce any tyrosine phosphorylation (Paper I, Figure 5). Thus the tyrosine phosphorylated protein was not connected to melanosome movement in general, but more specifically related to receptor-mediated, melatonin-induced melanosome aggregation.

It has been shown that dispersion of melanosomes was dependent on increasing cAMP concentration, while reduced cAMP levels led to aggregation of granules (reviewed by (Tuma and Gelfand 1999)). The



adenylyl cyclase (AC) activator forskolin inhibited melanosome aggregation in response to melatonin in microplate assays (Paper I, Figure 1d). In addition, forskolin reduced tyrosine phosphorylation in response to melatonin (Paper I, Figure 3). These results suggested that tyrosine phosphorylation was inhibited when active AC generated cAMP, which could activate PKA. We used the two PKA inhibitors H-89 and Rp-8-Cl-cAMPS to test if inhibited PKA generated tyrosine phosphorylation, as inactive PKA is assumed to be part of melatonin-induced aggregation. As shown in Paper III, Figure 2, neither of the two PKA inhibitors induced tyrosine phosphorylation in the cells. Perhaps tyrosine phosphorylation was not an outcome of the classical cAMP-regulated pathway. A possible alternative was that $G_{\beta\gamma}$ -subunits from activated $G_{i/o}$ -proteins could initiate tyrosine phosphorylation of the HMW protein. $\beta\gamma$ -subunits have been suggested to activate the MAPK cascade via β -arrestin mediated recruitment of c-Src to the plasma membrane (Luttrell, Ferguson et al. 1999). A recent study reports that a direct interaction between c-Src and the intracellular parts of the β_3 -adrenergic receptor is required for MAPK activation (Cao, Luttrell et al. 2000).

The possible involvement of c-Src and mitogen-activated protein kinase kinase (MEK) was investigated using the inhibitors herbimycin A and PD98059. Microplate assays showed that herbimycin A inhibited melanosome aggregation (Paper III, Figure 1B). Herbimycin A also inhibited melatonin-induced tyrosine phosphorylation in immunoblots (Paper III, Figure 1A). This suggested that c-Src may be needed for phosphorylation and aggregation. PD98059 also inhibited both tyrosine phosphorylation (Paper III, Figure 1A) and melanosome aggregation (Paper III, Figure 1C) by melatonin. Thus it seemed, as functional MEK was required in the signaling as well.

Some new interpretations of previously described results appeared, if MEK was involved in melanophore signaling. The results from our kinetic experiments, that tyrosine phosphorylation was most intense 5 minutes after melatonin-stimulation, and then declined (Paper I, Figure 2), might be due to the actions of PP2A on the MEK pathway. Active PP2A is needed for melanosome aggregation in melanophores (Reilein, 1998) and PP2A has been shown to dephosphorylate and inactivate both MEK and MAPK (Sontag, Fedorov et al. 1993), (Alessi, Gomez et al. 1995). The finding that tyrosine phosphorylation was inhibited by the AC activator forskolin (Paper I, Figure 3) may be mediated via inhibition of MEK activity. cAMP has been shown to inhibit MEK by uncoupling Ras from activation of Raf-1 (Cook and McCormick 1993).



It was interesting to note the possible involvement of c-Src in the process of melanosome aggregation. It has been shown in lymphoid cells that enrichment of actin in raft patches is dependent upon tyrosine phosphorylation in the patches (Harder and Simons 1999). It is believed that raft patches concentrate or separate proteins and lipids in the plasma membrane. Src-kinases are probably raft-associated (Simons and Ikonen 1997) and c-Src has been shown to coimmunoprecipitate with actin in synapses (Zhao, Cavallaro et al. 2000). Intracellular clusters of HMW protein, c-Src, actin and other proteins such as receptors, G proteins and kinases might well facilitate signaling in melanophores.

In summary, the described results suggested that melatonin-induced melanosome aggregation via a $G_{i/o}$ -coupled receptor involved c-Src, MEK and tyrosine phosphorylation of a HMW protein.

The identity of the tyrosine phosphorylated HMW protein

Mass spectrometric techniques were used to investigate the identity of the HMW protein. Melanophores were lysed and melanosomes removed by centrifugation. Proteins in lysates were separated on SDS-polyacrylamide gels. One gel was stained with coomassie to reveal protein bands of various size. Proteins in another gel were blotted onto a nitrocellulose membrane and labeled with antibodies towards phosphotyrosine, to reveal tyrosine phosphorylated proteins on a film. The gel and the film were put side by side and the 270-290 kDa protein was recognized on the gel. Bands of the 270-290 kDa protein were cut out and the gel plugs frozen in distilled water. The gel plugs were subjected to mass spectrometric analysis (Shevchenko, Wilm et al. 1996) by Protana A/S, Denmark. The gel plugs were washed and digested with trypsin and masses of the peptides were determined by matrix assisted laser desorption ionization (MALDI) mass spectrometry (Figure 2). Partial sequence information was obtained from ten of the peptides by nano-electrospray (ES) mass spectrometry. For the query against sequence databases, peptide mass and sequence were combined into sequence tags. Figure 5A and B (Paper III) show the regions where peptides with high mass accuracy matched sequenced proteins. The gel plugs contained protein homologous to human spectrin β chain and mouse talin.



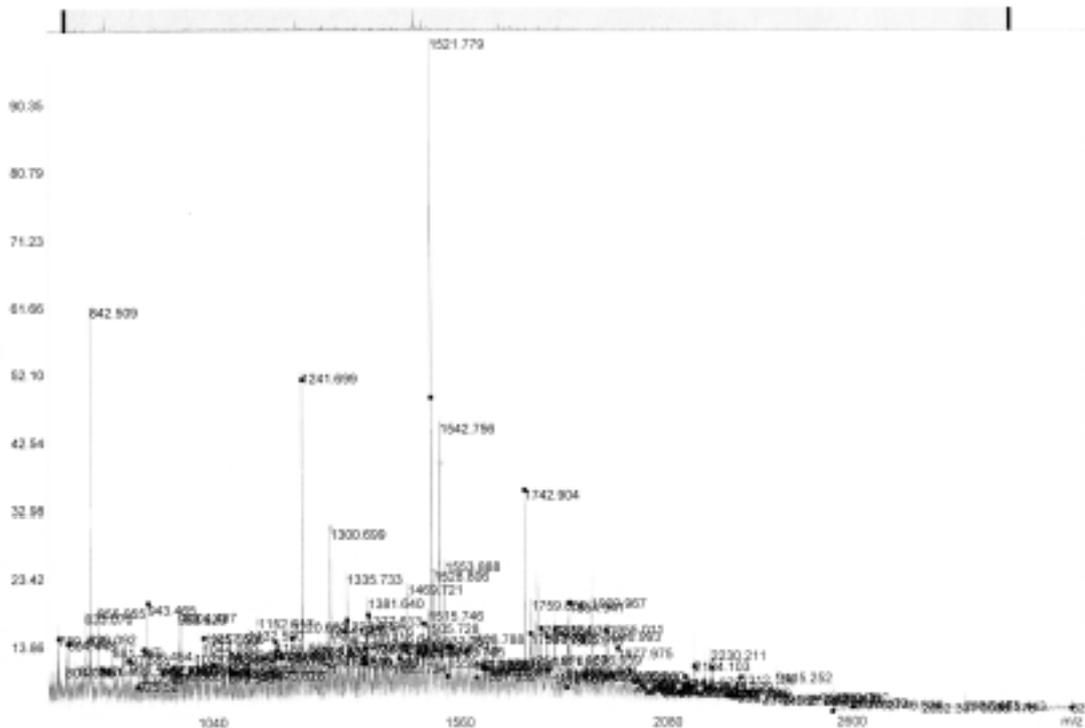


Figure 2. MALDI-mass spectrum results of the HMW SDS-PAGE band. Peaks are labeled □ (talin) and ■ (spectrin) to illustrate the origin of respective polypeptides.

Spectrin and talin are actin-binding proteins found on the cytoplasmic side of the plasma membrane (Li and Bennett 1996), (Critchley 2000), (De Matteis and Morrow 2000). Spectrin is also present on the membrane of intracellular organelles, and links them to motor proteins as well as to cytoskeleton fibers (De Matteis and Morrow 2000). Talin has been shown to be tyrosine phosphorylated by v-Src in v-Src transformed fibroblasts, though the specific site of phosphorylation is unknown (Sabe, Hamaguchi et al. 1997). The NetPhos 2.0 Protein Phosphorylation Prediction Server (Blom, Gammeltoft et al. 1999) was used to predict tyrosine phosphorylation sites in mouse talin and human spectrin β -chain. The predictions showed that both talin and β -spectrin have numerous potential tyrosine phosphorylation sites (Table 1).



Mouse talin			Human β -spectrin		
Position	Context	score	Position	Context	score
127	NHDEYSLVR	0.763	9	VATDYDNIE	0.917
199	RKFFYSDQN	0.689	18	IQQQYSDVN	0.614
298	AKVRYVKLA	0.900	79	ITDLYTDLR	0.836
373	DFGDYQDGY	0.914	190	KTAGYPNVN	0.804
377	YQDGYYSVQ	0.936	307	MIEKYESLA	0.884
570	AETDYTAVG	0.989	493	EAENYHDIK	0.971
1116	GNENYAGIA	0.900	601	DGEGYKPCD	0.754
1853	SFVDYQTTM	0.550	620	MEFCYQELC	0.574
1945	PSDVYTKKE	0.774	666	SSDDYGKDL	0.943
			831	IEERYKEVA	0.981
			1114	EIDNYEEDY	0.798
			1118	YEEDYQKMR	0.989
			1190	NNQEYVLAH	0.803
			1408	QSDDYGKHL	0.901
			1598	AQQYYFDAA	0.514
			1614	EQELYMMSE	0.892
			1644	AVEDYAETV	0.845
			2039	GQEPYLSSR	0.686
			2237	EMGFYKDAK	0.942

Table 1. Prediction of tyrosine phosphorylation sites in talin and β -spectrin by the NetPhos 2.0 Protein Phosphorylation Prediction Server. The output score values are in the range (0.000-1.000). In general the higher the score, the higher the confidence of the prediction (Blom, Gammeltoft et al. 1999).

The results from mass spectrometry analysis suggested that the 270-290 kDa protein band observed on SDS-polyacrylamide gels consisted of two proteins with similar molecular weights. To be able to confirm or rule out spectrin and talin as the HMW protein, we made immunoprecipitation experiments. Unfortunately, we did not find any β -spectrin antibody that crossreacted with *X. laevis* protein. Thus β -spectrin phosphorylation could not be either confirmed or excluded.

Two variants of immunoprecipitations were made. First, immunoprecipitation of talin was followed by immunoblots with phosphotyrosine antibodies. Then, immunoprecipitation of phosphotyrosine was followed by immunoblots with talin antibodies. Both these variants showed that talin was phosphorylated as a result of melatonin stimulation (Paper III, Figure 6). However, the immunoprecipitation experiments were difficult to reproduce. The study might be troubled due to talins sensitivity to proteolysis and generation of many different fragments (Bolton, Barry et al. 1997). Therefore, we used an additional technique.

A fusion protein was used to study *in vitro* phosphorylation of talin by activated melanophore lysate. The fusion protein corresponded to the N-terminal 47 kDa domain of chicken talin, contained an actin binding site (Hemmings, Rees et al. 1996), and was produced as a GST fu-



sion protein in *E. coli*. After incubation with cell lysate, the fusion protein was isolated and analyzed by immunoblots. Talin antibodies confirmed the presence of talin on the blots, but no tyrosine phosphorylations were detected (Paper III, Figure 7). Thus melatonin-activated melanophore lysate failed to phosphorylate the fusion protein.

There were some suggestions to explain the disparity between the results from talin immunoprecipitations and talin *in vitro* phosphorylation experiments. First, talin may not be the tyrosine phosphorylated HMW protein. Next, species differences could be important. The chicken talin fusion protein might not contain specific sites required for a *X. laevis* kinase substrate. Perhaps eucaryotic post-translational modifications of the fusion protein were needed. Furthermore, the talin domain in the studied fusion protein might not contain the specific site. Still, there are several potential phosphorylation sites in talin, as described in Table 1.

In conclusion, analysis of the HMW protein revealed two promising candidates, spectrin β -chain and talin, for the tyrosine-phosphorylated protein regulating melanosome aggregation.

The study of NO in melanosome aggregation

The possible involvement of NO in melanosome aggregation by melatonin was investigated in Paper II. NO has previously been shown to affect other cytoskeleton dependent processes such as smooth muscle relaxation (Carvajal, Germain et al. 2000) and paracellular permeability (Gorodeski 2000). We first studied if the enzyme NO synthase (NOS) was expressed in melanophores.

Detection of NOS expression and NO synthesis

Expression of NOS was studied by immunoblots of cell lysate. Antibodies raised against rat NOS1 and human NOS2 was used. Both antibodies bound to a protein of about 116 kDa, whereas the NOS2 antibody produced a more marked band (Paper II, Figure 1). Thus the melanophores produced proteins that were recognized by antibodies raised against NOS epitopes, and the labeled proteins were probably *X. laevis* NOS.

The cellpermeable probe 4,5-diaminofluorescein diacetate (DAF-2 DA) was used to detect NO production in the cells. DAF-2 DA becomes fluorescent upon reaction with NO (Kojima, Sakurai et al. 1998), (Nakatsubo, Kojima et al. 1998). Fluorescence intensity increased with time after addition of DAF-2 DA to melanophores. Most intense fluorescence was seen in cell centers and dendrites where few melanosomes were present (Paper II, Figure 2). This distribution might



not only reflect sites of NO production. Melanin could occlude staining in melanosome filled areas, as it is a light-absorbing pigment (Rogers, Karcher et al. 1999). The results indicated that NO was produced in melanophores, although specific locations for NO synthesis were not demonstrated.

Inhibition of NOS affected melanosome aggregation

Having shown that NOS was present and NO was produced in melanophores, we then tested if inhibition of NO synthesis had any effect on melanosome aggregation. Melanophores were incubated with the NOS substrate L-arginine or the NOS inhibitor L-NAME before stimulation with melatonin. Micrographs showed that melanosomes aggregated as in control cells after incubation with L-arginine (Paper II, Figure 3). On the contrary, melanosomes were hindered to aggregate when they had been incubated with L-NAME. L-NAME probably reduced NO production, and caused an intracellular NO deficiency that mysteriously inhibited aggregation. L-NAME was also shown to inhibit melatonin-induced aggregation in microplate assays, whereas L-arginine did not affect aggregation much (Paper II, Figure 4).

Was cGMP important in melanosome aggregation?

A number of substances were used to study if NO signaling via guanylyl cyclase (GC), guanosine 3',5'-cyclic monophosphate (cGMP), and protein kinase G (PKG) was involved in aggregation of melanosomes. The GC inhibitor 1H-(1,2,4) oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) reduced aggregation. This indicated that functional cGMP-producing GC was needed for aggregation. In contrast, the PKG inhibitor KT 5823 did not affect aggregation. This suggested that GC and cGMP could mediate the effect of NO in aggregation but not by PKG.

We used the nonhydrolysable cGMP analogue 8-Br-cGMP to mimic cGMP production. 8-Br-cGMP also inhibited aggregation. This result implied that elevated concentrations of cGMP could disturb aggregation. Perhaps low concentrations of cGMP were needed for melanosome aggregation, but higher concentrations inhibited aggregation. Even so, interpretation of the combined results of the experiments on ODQ, KT5823, and 8-Br-cGMP were not straightforward. We could not demonstrate that the effect of NOS inhibition was mediated via the route GC, cGMP or PKG.

Inhibition of NOS mysteriously reduced aggregation

Initially, we concluded that NOS was expressed, as antibodies towards NOS labeled proteins in melanophore lysate. For L-NAME to have an effect, NOS must have been active in the melanophores, either constantly or in response to a stimulus. The DAF-2 DA probe showed that



NO was present in cells which had not been stimulated with melatonin, L-arginine or L-NAME.

We studied if inhibition of NOS affected melatonin-induced tyrosine phosphorylation of the HMW protein. Immunoblots showed that treatment with L-NAME did not affect tyrosine phosphorylation (Figure 3). Thus the effect of NOS inhibition on melanosome aggregation was not mediated via changes in melatonin-induced tyrosine phosphorylation of the HMW protein.

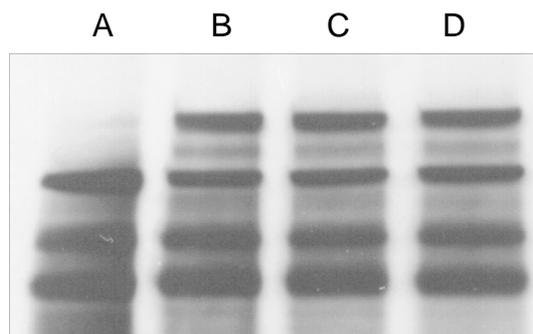


Figure 3. Immunoblot labeled with phosphotyrosine antibodies. Melanophores were pretreated with medium (A, B), 1 mM L-arginine (C), or 1mM L-NAME (D) for 15 minutes and then stimulated with 1 nM melatonin for 5 minutes (B, C, D). Melanophores in (A) were incubated in medium only.

Others have demonstrated NO effects that were not mediated via cGMP (reviewed by (Mateo and Artinano 2000)). Cysteine and tyrosine groups in proteins could be nitrosylated by NO. These modifications are suggested to account for NO inactivation of G_i proteins, NO inhibition of AC, and NO activation of PKC. Incorporation of 3-nitrotyrosine in α -tubulin resulted in microtubule dysfunction and possibly a decreased affinity for cytoplasmic dynein to α -tubulin (Eiserich, Estevez et al. 1999).

A possible scenario to clarify our findings could be that in the presence of NO, AC was nitrosylated and inactivated. If L-NAME was added, more AC could then become active and produce cAMP, which could lead to dispersion. This was contradicted by recent experiments where it was shown that L-NAME did not increase cAMP concentration (Nilsson 2000).

Another previously described cGMP-independent action of NO was stimulation of adenosine diphosphate (ADP)-ribosyltransferases, which transfer ADP-ribose groups from nicotine adenine dinucleotide (NAD^+) to proteins. NO could probably inhibit G_i proteins by activation of ADP-ribosyltransferases (Pozdnyakov, Lloyd et al. 1993). It has also been shown that NO initiated ADP-ribosylation of actin and thereby inhibited actin polymerization in neutrophils (Clancy, Leszczynska et



al. 1995). Rogers and Gelfand have shown that depolymerization of actin filaments caused melanosome aggregation (Rogers and Gelfand 1998). Perhaps NO disturbed actin polymerization, and enabled aggregation? Inhibition of NOS and subsequent reduced ADP-ribosylation would then allow more actin polymerization, which could limit aggregation. However, other studies have shown no difference in arrangement of actin filaments in dispersed and aggregated melanophores (Schliwa, Weber et al. 1981), (Rollag and Adelman 1993).

Biosensing with melanophores

The use of recombinant melanophores as a biosensor was considered in Paper IV. A human GPCR, opioid receptor 3, was inserted into melanophores by the transfection method electroporation. Melanophores and DNA were mixed and exposed to high voltage which made transient pores in the plasma membranes. This enabled the entry of foreign DNA into the cells, before the plasma membranes were resealed. Melanophores were electroporated with two DNA-plasmids, one coding for the opioid receptor and another coding for antibiotic resistance. The cells were selected by the presence of antibiotics in the culture medium after electroporation.

Melanophores aggregated their melanosomes in response to opioids

The melanophore response to opioids was studied by micrographs and aggregation assays. Micrographs showed that melanophores aggregated their melanosomes when DAMGO, a synthetic opioid peptide, was added (Paper IV, Figure 1A). DAMGO induced complete aggregation in the responding melanophores. However, all melanophores in the culture did not respond by aggregating their melanosomes. This could be due to the experimental setup. Melanophores which survived antibiotic treatment should have incorporated the antibiotic resistance plasmid, but not necessarily the plasmid coding for the opioid receptor. Thus, the non-responsive cells might lack the opioid receptor.

The electroporated melanophores were then studied in aggregation assays. Both DAMGO and morphine caused dose-dependent aggregation of melanosomes (Paper IV, Figure 1B). The effect of the opioid receptor inhibitor naloxone was also studied in aggregation assays. Increasing concentrations of naloxone gave competitive inhibition of the aggregation response to DAMGO (Paper IV, Figure 2). The naloxone dissociation constant pA_2 was estimated to 8.67. This value is consistent with the report of a naloxone-morphine dissociation constant of 8.56 in guinea pig ileum. Hence the pharmacology of



the expressed opioid receptors was very similar to its mammalian counterpart.

Prospective uses of melanophore biosensors

Transfection of melanophores with selected receptors enables the creation of a large number of melanophore biosensors, which could detect various substances. In favor of the melanophores is the measurement of a final physiological response with its inherent biochemical amplification. There is no need for additional fluorescent or radioactive compounds or reporter gene constructs. The simple format, the 96 well microtiter plates, enables simultaneous analysis of separate samples, or parallel detection of assorted substances in a single sample. The initial melanophore response is fast, although it takes about 1 hour to get maximal aggregation response (Paper I, Figure 2). Detection of melanosome movement could probably be done after 5 or 10 minutes. If an even faster response is desired, development of fish cell lines might be an interesting alternative. For example, melanophores from *Labrus ossifagus* (Svensson, Adolfsson et al. 1997) and erythrophores from *Myripristis occidentalis* (Karlsson, Andersson et al. 1988) aggregated within 5 seconds after sympathetic nerve stimulation. Xanthophores from *Oryzias latipes* responded to light by pigment aggregation within 30 seconds (Oshima, Nakata et al. 1998). Thus fish cell biosensors may enable more rapid detection of substances.

The human genome has been estimated to code for several thousands of GPCR (Kolakowski 1994), which might be transfected into melanophores. Even so, the potential melanophore biosensors are not limited to detecting ligands to GPCR. Cell surface receptors of another class, namely growth factor receptors, could also be expressed in melanophores (Graminski and Lerner 1994).

Transfected melanophores can be used for pharmacological studies, where the effect of an applied drug can be detected by means of the induced color change in the cells. For example, the cells can be used in drug design high-throughput screening in the search for "hits" in a library of chemical compounds, which might interact with defined "targets", e.g. GPCR. Substances that possibly could be detected by melanophore biosensors are for example narcotics, (as shown in Paper IV), odors, tastes, and pheromones, whose receptors belong to the GPCR family (Horn, Weare et al. 1998). Recently, the discovery of a putative pheromone receptor gene expressed in human olfactory mucosa was reported (Rodriguez, Greer et al. 2000). Pheromones are chemicals produced by an organism that signals its presence to other members of the same species. The pheromones are believed to have particular importance in sexual behavior. Potential pheromone sen-



sors can be very useful in future pheromone research. Their convenient high-throughput format enables rapid screening of substances in the exciting search for human pheromones.





More speculative thoughts on the function of the mysterious protein

This thesis reports that both talin and β -spectrin are probable candidates of the tyrosine phosphorylated HMW protein. Some tentative models are described in this section.

What if β -spectrin is the HMW protein?

Spectrin is a cytoskeletal protein that forms a membrane skeleton web of anti-parallel heterodimers of α -spectrin and β -spectrin (De Matteis and Morrow 2000). Neuronal spectrin is a tertiary complex with two heterodimers aligned head to head (Goodman, Zimmer et al. 1995). Spectrin was first characterized as a protein skeleton supporting the plasma membrane of erythrocytes (Yu, Fischman et al. 1973). In addition, spectrin isoforms are found on intracellular organelles such as the Golgi, endo-lysosomes, and synaptic vesicles as well as on the plasma membrane in the presynaptic compartment (Goodman, Zimmer et al. 1995), (De Matteis and Morrow 2000). Numerous ligands such as protein kinases, $G_{\beta\gamma}$ subunits, motor proteins, and integral membrane proteins bind selectively to spectrin, either directly or via adapter proteins (De Matteis and Morrow 2000). Actin can bind directly and via the adapter protein adducin, which also binds microtubules (Li and Bennett 1996), (De Matteis and Morrow 2000). Networks of linked spectrin dimers, with their respective associated proteins and lipids, form clusters where membrane proteins, cytosolic signaling molecules, and cytoskeleton fibers come together. Interactions concerning spectrin and its adapter molecules seem to be regulated by, for instance, phosphorylation, proteolysis, and small GTP-binding proteins (De Matteis and Morrow 2000).

There may be similarities between the directed melanosome movement along the cytoskeleton in melanophores and the regulated release of neurotransmitters from secretory vesicles in neurons. During synaptic transmission, vesicles are released from their cytoskeletal bonds and fused with the presynaptic plasma membrane. Brain spectrin associates to synaptic vesicles through a synapsin I attachment site on β -spectrin (Zimmer, Zhao et al. 2000). Synapsin I is a membrane protein on synaptic vesicles, which controls the availability of vesicles during synaptic transmission (De Camilli, Benfenati et al. 1990). Synapsin I and c-Src have been shown to coimmunoprecipitate (Onofri, Giovedì et al. 1997), (Foster-Barber and Bishop 1998), and the interaction of synapsin I with c-Src enhances c-Src tyrosine kinase activity (Onofri, Giovedì et al. 1997). Electron microscopy of presynaptic terminals has shown that the vesicles are closely associated to 100 nm



fibers, connecting vesicles to the plasma membrane. These strands are assumed to be spectrin (Landis, Hall et al. 1988). A “casting the line hypothesis” was formulated by Goodman and Zimmer five years ago (Goodman, Zimmer et al. 1995). Briefly, a connection between spectrin on the plasma membrane and synapsin I on the vesicle membrane may form when vesicles approach the plasma membrane. According to the hypothesis, the binding of a vesicle via synapsin I to spectrin would weaken a spectrin-actin bond, releasing one end of spectrin from the actin skeleton, while the other end is still tied to the plasma membrane via actin. The synapsin I attachment site on β -spectrin is shown to be close to an actin-binding domain (Zimmer, Zhao et al. 2000).

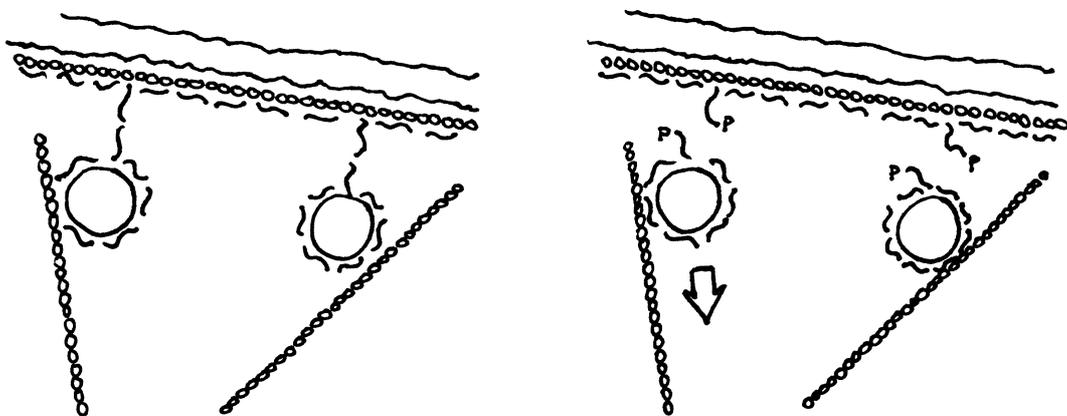


Figure 4. The spectrin cord model. A) Spectrin cords linking melanosomes to the cell membrane maintain the dispersed state. B) Melatonin signaling for aggregation induces tyrosine phosphorylation of β -spectrin. Phosphorylated spectrin adopts another conformation and the spectrin cord disrupts. Melanosomes are free to aggregate.

The spectrin cord model

Agents such as α -MSH and light induce the dispersed state in *X. laevis* melanophores. When the melanosomes are migrated centrifugally by kinesin along microtubules, they eventually come across actin filaments traversing the cytoplasm or coating the plasma membrane. Many melanosomes continue their dispersing movement along actin filaments, now driven by myosin. Spectrin is distributed on the melanosomes and on the inside of the plasma membrane. Some spectrins are connected to actin filaments that line the plasma membrane. When the melanosomes reach the plasma membrane, spectrins on melanosomes and plasma membrane may switch attachment points when they come in contact, causing a spectrin-spectrin-actin binding of melanosomes to the plasma membrane. A similar binding may also



be mediated by synapsin I or other membrane proteins on melanosomes, forming a synapsin I-spectrin-actin connection to the inside of the plasma membrane. In either case, spectrin would constitute a cord, connecting melanosomes to actin on the plasma membrane. There is also a possibility that spectrin and synapsin I molecules on adjacent melanosomes connect to each other, as well as spectrin molecules on adjacent melanosomes. Dispersed melanosomes are thus in a tethered position, bound via spectrin-spectrin-actin or synapsin I-spectrin-actin, and remain close to the plasma membrane.

When melanosomes are stimulated with melatonin, the signal is conveyed through the receptor to $G_{i/o}$ proteins, that separate into α and $\beta\gamma$ subunits and transfer the signal to effector proteins. The signal is possibly mediated within short distances in a protein cluster, or membrane raft, as protein kinases, $G_{\beta\gamma}$ subunits, motor proteins, cytoskeleton filaments, and integral membrane proteins such as receptors are all known to interact with spectrin at the cytoplasmic side of the plasma membrane. Besides other effects, the signaling results in tyrosine phosphorylation of β -spectrin, perhaps via c-Src and the MAPK cascade. Phosphorylated β -spectrin adopts a different conformation that causes synapsin I and actin to dissociate. This enables aggregational movement of melanosomes by dynein, as melanosomes are no longer tethered to actin filaments at the plasma membrane.

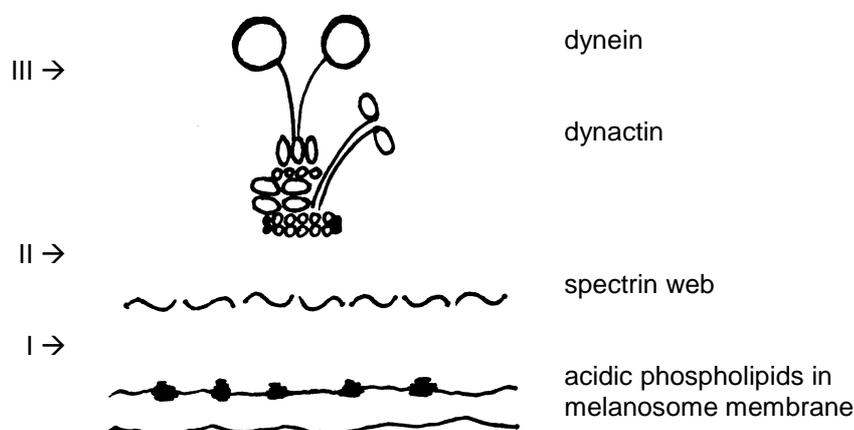


Figure 5. The spectrin dynactin dynein lipid model. Melatonin signaling for aggregation and tyrosine phosphorylation of β -spectrin could either I) improve spectrin binding to lipids and facilitate dynactin-dynein function, or II) increase spectrin affinity to dynactin, or III) indirectly increase dynein ATPase activity.

The spectrin dynactin dynein lipid model

Recent *in vitro* findings indicate that spectrin is necessary for dynactin-dependent dynein-driven transport on microtubules (Muresan, Stankewich et al. 2000). The investigators suggest that dynactin is recruited to a spectrin meshwork on organelles and that spectrin binds



to acidic phospholipids on organelles via its plextrin homology domain. Moreover, production of acidic phospholipids may regulate the recruitment of spectrin-dynactin-dynein to organelle membranes.

In melanophores, spectrin might recruit the dynein-dynactin complex to melanosomes. The melatonin-induced tyrosine phosphorylation of β -spectrin could either I) improve spectrin binding to lipids, to facilitate dynactin-dynein function on melanosomes, or II) increase spectrin affinity to dynactin, or III) increase dynein ATPase activity. Thereby aggregational movement of melanosomes by dynein would be enabled.

What if talin is the HMW protein?

Talin is a membrane-associated protein localized in cellular contacts with the extracellular matrix (Critchley 2000). The binding properties of talin make it an interesting candidate for linkage of signals from the outside via interactions with membrane proteins, to the inside of cells, where it connects to the cytoskeleton and signaling components. Talin binds *in vitro* to, for example, integrins, actin, and the protein tyrosine kinase called focal adhesion kinase (FAK) (Critchley 2000). Talin is very sensitive to proteolysis and generates many different peptide fragments (Bolton, Barry et al. 1997). Calpain II cleavage results in an N-terminal fragment of 47 kDa and a 190-200 kDa C-terminal domain (Rees, Ades et al. 1990).

Talin has been shown important in the organization of actin filaments and control of cell motility. Microinjection of talin antibodies disrupted actin filaments and inhibited motility in human fibroblasts (Bolton, Barry et al. 1997). Talin has also been shown to be tyrosine phosphorylated by v-Src in v-Src transformed fibroblasts (Sabe, Hamaguchi et al. 1997). If this is applicable to the actions of c-Src remains to be shown. We suggested that c-Src might be required for tyrosine phosphorylation of the HMW protein and aggregation of melanosomes in melanophores (Paper III).

Studies of myocytes reported that actin stress fibers were undetectable after platelet derived growth factor (PDGF) stimulation (Tidball and Spencer 1993). Immunoprecipitations showed that talin and the PDGF receptor were tyrosine phosphorylated. There were no changes in the distribution of talin, but the PDGF receptor was located on granules in the perinuclear region after PDGF stimulation. These results raises the possibility that tyrosine phosphorylation of talin prevents its binding to actin.

Furthermore, *in vitro* experiments showed that talin has a myosin-binding site in the N-terminal (Lin, Kishi et al. 1998). The 47 kDa N-terminal talin fragment could stimulate myosin ATPase activity, ir-



respective of the phosphorylation state of myosin. Intact talin did not stimulate myosin when actin was present. The investigators suggested that talin might crosslink myosin and actin.

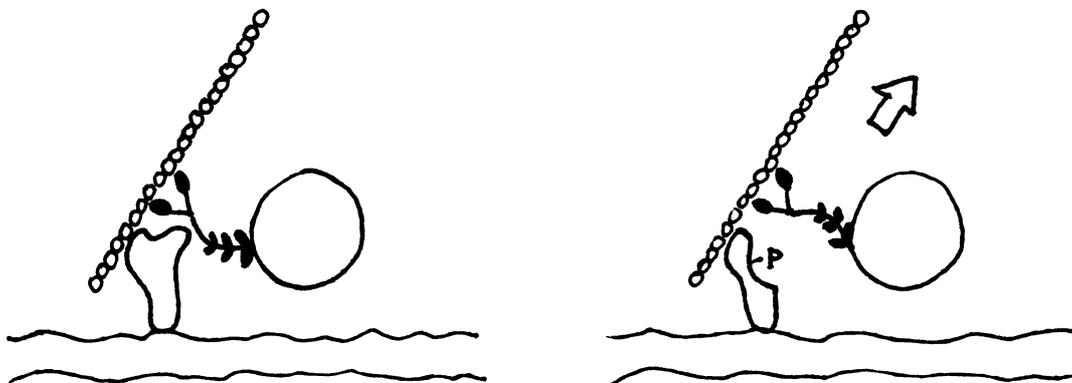


Figure 6. The talin-myosin model. A) Talin maintains the dispersed state by either I) binding of inactive myosin to the region close to the membrane, or II) activation of myosin. B) Signaling for aggregation and tyrosine phosphorylation of talin causes either I) detachment of myosin, or II) inactivation of myosin. Melanosomes are free to aggregate.

The talin-myosin model

Actin filaments are required to maintain the dispersed state in melanophores (Rogers and Gelfand 1998). Active myosin might be needed to disperse melanosomes. In the dispersed state, talin either I) crosslinks myosin and its connected melanosomes to actin filaments, or II) keeps myosin active. Both alternatives would hinder aggregation of melanosomes. In these two scenarios, melatonin-induced phosphorylation could have these effects. I) Phosphorylated talin releases myosin and its attached melanosomes from actin filaments. II) Phosphorylated talin adopts a conformation that does not activate myosin. These two alternatives enable aggregational movement of melanosomes by dynein, as melanosomes are no longer I) bound to actin or II) moved by myosin along actin filaments.



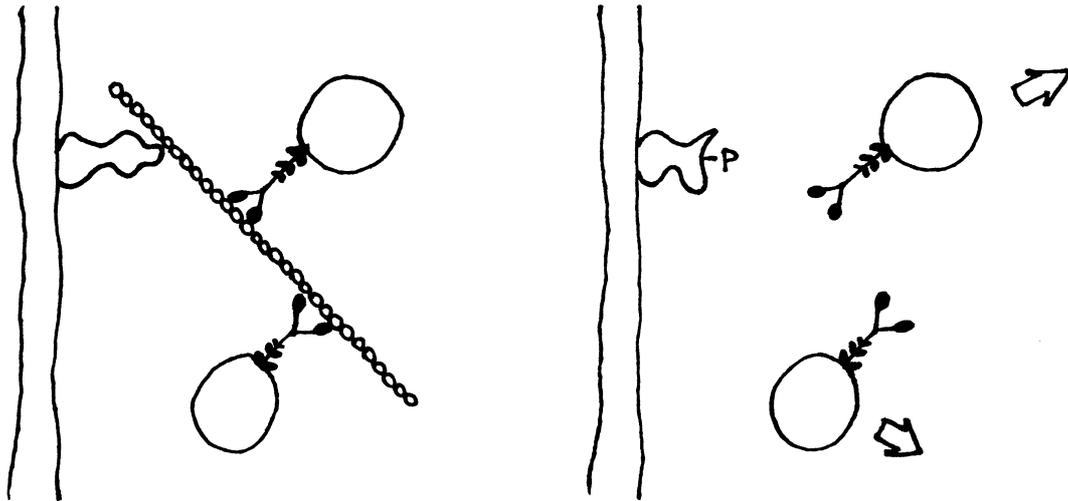


Figure 7. The talin-actin binding model. A) Talin maintains the dispersed state by stabilizing actin filaments. B) Signaling for aggregation induces tyrosine phosphorylation of talin. Phosphorylated talin releases actin filaments, which depolymerize. Melanosomes are free to aggregate.

The talin-actin binding model

When these reports are summarized, a second talin model suggests itself:

- Microinjection of talin antibodies disrupts actin filaments in human fibroblasts (Bolton, Barry et al. 1997).
- Latrunculin disrupts actin filaments and induces melanosome aggregation in melanophores (Rogers and Gelfand 1998).
- PDGF-stimulation of myocytes resulted in tyrosine phosphorylation of talin and disassembly of actin stress fibers (Tidball and Spencer 1993).

Maybe tyrosine phosphorylation of talin disrupts actin filaments and facilitates melanosome aggregation?

In this model, talin binds to actin filaments in the dispersed state. Melatonin-stimulation initiates phosphorylation of talin. Phosphorylated talin adopts a different conformation that causes release and subsequent depolymerization of actin filaments. This enables aggregational movement of melanosomes by dynein, as melanosomes are no longer tethered to actin filaments by myosin. There are reports that argue against this hypothesis. It has been shown in *X. laevis* as well as *Pterophyllum scalare* melanophores that melatonin does not cause rearrangement of actin filaments as it induces melanosome aggregation (Schliwa, Weber et al. 1981), (Rollag and Adelman 1993).



Conclusions

Signal transduction pathways controlling melatonin-induced melanosome aggregation in *X. laevis* melanophores were studied. The use of melanophores as biosensors has also been examined. The experimental results were interpreted as follows.

- Melanosome aggregation was accompanied by tyrosine phosphorylation of a HMW protein. Inhibition of tyrosine phosphorylation reduced melanosome aggregation as well. We believed that the phosphorylation preceded pigment aggregation. Two candidates for the HMW protein were presented, talin and β -spectrin.
- Tyrosine phosphorylation of the HMW protein was mediated via a $G_{i/o}$ protein coupled receptor, probably the melatonin receptor Mel1c.
- Increased AC activity reduced tyrosine phosphorylation of the HMW protein. On the other hand, inhibition of PKA did not induce phosphorylation. We assumed that the phosphorylation was not a result of the classical $G_{i/o}$ protein pathway, as Src-kinase and MEK seemed required for phosphorylation and melanosome aggregation.
- NO appeared to be necessary for melanosome aggregation. The inhibitory effect of decreased NO concentration was probably not mediated via GC, cGMP or PKG. We speculated that NO could affect melanosome distribution via modifications of the actin cytoskeleton.
- The effect of NOS inhibition on melanosome aggregation was not mediated via changes in tyrosine phosphorylation of the HMW protein.
- The enzyme NOS was expressed in melanophores, as NOS antibodies recognized proteins in melanophore lysate.
- Melanophores could be used for detection of opioids. The melanophore response was dose-dependent to opioids. An inhibitor of opioid receptors reduced the aggregation response.





Comments on future research

Along with the findings in this study, many new questions have appeared, of which a few are discussed here.

- We have occasionally observed tyrosine phosphorylation of a protein about 110 kDa after melatonin stimulation. What is this protein and does it have any function in melanosome movement? Mass spectrometry might reveal the identity of the protein.
- To confirm the identity of the tyrosine-phosphorylated protein, 2D-gel electrophoresis and mass spectrometry analysis should be useful.
- Where is the HMW protein located in melanophores? On the granules? At the plasma membrane? Centrifugation into cellular fractions and immunoblotting might reveal the locations of the HMW protein. This could also be studied by microscopic examination of immunolabeled cells.
- Perhaps the HMW protein is part of a signaling cluster? Immunoprecipitation might reveal interacting proteins such as receptors, G proteins, kinases, phosphatases, motors, or cytoskeleton components.
- It would be valuable to make antibodies directed towards *X. laevis* β -spectrin and talin, especially since we have not found any antibody crossreacting with *X. laevis* β -spectrin. Antibodies directed towards purified protein, or to *in vitro* synthesized peptide sequences could be produced. These antibodies would be important in the study of intracellular localization, protein interactions via immunoprecipitation, and the function of β -spectrin and talin in melanophores.
- Is talin the tyrosine phosphorylated HMW protein? Talin is sensitive to proteolysis. Treatment of cell lysate with thrombin or calpain II, and labeling of immunoblots with talin and phosphotyrosine antibodies should reveal a 47 kDa or a 190-200 kDa protein.
- What site, which aminoacid(s) in β -spectrin or talin is phosphorylated? *In vitro* phosphorylation of short peptides could locate the phosphorylation(s).
- Is myosin active to maintain dispersion? Actin filaments are required to maintain the dispersed state in melanophores (Rogers and Gelfand 1998). Perhaps active myosin is necessary to keep melanosomes dispersed? Motor activity could be measured by myosin immunoprecipitation followed by determination of the amount of ATP hydrolyzed over time.



- It would also be really interesting to know when the motors dynein and kinesin are active in melanophores. Immunoprecipitation of motors followed by ATP hydrolysis measurements should reveal exciting findings.

We must not conceal from ourselves the fact that the causal investigation of organisms is one of the most difficult, if not the most difficult problem which the human intellect has attempted to solve, and that this investigation, like every causal science, can never reach completeness, since every new cause ascertained only gives rise to fresh questions concerning the cause of this cause.

WILHELM ROUX (1894)



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Lessing, G. E. 1778. "Eine Duplik." Reprinted in F. Muncker (ed.), *Sämtliche Schriften* 13, Göschen, Leipzig, 1897, p. 23.

Rostand, J. 1960. *Carnets d'un Biologiste*. Librairie Stock, Paris.

Roux, W. 1894. The problems, methods, and scope of developmental mechanics. *Biol. Lect. Woods Hole* 3:149-190.



References

- Alessi, D. R., N. Gomez, et al. (1995). "Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines." *Curr Biol* 5(3): 283-295.
- Blom, N., S. Gammeltoft, et al. (1999). "Sequence and structure-based prediction of eukaryotic protein phosphorylation sites." *J mol biol* 294(1351-1362).
- Bolton, S. J., S. T. Barry, et al. (1997). "Monoclonal antibodies recognizing the N- and C-terminal regions of talin disrupt actin stress fibers when microinjected into fibroblasts." *Cell Motil Cytoskeleton* 36: 363-376.
- Byers, H. R., M. Yaar, et al. (2000). "Role of cytoplasmic dynein in melanosome transport in human melanocytes." *J Invest Dermatol* 114(5): 990-7.
- Cao, W., L. M. Luttrell, et al. (2000). "Direct binding of activated c-Src to the β_3 -adrenergic receptor is required for MAP kinase activation." *J Biol Chem* 275(49): 38131-4.
- Carvajal, J. A., A. M. Germain, et al. (2000). "Molecular mechanism of cGMP-mediated smooth muscle relaxation." *J Cell Physiol* 184: 409-420.
- Clancy, R., J. Leszczynska, et al. (1995). "Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils." *J Leukoc Biol* 58(2): 196-202.
- Cook, S. J. and F. McCormick (1993). "Inhibition by cAMP of Ras-dependent activation of Raf." *Science* 262: 1069-1072.
- Cozzi, B. and M. D. Rollag (1992). "The protein-phosphatase inhibitor okadaic acid mimics MSH-induced and melatonin-reversible melanosome dispersion in *Xenopus laevis* melanophores." *Pigment Cell Res* 5: 148-154.
- Critchley, D. R. (2000). "Focal adhesions - the cytoskeletal connection." *Curr Opin Cell Biol* 12: 133-139.
- Daniolos, A., A. B. Lerner, et al. (1990). "Action of light on frog pigment cells in culture." *Pigment Cell Res* 3: 38-43.
- De Camilli, P., F. Benfenati, et al. (1990). "The synapsins." *Annu Rev Cell Biol* 6: 433-60.
- de Graan, P. N. E., A. B. Oestreicher, et al. (1985). "Characterization of α -MSH-induced changes in the phosphorylation of a 53 kDa protein in *Xenopus* melanophores." *Mol Cell Endocrinol* 42: 127-133.



- De Matteis, M. A. and J. S. Morrow (2000). "Spectrin tethers and mesh in the biosynthetic pathway." *J Cell Sci* 113: 2331-2343.
- deOliveira, A. R., A. M. L. Castrucci, et al. (1996). "Cellular signalling in vertebrate pigment cells." *Braz J Med Biol Res* 29: 1743-1749.
- Dikic, I. and A. Blaukat (1999). "Protein tyrosine kinase-mediated pathways in G protein-coupled receptor signaling." *Cell Biochem and Biophys* 30(3): 369-387.
- Ebisawa, T., S. Karne, et al. (1994). "Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores." *Proc Natl Acad Sci U S A* 91: 6133-6137.
- Eiserich, J. P., A. G. Estevez, et al. (1999). "Microtubule dysfunction by posttranslational nitrotyrosination of α -tubulin: a nitric oxide-dependent mechanism of cellular injury." *Proc Natl Acad Sci USA* 96(11): 6365-70.
- Euteneuer, U. and J. R. McIntosh (1981). "Polarity of some motility-related microtubules." *Proc Natl Acad Sci USA* 78(1): 372-376.
- Filadelfi, A. M. C. and A. M. d. L. Castrucci (1996). "Comparative aspects of the pineal/melatonin system of poikilothermic vertebrates." *J Pineal Res* 20: 175-186.
- Foster-Barber, A. and J. M. Bishop (1998). "Src interacts with dynamin and synapsin in neuronal cells." *Proc Natl Acad Sci USA* 95: 4673-4677.
- Fujii, R. (2000). "The regulation of motile activities in fish chromatophores." *Pigment cell res* 13: 300-319.
- Ganong, W. F. (1993). Review of medical physiology. Englewood Cliffs, New Jersey, Prentice Hall.
- Gilbert, S. F. (1994). Developmental biology. Sunderland, Sinauer Associates Inc.
- Goodman, S. R., W. E. Zimmer, et al. (1995). "Brain spectrin: of mice and men." *Brain Res Bull* 36(6): 593-606.
- Gorodeski, G. I. (2000). "NO increases permeability of cultured human cervical epithelia by cGMP-mediated increase in G-actin." *Am J Physiol Cell Physiol* 278: C942-C952.
- Graminski, G. F., C. K. Jayawickreme, et al. (1993). "Pigment dispersion in frog melanophores can be induced by a phorbol ester or stimulation of a recombinant receptor that activates phospholipase C." *J Biol Chem* 268(8): 5957-5964.



- Graminski, G. F. and M. R. Lerner (1994). "A rapid bioassay for platelet-derived growth factor β -receptor tyrosine kinase function." *Bio/Technology* 12: 1008-1011.
- Gutkind, J. S. (2000). Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors, *Science's stke*.
- Gyoeva, F. K. and V. I. Gelfand (1991). "Coalignment of vimentin intermediate filaments with microtubules depends on kinesin." *Nature* 353(6343): 445-8.
- Gyoeva, F. K., E. V. Leonova, et al. (1987). "Vimentin intermediate filaments in fish melanophores." *J Cell Sci* 88: 649-655.
- Harder, T. and K. Simons (1999). "Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation." *Eur J Immunol* 29: 556-562.
- Hemmings, L., D. J. G. Rees, et al. (1996). "Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site." *J Cell Sci* 109: 2715-2726.
- Hirokawa, N. (1998). "Kinesin and dynein superfamily proteins and the mechanism of organelle transport." *Science* 279: 519-526.
- Horn, F., J. Weare, et al. (1998). "GPCRDB: an information system for G protein-coupled receptors." *Nucleic Acids Res* 26(1): 275-279.
- Karlsson, J. O., R. G. Andersson, et al. (1988). "Pigment migration in fish erythrophores is controlled by α_2 -adrenoceptors." *Comp Biochem Physiol C* 91(2): 513-6.
- Kojima, H., K. Sakurai, et al. (1998). "Development of a fluorescent indicator for nitric oxide based on the fluorescein chromophore." *Chem Pharm Bull* 46(2): 373-375.
- Kolakowski, L. F. J. (1994). "GCRDb: a G-protein-coupled receptor database." *Receptors Channels* 2(1): 1-7.
- Landis, D. M. D., A. K. Hall, et al. (1988). "The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse." *Neuron* 1: 201-209.
- Lerner, A. B., J. D. Case, et al. (1958). "Isolation of melatonin, the pineal gland factor that lightens melanocytes." *J Am Chem Soc* 80: 2587.
- Li, X. and V. Bennett (1996). "Identification of the spectrin subunit and domains required for formation of spectrin/adducin/actin complexes." *J Biol Chem* 271(26): 15695-15702.



- Lin, Y., H. Kishi, et al. (1998). "N-terminal myosin-binding fragment of talin." *Biochem Biophys Res Commun* 249: 656-659.
- Luttrell, L., Y. Daaka, et al. (1999). "Regulation of tyrosine kinase cascades by G-protein-coupled receptors." *Curr Opin Cell Biol* 11(2): 177-83.
- Luttrell, L. M., S. S. G. Ferguson, et al. (1999). "β-arrestin-dependent formation of β₂ adrenergic receptor-Src protein kinase complexes." *Science* 283: 655-661.
- Malbon, C. C. and V. Karoor (1998). "G-protein-linked receptors as tyrosine kinase substrates: new paradigms in signal integration." *Cell Signal* 10(8): 523-527.
- Martin, V., P. Ronde, et al. (1999). "Leukotriene Binding, Signaling, and Analysis of HIV Coreceptor Function in Mouse and Human Leukotriene B₄ Receptor-transfected Cells." *J Biol Chem* 274(13): 8597-8603.
- Mateo, A. O. and M. A. A. d. Artinano (2000). "Nitric oxide reactivity and mechanisms involved in its biological effects." *Pharm Res* 42(5): 421-427.
- McClintock, T. S. and M. R. Lerner (1997). "Functional analysis by imaging of melanophore pigment dispersion of chimeric receptors constructed by recombinant polymerase chain reaction." *Brain Res Brain Res Protoc* 2(1): 59-68.
- McClintock, T. S., J. P. Rising, et al. (1996). "Melanophore pigment dispersion responses to agonists show two patterns of sensitivity to inhibitors of cAMP-dependent protein kinase and protein kinase C." *J Cell Physiol* 167: 1-7.
- McCord, C. P. and F. P. Allen (1917). "Evidences associating pineal gland function with alterations in pigmentation." *J Exp Zool* 23: 207-24.
- Muresan, V., M. C. Stankewich, et al. (2000). "Dynactin-dependent, dynein-driven vesicle transport in the absence of membrane proteins: a role for spectrin and acidic phospholipids." The American Society for Cell Biology, Fortieth Annual Meeting, San Francisco, CA.
- Nakatsubo, N., H. Kojima, et al. (1998). "Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins." *FEBS Letters* 427: 263-266.
- Nery, L. E. M. and A. M. d. L. Castrucci (1997). "Pigment cell signaling for physiological color change." *Comp Biochem Physiol* 118A(4): 1135-1144.



- Nilsson, H. (2000). Personal communication.
- Nilsson, H. (2000). Pigment organelle localization in fish melanophores. *Zoophysiology, Zoology*. Göteborg, Göteborg University: 42.
- Nilsson, H., M. Rutberg, et al. (1996). "Localization of kinesin and cytoplasmic dynein in cultured melanophores from Atlantic cod, *Gadus morhua*." *Cell Motil Cytoskeleton* 33: 183-196.
- Nilsson, H. and M. Wallin (1997). "Evidence for several roles of dynein in pigment transport in melanophores." *Cell Motil Cytoskeleton* 38(4): 397-409.
- Obika, M. (1986). "Intracellular transport of pigment granules in fish chromatophores." *Zool Sci* 3: 1-11.
- Obika, M., D. G. Menter, et al. (1978). "Actin filaments in melanophores of *Fundulus heteroclitus*. Their possible involvement in melanosome migration." *Cell Tissue Res* 193(3): 378-97.
- Onofri, F., S. Giovedì, et al. (1997). "Synapsin I interacts with c-Src and stimulates its tyrosine kinase activity." *Proc Natl Acad Sci USA* 94: 12168-12173.
- Oshima, N., E. Nakata, et al. (1998). "Light-induced pigment aggregation in xanthophores of the medaka, *Oryzias latipes*." *Pigment Cell Res* 11(6): 362-7.
- Potenza, M. N., G. F. Graminski, et al. (1994). "Functional expression and characterization of human D₂ and D₃ dopamine receptors." *J Neurosci* 14(3): 1463-1476.
- Potenza, M. N. and M. R. Lerner (1992). "A rapid quantitative bioassay for evaluating the effects of ligands upon receptors that modulate cAMP levels in a melanophore cell line." *Pigment Cell Res* 5: 372-378.
- Pozdnyakov, N., A. Lloyd, et al. (1993). "Nitric oxide-regulated endogenous ADP-ribosylation of rod outer segment proteins." *Biochem biophys res commun* 192(2): 610-5.
- Provencio, I. (1998). "Melanopsin: an opsin in melanophores, brain, and eye." *Proc Natl Acad Sci USA* 95: 340-345.
- Rees, D. J. G., S. E. Ades, et al. (1990). "Sequence and domain structure of talin." *Nature* 347: 685-689.
- Reese, E. L. and L. T. Haimo (2000). "Dynein, dynactin, and kinesin II's interaction with microtubules is regulated during bidirectional transport." *J Cell Biol* 151(1): 155-165.
- Reilein, A. R., I. S. Tint, et al. (1998). "Regulation of organelle movement in melanophores by protein kinase A (PKA), protein kinase C



- (PKC), and protein phosphatase 2A (PP2A)." *J Cell Biol* 142(3): 803-813.
- Reppert, S. M. (1997). "Melatonin receptors: molecular biology of a new family of G protein-coupled receptors." *J Biol Rhythms* 12(6): 528-531.
- Rodionov, V. I., A. J. Hope, et al. (1998). "Functional coordination of microtubule-based and actin-based motility in melanophores." *Curr Biol* 8: 165-168.
- Rodriguez, I., C. A. Greer, et al. (2000). "A putative pheromone receptor gene expressed in human olfactory mucosa." *Nat Genet* 26: 18-19.
- Rogers, S. L. and V. I. Gelfand (1998). "Myosin cooperates with microtubule motors during organelle transport in melanophores." *Curr Biol* 8: 161-164.
- Rogers, S. L., R. L. Karcher, et al. (1999). "Regulation of melanosome movement in the cell cycle by reversible association with myosin V." *J Cell Biol* 146(6): 1265-1275.
- Rogers, S. L., I. S. Tint, et al. (1997). "Regulated bidirectional motility of melanophore pigment granules along microtubules *in vitro*." *Proc Natl Acad Sci U S A* 94: 3720-3725.
- Rollag, M. D. and M. R. Adelman (1993). "Actin and tubulin arrays in cultured *Xenopus* melanophores responding to melatonin." *Pigment Cell Res* 6: 365-371.
- Sabe, H., M. Hamaguchi, et al. (1997). "Cell to substratum adhesion is involved in v-Src-induced cellular protein tyrosine phosphorylation: implication for the adhesion-regulated protein tyrosine phosphatase activity." *Oncogene* 14: 1779-1788.
- Schliwa, M., K. Weber, et al. (1981). "Localization and organization of actin in melanophores." *Journal of Cell Biology* 89(2): 267-75.
- Shevchenko, A., M. Wilm, et al. (1996). "Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels." *Anal Chem* 68(5): 850-858.
- Simons, K. and E. Ikonen (1997). "Functional rafts in cell membranes." *Nature* 387: 569-572.
- Sontag, E., S. Fedorov, et al. (1993). "The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation." *Cell* 75(5): 887-897.



Sugden, D. (1991). "Aggregation of pigment granules in single cultured *Xenopus laevis* melanophores by melatonin analogues." *Br J Pharmacol* 104: 922-927.

Sugden, D. and S. J. Rowe (1992). "Protein kinase C activation antagonizes melatonin-induced pigment aggregation in *Xenopus laevis* melanophores." *J Cell Biol* 119(6): 1515-1521.

Svensson, S. P. S., P. I. Adolfsson, et al. (1997). "Multiple α_2 -adrenoceptor signalling pathways mediate pigment aggregation within melanophores." *Pigment cell res* 10: 395-400.

Tidball, J. G. and M. J. Spencer (1993). "PDGF stimulation induces phosphorylation of talin and cytoskeletal reorganization in skeletal muscle." *J Cell Biol* 123(3): 627-635.

Tuma, M. C. and V. I. Gelfand (1999). "Molecular mechanisms of pigment transport in melanophores." *Pigment Cell Res* 12: 283-294.

Tuma, M. C., A. Zill, et al. (1998). "Heterotrimeric kinesin II is the microtubule motor protein responsible for pigment dispersion in *Xenopus* melanophores." *J Cell Biol* 143(6): 1547-1558.

van de Veerdonk, F. C. G. and T. M. Konjin (1970). "The role of adenosine 3',5'-cyclic monophosphate and catecholamines in the pigment migration process in *Xenopus laevis*." *Acta Endocrinol* 64: 364-376.

Vancoillie, G., J. Lambert, et al. (2000). "Kinesin and kinectin can associate with the melanosomal surface and form a link with microtubules in normal human melanocytes." *J Invest Dermatol* 114(3): 421-9.

White, B. H., R. D. Sekura, et al. (1987). "Pertussis toxin blocks melatonin-induced pigment aggregation in *Xenopus* dermal melanophores." *J Comp Physiol [B]* 157: 153-159.

Yu, J., D. A. Fischman, et al. (1973). "Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents." *J Supramol Struct* 1(3): 233-248.

Zhao, W., S. Cavallaro, et al. (2000). "Nonreceptor tyrosine protein kinase pp60c-src in spatial learning: synapse-specific changes in its gene expression, tyrosine phosphorylation, and protein-protein interactions." *PNAS* 97(14): 8098-8103.

Zimmer, W. E., Y. Zhao, et al. (2000). "The domain of brain β -spectrin responsible for synaptic vesicle association is essential for synaptic transmission." *Brain Res* 881: 18-27.

