Physiological manipulation of cellular activity tunes protein and ultrastructural profiles in a neuroendocrine cell

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Abstract

To study *in vivo* the dynamics of the biosynthetic and secretory processes in a neuroendocrine cell, we use the proopiomelanocortin-producing intermediate pituitary melanotrope cells of *Xenopus laevis*. The activity of these cells can be simply manipulated by adapting the animal to a white or a black background, resulting in inactive and hyperactive cells respectively. Here, we applied differential display proteomics and field emission scanning electron microscopy (FESEM) to examine the changes in architecture accompanying the gradual transition of the inactive to the hyperactive melanotrope cells.

The proteomic analysis showed differential expression of neuroendocrine secretory proteins, endoplasmic reticulum (ER)-resident chaperones, and housekeeping and metabolic proteins. The FESEM study revealed changes in the ultrastructure of the ER and Golgi and the number of secretory granules. We conclude that activation of neuroendocrine cells tunes their molecular machineries and organelles to become professional secretors.

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Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) and Golgi apparatus play a pivotal role in the quality control of secretory proteins. The ER contains enzymes and molecular chaperones ensuring that secretory proteins are folded and assembled to achieve their correct tertiary structures, whereas the Golgi controls a number of post-translational modifications and the sorting event. In the secretory granules of neuronal and endocrine cells, prohormones are proteolytically cleaved to biologically active peptides. Our model system, the proopiomelanocortin (Pomc)-producing intermediate pituitary melanotrope cells of *Xenopus laevis*, is ideal to study the quality control of synthesis and transport of secretory proteins in physiologically inactive and activated neuroendocrine cells. The model concerns a well-characterized physiological neuroendocrine reflex, namely skin color change as a result of adaptation to a changed background condition. By placing the animal on a black background, the melanotrope cells are triggered to produce and release α -melanophore stimulating hormone (α -MSH) that stimulates the dispersion of melanin in the melanophores of the skin, causing a darkening of the animal. An elevated release of α -MSH is accompanied by a high expression rate of Pomc, the prohormone for α -MSH. Consequently,

melanotrope cells of black-background-adapted *Xenopus* contain ~ 30 times more Pomc mRNA and display a much higher biosynthetic and secretory activity than those of white-background-adapted animals (Jenks *et al.* 1993). The 37 kDa precursor form of POMC is processed to the intermediate N-terminal 18 kDa cleavage fragment and a number of peptides, including α -MSH. The prohormone convertase 2 (PC2) plays a primary role in the proteolytic processing of POMC (e.g. Braks *et al.* 1992) and occurs in an inactive proenzyme form and an enzymatically active form. Following activation through an autocatalytic reaction, active PC2 cleaves the prohormone at pairs of basic amino acid residues (e.g. Benjannet *et al.* 1991).

Earlier studies have focused on the extreme situations, namely on the inactive melanotrope cells in fully white-background-adapted *Xenopus* and on the hyperactive cells of animals fully adapted to a black background. In the inactive and hyperactive *Xenopus* melanotrope cells, differential expression has been found for a number of gene transcripts encoding proteins involved in prohormone biosynthesis (Holthuis *et al.* 1995) and protein expression of POMC and a number of ER chaperones (Devreese *et al.* 2002). Cytological differences in the inactive and hyperactive cells have been studied by transmission electron microscopy (TEM; Hopkins 1970, de Rijk *et al.* 1990), a morphometric

TEM approach (Weatherhead & Whur 1972) and field emission scanning electron microscopy (FESEM; Van Herp *et al.* 2005). The latter technique provides a fast and detailed 3D survey of organelles.

Here, we decided to use differential display proteomics and FESEM to study the dynamics of the melanotrope cell model by examining the protein and ultrastructural patterns not only in melanotrope cells from fully white- and fully black-background-adapted *Xenopus*, but also from white animals periodically adapted to a black background and thus with various states of cellular activity. The combination of the two methods permitted us to compare the molecular and cellular events in inactive, gradually activated and hyperactive *Xenopus* melanotrope cells.

Materials and Methods

Chemicals

The chemicals for the buffer solutions were purchased from Merck. The chemicals specifically used for the proteomics study were: Tris base (from Invitrogen), Pefablock (Roche), trichloroacetic acid, diethyl ether, ethanol, sodium thiosulfate, formaldehyde and EDTA (from Merck), deoxycholate, thiourea, CHAPS (3-cholamidopropyl dimethylamino-1-propanesulphonate), 1,4-dithiothreitol and iodoacetamide (from Sigma-Aldrich), urea and glutaraldehyde (from Fluka, Sigma-Aldrich), SDS (from LKB, Bromma, Sweden), acrylamide/bisacrylamide (from MP Biochemicals, Solon, OH, USA), and acetic acid (from Acros Organics, Geel, Belgium). Chemicals specifically for the FESEM method were: glutaraldehyde (25% solution (from Agar Scientific, Stansted, UK), 1-hexadecene (from Merck), and gold-palladium (from Cressington Scientific Instruments Ltd, Watford, UK).

Preparation of *Xenopus* melanotrope cells under different physiologically (hyper)active situations

South African claw-toed frogs *X. laevis* were bred, reared, and maintained in the Central Animal Facility of the Radboud University (Nijmegen, The Netherlands). All animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare and the RU-DEC 2003-53 and 2007-027 from the animal experiment committee of the Radboud University for enabling the use of *X. laevis* frogs. Animals adapted to a grey background were put on a white or a black background for various time periods to obtain melanotrope cells with different degrees of (hyper)activity, namely 1) inactive melanotrope cells: longer than 3 weeks adaptation of grey-background-adapted animals to a white background; 2) short-term-activated melanotrope cells: 6, 12, and 24 h (1 day) adaptation of white-background-adapted animals to a black background and 3) long-term-activated melanotrope cells: 2, 3, 6, 12, and 24 days

adaptation of white-background-adapted animals to a black background. The virtually inactive melanotrope cells of long-term white-adapted animals were used as a reference point for cellular inactivity. A number of animals were adapted for the indicated time points and a selection between these animals was made using the melanophore index (i.e. melanosome aggregation in the skin; white: 1; black: 5). For the differential display proteomics approach, we used three animals per time point, while for the FESEM approach at each time point the number of animals varied between three and five depending on the quality of the sample preparation.

Identification of proteins in the melanotrope cells of *X. laevis*

The identification of the protein spots selected for our proteomics study was performed by a combination of 2D electrophoresis and mass spectrometry, and details of the approach have been described by Devreese *et al.* (2002). The protein spots were cut from the gel and subjected to in-gel protein digestion followed by mass spectrometric characterization. All spots were submitted to MALDI TOF/TOF analysis. For spots for which MALDI-MS(/MS) did not provide a significant hit, we used the automated nano-LC-MS-MS system (Devreese *et al.* 2002). Mascot was used for protein identification using the non-redundant NCBI database. For some protein spots, the *Xenopus* EST database embedded in the NCBI databases was interrogated.

Differential display proteomics

Sample preparation and protein extraction Each dissected *Xenopus* neurointermediate lobe was collected in a glass/glass potter. Homogenization was performed in 250 µl of a solution containing 40 mM Tris base (pH 9.5) and 10 mM Pefablock. The proteins were precipitated by a trichloroacetic acid–deoxycholate (TCA–DOC) procedure based on the method described by Molloy *et al.* (1998). The homogenized sample was adjusted to 900 µl with ice-cooled 10 mM PBS (pH 7.4), mixed by vortex with 100 µl ice-cooled 3% DOC, and incubated for 30 min on ice. Then, 110 µl 100% TCA was added, mixed, and left on ice for 15 min and then centrifuged at 21 000 *g* at 4 °C. The precipitated material was washed in 1 ml diethyl ether–ethanol (1:1), thoroughly mixed (by vortex) and centrifuged again at 21 000 *g* for 20 min. After three further washing steps, the recuperated precipitate was air dried for about 2 h. The dry material was then dissolved in 50 µl lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% 1,4-dithiothreitol (DTT), and 0.8% IPGphor buffer (pH 3–10L) and sonicated three times for 5 seconds on ice-cooled water.

Two-dimensional gel electrophoresis The 2D gel electrophoretic analysis was performed on an immobilized pH gradient (IPGPhor) system and the SDS–PAGE (Etan DALT six) electrophoresis unit (Amersham-Bioscience). For electrophoresis in the first dimension, each premade IPG drystrip

(18 cm, pH 3–10L) was placed in the porcelain electrophoretic holder containing the 50 µl sample and 310 µl rehydration buffer (8 M urea, 0.5% CHAPS, 0.5% IPGphor (pH 3–10L), 0.28% DTT, and 5 µl bromophenol blue solution (1 mg/ml), which was primarily centrifuged for 15 min at 1000 *g* at room temperature). The strips were then covered with IPGphor cover fluid and put under an electric field of 50 µA per strip at 20 °C. Further rehydration/separation took place at 30 V for 12 h (360 Vh), 500 V and 1000 V each for 1 h (respectively 500 and 1000 Vh), and 8000 V for 4½ h (36 000 Vh). After this electrofocusing step, the strips were washed for a few seconds in distilled water and prepared for the second electrophoretic run by incubation under shaking for 15 min in equilibration buffer (6 M urea, 30% (w/v) glycerol, and 2% (w/v) SDS dissolved in 0.05 M Tris–HCl buffer (pH 8.8)), containing 2 µl bromophenol blue and 10 mg DTT per ml equilibration buffer, and 15 min in equilibration buffer containing 25 mg iodoacetamide per ml. Then, they were dried on filter paper and placed on a self-made 12.5% SDS–PAGE gel (25 by 21 cm) to separate the proteins according to their molecular weight. After the second electrophoretic run, the SDS–PAGE gels were fixed during 30 min in fixation solution (40 ml ethanol, 10 ml acetic acid, and 50 ml distilled water) and silver stained according to the following standardized procedure: 30 min in sensitization solution (30 ml ethanol, 292 µl glutaraldehyde, 4 ml 5% sodium thiosulfate, 6.8 g sodium acetate, and 66 ml distilled water), three times 5 min washing in distilled water, 20 min in staining solution (100 ml distilled water, 40 µl formaldehyde, and 0.25 g AgNO₃), twice rinsing for exactly 1 min each in distilled water, exactly 4 min in developing solution (6.74 g Na₂CO₃ 10 H₂O, 20 µl formaldehyde in 100 ml distilled water), and finally rinsing for 10 min in EDTA solution (1.42 g EDTA in 100 ml distilled water) and for 10 min in distilled water.

Analysis of differentially expressed proteins For analyzing the differential expression of the selected proteins (see Table 1 and Fig. 2), we used the electropherograms of three

individual neurointermediate lobes for each time point. The relative amounts of the selected proteins were measured by densitometric scanning of the gels using the Umax Powerlook III (Amersham). Spot detection and matching of spots between gels was performed with the PDQuest software package (version 7.2 of Bio–Rad). Normalization between the gels was based on the total signal of specific melanotrope cell spots (i.e., total protein amount) per gel and all scanned gels were corrected for background signals. Quantification of each protein family was based on the total sum of the selected protein spots. The statistical evaluation of the relative changes was carried out using the program SPSS (version 12.0.1; Chicago, IL, USA). To test homogeneity of variances, we used Levene statistics, when appropriate, log transformation was performed. The expression profile data of PC2, immunoglobulin heavy chain-binding protein or 76 kDa glucose-regulated protein precursor (BiP), calreticulin, protein disulfide isomerase (PDI), 90 kDa heat shock protein (HSP90), actin, enolase, and aldolase were analyzed using one-way ANOVA followed by *post hoc* analysis with Dunnett *t* (two-sided). Since no homogeneity of variance was reached after log transformation for the expression profiles of POMC 37 kDa, POMC 18 kDa, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Kruskal–Wallis followed by Mann–Whitney *U* tests were used for statistical analysis.

FESEM

To explore the time course of the ultrastructural changes during the transition from one activity state of the melanotrope cells to another state, we applied the fast cryo-FESEM approach described by Van Herp *et al.* (2005). Following dissection of the pituitary and separation of the pars intermedia from the pars nervosa, a tissue consisting predominantly of melanotrope cells was obtained. Each preparation was immediately fixed in 0.5% glutaraldehyde (prepared in 0.1 M cacodylate buffer, pH 7.4) for 1 h, three times rinsed in cacodylate buffer for 30 min each, and

Table 1 Protein identification in the melanotrope cells of *Xenopus laevis*

	Protein name	NCBI acc. no.	Mr kDa (theor.)	pI (theor.)
Protein family				
	Regulated secretory proteins			
	37 kDa POMC	gi 5931776	29.9	6.2
	18 kDa POMC	–	12.6	4.8
ER-resident proteins	PC2	gi 64972	70.5	5.6
	BiP	gi 27370850	72.3	5.0
	Calreticulin	gi 64609	48.3	4.4
	PDI	gi 28302197	56.6	5.6
Housekeeping and metabolic proteins	β-Actin	gi 537596	41.8	5.3
	GAPDH	gi 24415375	35.9	8.5
	α-Enolase	gi 32450571	47.4	6.2
	Aldolase	gi 1944025	39.4	8.5
	HSP90	gi 27803582	82.9	5.0

BiP, immunoglobulin heavy chain-binding protein or 76 kDa glucose-regulated protein precursor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP90, 90 kDa heat shock protein; PC2, prohormone convertase 2; PDI, protein disulfide isomerase; 37 K POMC (ProPOMC), precursor of proopiomelanocortin; 18 K POMC, processed form of POMC. Details of the identification are given in Supplementary Tables 1 and 2, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol198/issue3/>.

incubated in 30% glycerol/cacodylate buffer for at least 1 h. After this light fixation step, each sample was mounted in 1-hexadecene into the cavity of a cylindrical carrier (Oxford; 3 mm×1.4 mm outer diameter and 1 mm inner diameter) leaving a part of the tissue free on top of the tube and 'slush' cryofixed in liquid nitrogen. The obtained fragile frozen sample was then mounted on a sample holder of the freeze-fracturing device from Oxford Alto and immediately transferred to the Alto 2500 cryo-preparation chamber using the vacuum transfer device for fracturing, etching, and coating of the samples. All tissue samples were fractured at -120°C , etched for 3 min at -95°C , coated with a 2–3 nm layer of gold–palladium, and subsequently transferred to the high vacuum area of a coupled JEOL 6330 FESEM microscope. The results of the FESEM observations were analyzed after processing and archiving of the images on a computer.

Results

*Dynamics of the protein expression profiles in *Xenopus* melanotrope cells with different states of activity*

To examine the changes in protein expression during the gradual transition of the inactive to the hyperactive *Xenopus* melanotrope cells, we selected a number of proteins that we have previously found to be differentially expressed in fully white- and fully black-background-adapted animals (Table 1 and Fig. 1A; Devreese *et al.* 2002). As we were primarily interested in protein processing, folding, maturation, and trafficking in the melanotrope cells, we used regulated secretory proteins (37 kDa POMC, 18 kDa POMC, and PC2) to follow the processing activity in the cells and ER-resident proteins (BiP, calreticulin and PDI) to examine the folding and maturation activity in the luminal part of the ER. The housekeeping (β -actin) and metabolic (GAPDH, α -enolase, and aldolase) proteins were selected to obtain information on the status of the cytoskeleton and the basal energy metabolism respectively. With HSP90, we wanted to follow protein-folding activity in the cytoplasm. An overview of the changes in protein expression levels of the selected family members at the various time points is shown in Fig. 1B. Together, these proteins allowed us to follow the quality control of the biosynthetic and secretory activities of the melanotrope cells in white animals adapting to a black background.

Figure 2, showing the endocrine response during black-background adaptation, provides the protein expression profiles of the POMC precursor (37 kDa) and the cleaved 18 kDa form of POMC as well as of the PC2 (the precursor and mature forms of this POMC cleavage enzyme). Significantly, higher amounts of 37 kDa POMC were measured only after 12 days of black-background adaptation ($P<0.05$). In contrast, the processed 18 kDa form of POMC showed similar amounts during the whole adaptation period. The amounts of PC2 were the highest in white-adapted

animals and gradually decreased in the first week of black-background adaptation. During the longer adaptation periods, the level of PC2 increased again but remained 2.5 times lower than in fully white-adapted animals. At all time points examined, the various levels of 18 kDa POMC expression were not significantly different nor were those of PC2 expression.

As components of the ER play an essential role in the proper folding and maturation of newly synthesized proteins in the secretory pathway, we followed in the melanotrope cells the changes in the levels of the ER-resident proteins BiP, calreticulin, and PDI during black-background adaptation of the animal (Fig. 3). After normalization of the density of the corresponding protein spots in the 2D electropherograms (Fig. 1), we found that the relative amounts of the three selected proteins remained low up to the third day of adaptation to a black background. However, the levels of BiP, calreticulin, and PDI increased gradually when the cells became activated by longer adaption of the animals to a black background. The amounts of the ER chaperones present in the hyperactive melanotrope cells, when the animals were adapted for 2–3 weeks, were significantly different from those in the inactive cells (BiP ($P<0.005$), calreticulin ($P<0.05$), and PDI ($P<0.005$)).

To obtain insight into their structural and metabolic states during activation of the melanotrope cells, we selected the following housekeeping proteins: actin as a marker for the cytoskeletal changes, GAPDH, enolase, and aldolase for the metabolic changes and HSP90 for the changes in cytoplasmic folding activity (Fig. 4). In contrast to the proteins involved in folding and maturation of newly synthesized proteins (Fig. 3), no clear trend was observed for the proteins involved in housekeeping functions. The relative amount of actin was constant during the time period examined. Relatively large changes in the relative amounts of the GAPDH, enolase, and aldolase proteins were observed during the adaptation period, but these changes were only significantly different for enolase ($P<0.05$ and $P<0.01$).

The cytosol-localized HSP90, a protein with chaperone activity, influences the regulation of transcription of a discrete set of genes. The amount of HSP90 remained low during the first week of adaptation but increased afterwards to levels that were 2.5 and 6 times higher after 12 and 24 days of adaptation respectively ($P<0.005$; Fig. 4). Therefore, the protein expression of HSP90 showed a trend similar to those of the chaperones operational in the secretory pathway (Fig. 3).

*Ultrastructural differences between physiologically inactive, gradually activated, and hyperactivated *Xenopus* melanotrope cells*

The cryo-FESEM method was used to allow a fast and detailed study on the biosynthetic and secretory activities in the melanotrope cells of *Xenopus* adapted to a white or black background for extended time series (longer than 3 weeks adapted to a white background and adapted for 6, 12, and 24 h, and for 2, 3, 6, 12, and 24 days to a black background).

Figure 1 (A) 2D PAGE electropherogram of proteins expressed in melanotrope cells of 24-day black-background-adapted *Xenopus*. Indicated are the locations of the proteins selected for detailed analysis. (B) Overview of the changes in protein expression levels of the selected family members at various time points.

Figure 2 Quantification of relative protein amounts of 37 and 18 kDa POMC and PC2 in the melanotrope cells of *Xenopus* during short- and long-term adaptation to a black background (values are expressed as the mean $\pm$ S.E.M.; $n=3$ individual protein samples each analyzed on a separate gel; * $P<0.05$ different from the values of long-term white-adapted (LTWA) animals). The dotted line indicates the expression level in inactive melanotrope cells.

After fracturing of the high-pressure-frozen melanotrope cells, the fracture plane was frequently found in regions containing ER and secretory granules, and the nucleus was mostly flatly broken or fractured through the region of the nuclear envelope. The quality of the ultrastructural analysis was such that the cytoplasmic microstructures were readily visible in the finely granulated cytosolic matrix (Fig. 5A–H). The ultrastructural information obtained could therefore be directly combined with the described molecular information.

The population of inactive melanotrope cells from white-adapted *Xenopus*, studied at low and high magnifications, was dense and no or only a few intercellular spaces were found with most of the cells being round to oval and completely loaded with round to oval granules. In the inactive cells, the ER and Golgi were poorly developed (Fig. 5A).

Figure 3 Quantification of relative protein amounts of BiP, calreticulin, and PDI in melanotrope cells of *Xenopus* during short- and long-term adaptation to a black background (values are expressed as the mean $\pm$ S.E.M.; $n=3$ individual protein samples each analyzed on a separate gel; * $P<0.05$; ** $P<0.01$ and *** $P<0.005$ different from the values of long-term white-adapted (LTWA) animals). The dotted line indicates the expression level in inactive melanotrope cells.

In white-adapted animals adapted to a black background for 6 or 12 h, the melanotrope cells showed their first cytological changes in that the density of the secretory granules decreased (Fig. 5B) and the cells started to become elongate (Fig. 5C). In this short-term adaptation period, the cells became activated and started to release the contents of their storage granules. The melanotrope cells of 24-h black-background-adapted exhibited a remarkable organelle reorganization in that the number of granules was gradually reduced and larger vesicular structures were present in the cytoplasm of the activated cells (Fig. 5D).

Characteristics of cellular activation became visible following adaptation of the animal for a longer time period. Around the second day, tubular structures of the ER surrounding the nuclei

of the melanotrope cells became visible and the amounts of ER and Golgi gradually increased (Fig. 5E). Between 3 and 6 days of adaptation, fenestrated saccule/tubular networks of the Golgi apparatus, surrounded by cysternae of rough ER, were frequently observed in the melanotrope cells (Fig. 5F). The number of active melanotrope cells gradually increased between 6 and 12 days of black-background adaptation and, after 24 days, all cells were hyperactivated and contained high levels of ER and Golgi activities. In these fully black-background-adapted animals (24 days), the hyperactive melanotrope cells were present as a loosely attached group of elongated cells and the cytoplasm of these cells was filled with rough ER and contained only a low number of secretory granules (Fig. 5G) and well-developed Golgi (Fig. 5H).

Discussion

Dynamics of the protein expression profiles in *Xenopus* melanotrope cells with different activity states

One of the main focuses of this study was an analysis of the protein expression patterns of 37 and 18 kDa POMC and of the proteolytic cleavage enzyme PC2. Our data indicate that the 18 kDa cleaved form of POMC and not the 37 kDa precursor is the major storage product in the melanotrope cells of animals adapted to a white background. During the adaptation period the levels of this cleaved form remained similar, while only after a prolonged adaptation to a black background the cellular amount of 37 kDa POMC started to increase. These results are in line with the Western blot data previously described by Dotman *et al.* (1998). During the adaptation period, the expression profiles of 18 kDa POMC and PC2 showed a similar tendency. Coexpression of POMC and the PC2 genes has been described at the mRNA level in *X. laevis* (Martens *et al.* 1987, Braks *et al.* 1992, Holthuis *et al.* 1995) and in *Rana ridibunda* (Peinado *et al.* 2006). In white frogs, the melanotrope cells are loaded with storage products (18 kDa form of POMC and PC2) and, once triggered, the cells are ready to rapidly release the stored material. When the cells are activated, the cellular level of 18 kDa POMC decreases due to a higher rate of secretion. The newly synthesized 37 kDa POMC molecules are rapidly cleaved by PC2 and only after long-term black-background adaptation the amount of the 37 kDa prohormone increases to detectable levels.

The expression of the ER chaperones is increased in the activated cells and is very high in the hyperactive cells when the cells exhibit markedly increased biosynthetic and

Figure 4 Quantification of relative protein amounts of actin, GAPDH, enolase, aldolase, and HSP90 in melanotrope cells of *Xenopus* during short- and long-term adaptation to a black background (values are expressed as the mean $\pm$ s.e.m.; $n=3$ individual protein samples each analyzed on a separate gel; * $P<0.05$; ** $P<0.01$ and *** $P<0.005$ different from the values of long-term white-adapted (LTWA) animals). The dotted line indicates the expression level in inactive melanotrope cells.

Figure 5 (A–H) FESEM observations. (A) FESEM at high magnification of an inactive melanotrope cells of a fully white-background-adapted *Xenopus*. Bar, 1 μ m. (B–D) FESEM at high magnification showing melanotrope cells of *Xenopus* after short-term adaptation to a black background (B, 6 h; C, 12 h; and D, 24 h). Bar, 1 μ m. (E–F) FESEM at high magnification showing melanotrope cells of *Xenopus* after long-term adaptation to a black background (E, 2 days; F, 6 days). Bar, 1 μ m. (G–H) FESEM at high magnification of hyperactive melanotrope cells of a fully black-background-adapted *Xenopus* (24 days adaptation to a black background). Bar, 1 μ m. fil, filament-like structures forming a matrix around the secretory granules; G, some cysternae of the Golgi complex; lvs, large vesicular structure; N, nucleus; ne, nuclear envelope; opl, outer side of the plasma membrane; pl, plasma membrane; rER, well-developed rough endoplasmic reticulum; s, secretory granule; undev ER, poorly developed ER.

secretory activities characterized by a high level of POMC biosynthesis, and α -MSH acetylation and release as shown by Jenks *et al.* (1993) and Vázquez-Martínez *et al.* (2005). Since ER chaperones play a role in protein folding, facilitate the assembly of multimeric protein complexes or participate in the folding of proteins containing disulfide bonds (Benham & Braakman 2000, Ellgaard & Helenius 2001, Schröder & Kaufman 2005), the higher expression of BiP, calreticulin, and

PDI in long-term black-background-adapted animals reflects their key function in POMC biosynthesis and the generation of the final secretory peptide signals under (hyper)activated conditions.

The housekeeping proteins actin, GAPDH, enolase, and aldolase were selected to provide an insight into the metabolic activity of the cells. These proteins are not directly involved in secretory pathway processes, but actin is an important protein in the cytoskeleton, and GAPDH, aldolase, and enolase are key enzymes in energy metabolism. Although the melanotrope cells changed their activity from an inactive to a hyperactive state during the adaptation period, the amount of actin remained about the same. The variations in the levels of the metabolic enzymes may reflect fluctuations in the metabolic activities of the melanotrope cells under various states of activity and may thus represent biological sample variations.

The changes in HSP90 protein levels were comparable with the differences in expression of the ER-resident proteins, illustrating a functional link between this heat shock protein and the ER folding catalysts.

Ultrastructural differences between physiologically inactive, gradually activated, and hyperactivated Xenopus melanotrope cells

We recently applied FESEM to study the ultrastructural differences in the inactive and hyperactive *Xenopus* melanotrope cells (Van Herp *et al.* 2005). These results were similar to the TEM ultrastructural data described by Hopkins (1970), Weatherhead & Whur (1972) and de Rijk *et al.* (1990). Now we have extended our cryo-FESEM observations with the description of the ultrastructural changes in melanotrope cells under various states of cellular activity. A drawback of the freeze-fracturing procedure, being part of the FESEM technique, is the fact that flat block faces are created on which only a limited number of fractured areas of high quality can be obtained. For this reason, morphometric measurements on a high number of equally sectioned cells were not possible, and we thus studied the differences on a broad series of samples to confirm the reproducibility of the ultrastructural characteristics for each activity state. We found that the melanotrope cells of white-adapted animals are in a secretory inactive state, containing numerous storage granules and poorly developed Golgi and ER, that the cells are activated during a short-term adaption of the white *Xenopi* to a black background and that the cells become hyperactive and have large amounts of rough ER and a well-developed Golgi in a long-term black-background-adapted situation. In fully white-background-adapted animals, the far majority of the melanotrope cells were inactive, whereas most melanotrope cells in fully black-background-adapted animals were in a hyperactive state. These observations are in line with earlier morphometric TEM observations showing that nearly all melanotrope cells (95%) of white-background-adapted *Xenopus* animals are inactive and that about 80% of the

melanotropes in black-background-adapted animals are hyperactive (de Rijk *et al.* 1990).

Intriguingly, the drastic reorganization that we observed following 24 h of black-background adaptation has not been previously found. Hopkins (1970) detected one or two multivesicular bodies in the melanotrope cells of fully white-adapted animals. We, however, found high numbers of multivesicular bodies in cells of animal adapted for 24 h. The significance of these multivesicular bodies is at present unclear. The presence of the bodies may be related to the formation of the peripheral cisternae of the Golgi complex, and their contents may be comparable with the contents in the cisternal complex of the Golgi (Hopkins 1970). Alternatively, the large multivesicular structures may be linked to the formation of lysosomal bodies recycling membranous material that is present following the high levels of release of the storage granular content at the beginning of black-background adaptation.

Biological relevance of the dynamics in protein and ultrastructural patterns of Xenopus melanotrope cells with different cellular activity states

The combination of differential display proteomics and FESEM enabled us to compare the molecular and cellular events in inactive, physiologically activated, and hyperactive melanotrope cells of *X. laevis*. The inactive melanotrope cells are characterized by a high density of storage granules and a poorly developed ER and Golgi; their secretory activity and the level of POMC is low, but their hormonal content in the storage granules is high. In this situation, the amounts of the processed 18 kDa POMC form and of the POMC-processing enzyme PC2 are high, whereas the amounts of the 37 kDa POMC precursor form and of the ER chaperones and HSP90 are low. The biosynthetic and secretory activities of the melanotrope cells are induced when the animals adapt to a black background. The first ultrastructural changes are observed already after a short adaptation period, namely after 6–12 h of adaptation the density of the secretory granules in the cells was reduced and after 24 h a dramatic cytological reorganization occurred. The induction of the release process at 24 h is reflected by the reduced amounts of 18 kDa POMC and PC2. On the second day of black-background adaptation, ER laminar structures become visible and, between 3 and 6 days of black-background adaptation, these structures gradually develop in more and more cells. During that period, the amounts of BiP, calreticulin, and PDI increase significantly. Following an adaptation period of 12–24 days, the biosynthetic and secretory activities greatly increase in all melanotrope cells of black-background-adapted animals. The activated cells are filled with large amounts of rough ER and well-developed Golgi structures are frequently seen. At the protein expression level, the hyperactivity is first obvious after 12 days, namely the presence of a high amount of the 37 kDa precursor form of POMC. The need for folding and maturation of the precursor is reflected by the high levels of

the ER chaperones BiP, calreticulin, and PDI. Thus, the reorganization of the organelles is accompanied by differences in the expression patterns of the selected protein families and the tuning of the processes is relatively slow.

Despite the cytological changes, the levels of expression of the housekeeping and metabolic proteins remained remarkably the same. For instance, the shape of the melanotrope cells and the density of the cell population changed from oval/round cells at a high density in white-background-adapted animals to elongated cells at a lower density in black-background-adapted animals, while the amounts of actin were similar. The fact that the protein expression profiles of the metabolic enzymes were not significantly different in inactive and activated cells indicates that these enzymes are important for general melanotrope cell functioning.

In conclusion, the combination of differential display proteomics and FESEM allowed us to link the differences in expression of various protein family members with the changes in the architecture of the *Xenopus* melanotrope cells, illustrating that neuroendocrine cells tune their protein and ultrastructural patterns such that they become professional secretors.

Declaration of interest

The authors declare there is no conflict of interest which would prejudice the impartiality of this scientific work.

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