# Dynamics of proopiomelanocortin and prohormone convertase 2 gene expression in *Xenopus* melanotrope cells during long-term background adaptation

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## Abstract

The toad *Xenopus laevis* is able to adapt its skin color to background light intensity. In this neuroendocrine reflex, the proopiomelanocortin (POMC)-derived peptide α-melanophore-stimulating hormone (αMSH) is a key regulatory factor. In animals adapting to a black background, release of αMSH from the pituitary pars intermedia causes dispersal of melanin in skin melanophores. To investigate the long-term *in vivo* dynamics of αMSH production during black background adaptation, the biosynthetic rate of POMC and the contents of POMC, αMSH and the POMC processing enzyme precursor convertase 2 (PC2) have been studied in the pars intermedia using pulse-labeling, Western blot and radioimmunoassay. In control animals, adapted to a white background, the rate of POMC biosynthesis and the POMC content were low, while high αMSH and PC2 contents were found. After 1 week of adaptation to a black background, the rate of POMC biosynthesis and the POMC protein content had increased 19- and 3·7-fold respectively. These parameters attained a maximum level (28- and 5·8-fold higher than control) after 3 weeks and remained at these elevated levels for at least 12 weeks. After 1 week, the pars intermedia content of αMSH was only 30% of the control level, but after 6 and 12 weeks, the αMSH level had increased to the control level. The PC2 content decreased to 52% of control after 1 week and stabilized after 3 weeks at a level slightly lower than the control value. The results show that during long-term background adaptation a steady-state situation is reached, with a balance between the biosynthesis, enzymatic processing and release of αMSH. The *in vivo* dynamics of the processing enzyme PC2 suggest a parallel storage and release of αMSH and mature PC2 in the *Xenopus* pituitary pars intermedia.

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## Introduction

Most amphibian species possess the ability to adapt the melanin pigment distribution in their skin melanophores to the light intensity of the background. In white background-adapted animals, melanin is aggregated around the nucleus, resulting in a pale skin color, while black background-adapted animals have a dark skin color, caused by pigment dispersal (Bagnara & Hadley 1973). This process is a neuroendocrine reflex, mediated by the peptide α-melanophore-stimulating hormone (αMSH) (Wilson & Morgan 1979, Jenks *et al.* 1988, van Zoest *et al.* 1989), which is produced in melanotrope cells of the pituitary pars intermedia from the precursor protein proopiomelanocortin (POMC) (Martens *et al.* 1985).

Differences in the physiologic demand for the amount of αMSH released into the circulation are accompanied by plastic changes in the activity of melanotrope cells. Because melanotrope cell activity can be readily influenced in a physiological way by changing the light intensity, amphibian background adaptation is an ideal model to study various structural and biochemical aspects of long-term plasticity in a secretory cell. In the toad *Xenopus laevis* some of the plastic cellular changes occurring in response to background adaptation have been studied in detail (for reviews see Jenks *et al.* 1993, Roubos 1997). The ultrastructure of melanotrope cells in white background-adapted animals reflects low biosynthetic activity, with the abundance of secretory granules indicating storage of αMSH. Transfer of animals from a white to a black background leads to an increase in the volume of rough endoplasmic reticulum, the extent of Golgi apparatus and the number of mitochondria, indicative of a high rate of POMC and αMSH biosynthesis. These changes are accompanied by a decrease in the number of secretory granules, suggesting enhanced αMSH release

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(Weatherhead & Whur 1972, de Rijk *et al.* 1990). Such plastic changes in melanotrope cell activity have been confirmed in biochemical *in vitro* studies (Thornton 1971, Whur & Weatherhead 1971, Jenks *et al.* 1977, Loh & Gainer 1977, van Zoest *et al.* 1989).

In mammalian melanotropes, the processing enzyme prohormone convertase 2 (PC2) is responsible for the biosynthesis of αMSH from the precursor protein POMC (Smith & Funder 1988, Benjannet *et al.* 1991, Thomas *et al.* 1991, Day *et al.* 1992, Marcinkiewicz *et al.* 1993). POMC and PC2 are also coexpressed in *Xenopus* melanotropes. Much higher levels of POMC mRNA and PC2 mRNA occur in the pars intermedia of animals adapted to a black background compared with white background-adapted animals (Martens *et al.* 1987, Braks *et al.* 1992, Holthuis *et al.* 1995). At the subcellular level, PC2 has been localized in αMSH-containing secretory granules in *Xenopus* melanotropes, suggesting that also in amphibians PC2 is involved in proteolytic cleavage of POMC (Berghs *et al.* 1997, Kurabuchi & Tanaka 1997). Processing of *Xenopus* POMC yields the same spectra of peptide products as in mammals (Martens *et al.* 1982*a,b,* 1983, van Strien *et al.* 1995, 1996).

Obviously, efficient functioning of the melanotrope cell during background adaptation requires a well-coordinated interplay between biosynthesis of POMC, POMC processing by PC2, and storage and release of αMSH. Up to now this interplay has been studied only fragmentarily. POMC biosynthesis and αMSH release have been examined in short adaptation studies only (maximally 3–6 weeks; Maruthainar *et al.* 1992, Jenks *et al.* 1993, van Strien *et al.* 1995, 1996), whereas the dynamics of PC2 during background adaptation are unknown. In the present study the dynamics of POMC biosynthesis and processing and αMSH storage and release have been examined in a long-term *in vivo* approach to monitor the physiological steady-state situation. To this end, the rate of POMC biosynthesis and the amounts of POMC and αMSH were determined in the pars intermedia of the *Xenopus* pituitary for up to 12 weeks after transfer of the animals from a white to a black background. Furthermore, the *in vivo* role of PC2 in POMC processing was investigated by determining the PC2 protein content during this period.

## Materials and Methods

### *Animals*

*Xenopus laevis* were bred and reared under laboratory conditions at 22 °C. Before the experiments, male animals of the same age and weight were kept for 3 weeks on a white background (12 l white plastic buckets, four animals per bucket) under constant illumination. Lighting was with overhead daylight tube lamps (Osram L58 W/11) generating 3000 Lux at the water surface. Following adaptation to white background animals were either immediately killed (controls), or placed on a black background (12 l black plastic buckets) for 1, 3, 6 or 12 weeks, again under constant illumination. The transfer time schedule was designed in such a way that all animals could be killed on the same day. After decapitation, neurointermediate lobes were rapidly dissected and immediately subjected to pulse-labeling with radioactive amino acids or collected on dry ice and stored at −70 °C until further processing for Western blot or radioimmunoassay.

### *Radioactive amino acid incorporation (POMC biosynthesis)*

Neurointermediate lobes were individually rinsed in Ringer's solution containing 112 mM NaCl, 2 mM $CaCl_2$, 2 mM KCl, 15 mM Ultral-Hepes (Calbiochem, La Jolla CA, USA), 0·3 mg/ml BSA and 2 mg/ml glucose (pH 7·4), and pulse-labeled for 30 min in 10 µl Ringer's solution containing 1 mCi/ml [$^3$H]lysine (Amersham, Little Chalfont, Bucks, UK). Then they were washed in Ringer's solution and lyzed by boiling for 5 min in 100 µl sample buffer containing 62·5 mM Tris (pH 6·8), 12·5% glycerol, 1·25% SDS, 2·5% β-mercapto-ethanol and 0·0125% bromophenol blue, prior to subjecting 20 µl lobe extracts to 12·5% SDS–PAGE.

### *SDS–PAGE*

SDS–PAGE was performed according to Laemmli (1970). Subsequently, the gels were fixed (40% methanol, 10% acetic acid), saturated with 100% dimethylsulfoxide (DMSO) and treated with 20% 2,5-diphenyloxazol (PPO) in 100% DMSO for fluorography (Bonner & Laskey 1974). For analysis of results of pulse-labeling experiments, the 38 kDa POMC protein can readily be identified in the electrophoretic pattern of a lobe extract (Ayoubi *et al.* 1990). Quantification of the amount of POMC protein (in arbitrary units) was performed with an UltroScan XL laser densitometer (Pharmacia LKB, Uppsala, Sweden).

### *Western blot analysis (POMC and PC2 contents)*

Neurointermediate lobes were lysed by boiling in sample buffer, diluted 1:20 for POMC detection or 1:4 for PC2 detection, and subjected to 12·5% SDS–PAGE. For immunoblotting, the proteins were electrophoretically transferred to nitrocellulose membranes using a Mini-Protean II Cell system (Biorad, Segrate, Italy). Blots were incubated for 1 h in blocking buffer (5% BSA in tris-buffered saline (TBS) with 0·2% Tween-20), washed in TBST (TBS with 0·2% Tween-20) and incubated for 16 h at 4 °C in blocking buffer with antisera against POMC or PC2. For POMC detection 1:10 000 diluted polyclonal rabbit anti-*Xenopus* POMC was used (ST-62; see Berghs *et al.* 1997, for details showing the high specificity of the antiserum). For PC2 detection a 1:1000 diluted polyclonal



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rabbit antiserum was used, produced against recombinant human PC2. This antiserum exclusively recognized the precursor and mature form of PC2 (see Fig. 2). Following incubation with antisera the blots were washed in TBST, incubated for 1 h at 20 °C with 1:1000 diluted goat anti-rabbit IgG conjugated to peroxidase (Nordic, Tilburg, The Netherlands) in blocking buffer, and finally washed in 0·3 M $MgCl_2$. Antigen was detected by the enhanced chemiluminescence method (Amersham). Quantification of the amounts of POMC and PC2 protein was performed as described above.

*Radioimmunoassay (αMSH contents)*

Neurointermediate lobes were homogenized in 0·1 M HCl, vacuum dried, resuspended, and subjected to a radioimmunoassay for αMSH as described previously, using a polyclonal rabbit antiserum raised in our laboratory, at a final dilution of 1:30 000. Antibody-bound and free αMSH were separated by polyethylene glycol/ovalbumin precipitation (for details, see van Zoest *et al.* 1989).

*Statistics*

Data were analyzed by one-way analysis of variance (α=5%) (Bliss 1967) followed by Duncan's multiple range test (Steel & Torrie 1960), to compare groups at different time points with each other. (These differences have been indicated by characters in the respective figures.) The analysis was preceded by tests for the joint assessment of normality (Shapiro & Wilk 1965) and for the homogeneity of variance (Bartlett's test; Bliss 1967).

## Results

*POMC biosynthesis*

The biosynthetic activity was measured by [$^3$H]lysine labeling of POMC protein. The newly synthesized POMC was not detectably processed to smaller products during the short pulse incubation time of 30 min, and thus the rate of precursor biosynthesis could be accurately determined. In white background-adapted animals, a very low level of POMC biosynthesis was observed, which increased after 3 weeks of black background adaptation to a level 28 times higher than the control level (animals adapted to a white background; Fig. 1A). The biosynthetic activity appeared to increase slightly up to 12 weeks, but this increase was not statistically significant.

*POMC contents*

Determination of the pars intermedia contents of the POMC precursor, excluding detection of POMC processing products, was possible by Western blot analysis using an antibody directed against the first cleavage site of *Xenopus* POMC. In the 3-week white background-adapted control animals, POMC precursor protein was present in a low amount (Fig. 1B, t=0). During the first 3 weeks of black background adaptation, the POMC protein content increased to a maximum level. At 3 weeks, the amount of POMC was 5·8 times higher than that in white background-adapted animals. Subsequently, the content did not change significantly for up to 12 weeks (Fig. 1B).

*αMSH contents*

The pars intermedia of animals adapted to a white background contained a high amount of αMSH, as determined by radioimmunoassay (Fig. 1C; t=0). After 1 week of adaptation to a black background, the tissue content of αMSH was reduced by 70% compared with the control value. After 3 weeks, the αMSH amount had increased again, to reach control level after 6 weeks. This level did not change up to 12 weeks (Fig. 1C).

*PC2 contents*

The pars intermedia contents of the processing enzyme PC2 were measured by Western blot analysis (Fig. 2). The antibody used for PC2 detection is directed against recombinant PC2 and recognizes both the precursor and the mature form of PC2. In white-adapted animals, no pro-PC2 could be detected and at all stages of black-adaptation the amount of pro-PC2 was 10- to 30-fold lower than the amount of mature PC2 (e.g. Fig. 2). In determining the dynamics of PC2 contents during background adaptation we considered only the mature active form of PC2, which is available to process POMC (Fig. 1D). In animals adapted to a white background a high level of PC2 was present, which diminished to 52% of the control value after 1 week of black background adaptation and stabilized after 3 weeks at a level slightly lower than the control value (Fig. 1D).

## Discussion

*Dynamics of POMC biosynthesis and αMSH release*

In this study, long-term background adaptation has been investigated in *Xenopus laevis* by determining the time course of POMC biosynthesis and processing in the pituitary pars intermedia. In animals that had been adapted to a white background, biosynthetic activity is hardly detectable and a very low amount of POMC precursor protein is present in the pars intermedia, whereas the POMC peptide product αMSH is present in high amounts. In response to black background adaptation, the



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**Figure 1** Animals were adapted to a white background for 3 weeks and either immediately killed (t=0, controls), or subsequently adapted to a black background for 1, 3, 6 or 12 weeks. The average value of controls was set at 1 (A,B) or 100% (C,D). Data represent means ± S.E.M. Data with a common superscript do not differ significantly ($P$<0·05). (A) Amount of newly synthesized POMC (expressed as fold increase relative to the control, $n$=5) determined by labeling with [$^3$H]lysine. (B) Amount of POMC (expressed as fold increase relative to the control, $n$=5) determined by Western blot. (C) Amount of αMSH (expressed as a percentage of the control value, $n$=3) determined by radioimmunoassay. (D) Amount of PC2 (expressed as percentage control, $n$=5) determined by Western blot.

rate of POMC biosynthesis and the POMC content become much higher than in white background-adapted animals, while initially the αMSH content is strongly reduced. This indicates that the end product αMSH and not the precursor POMC is the major storage product in animals on a white background. The initial reduction of αMSH in animals adapting to a black background probably reflects secretion from 'mature' secretory granules before the biosynthetic machinery can fully compensate for this release. The observed difference in POMC protein contents of the pars intermedia of white and black background-adapted animals is in line with previous studies that showed a much lower POMC mRNA level in white-adapted than in black-adapted animals (Martens *et al.* 1987). Prolonged adaptation of 12 weeks to a black background does not significantly change the situation seen at 3–6 weeks, indicating that steady-state levels are reached at about 3 weeks of adaptation.

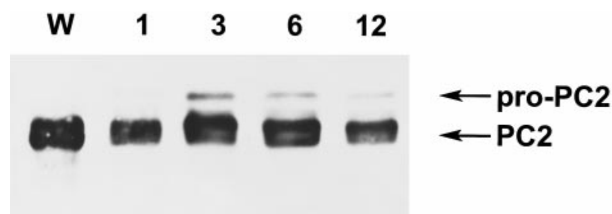


**Figure 2** Example of Western blot analysis of PC2 in individual neurointermediate lobes of animals adapting to black background. W is the white background control; 1, 3, 6, and 12 indicate the number of weeks adaptation to black background. Pro-PC2 is the precursor form of active PC2.

The *Xenopus* background adaptation process offers a very good medium to study the *in vivo* dynamics of endocrine peptide biosynthesis in response to chronic physiological demands. One might suppose that long-term adaptation would lead to an increased effectiveness of melanotrope functioning, with the same degree of skin color adaptation resulting from a lower level of biosynthetic activity. However, the present study shows that the high steady-state level of biosynthetic activity



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reached after 3 weeks is maintained for up to 12 weeks. Apparently, three phases in *Xenopus* background adaptation can be distinguished. The initial phase is regulated by a β-adrenergic mechanism, acting at the level of the skin melanophore (van Zoest *et al.* 1989); within the first few hours of transfer of an animal from a white to a black background pigment dispersion in skin melanophores is induced by (nor)adrenaline. The second phase adaptive response is controlled by αMSH release. It starts up slowly during the first days of adaptation, relying first on stores of αMSH, and ultimately leads to a balance between the biosynthesis, enzymatic processing and release of αMSH after about 3 weeks of adaptation. In the third phase, lasting at least up to 12 weeks, steady-state levels of αMSH biosynthesis, processing and release maintain the state of black-background adaptation.

*The role of PC2 in POMC processing* in vivo

In animals adapted to a white background, a high level of mature PC2 was found, while after 1 week of black background adaptation the PC2 content was reduced. This reduction parallels the reduction in αMSH, suggesting co-storage of αMSH and the mature form of PC2. The concomitant decreases of the αMSH and PC2 contents during early adaptation suggest a coordinate release of the two molecules in response to transfer of the animal to a black background. This observation is in agreement with the demonstrated coexistence of αMSH and PC2 in secretory granules in *Xenopus* melanotropes (Kurabuchi & Tanaka 1997). Release of PC2 from neurointermediate pituitary lobes was previously reported in *in vitro* studies (Braks & Martens 1994). The present study indicates that this release also occurs in the *in vivo* situation and, therefore, may have physiological relevance. This is the first time that PC2 protein dynamics in response to an environmental factor are demonstrated.

The co-expression of the POMC and PC2 genes has been well documented, i.e. high expression in fully black adapted animals and low expression in fully white adapted animals (Martens *et al.* 1987, Braks *et al.* 1992, Holthuis *et al.* 1995). In the present study, where the relative steady-state levels of active PC2 were determined during background adaptation, a partial 'depletion' of the enzyme was noted at 1 week adaptation, before its level stabilized with longer adaptations to black background. At 1 week adaptation the level of POMC biosynthesis and the steady-state level of POMC were also lower than the level they ultimately achieved with long adaptations. These observations indicate that the genes for both POMC and PC2 are only very slowly activated during black background adaptation. This indicates that the POMC and PC2 genes may be co-regulated. In the *Xenopus* pars intermedia, several inhibitory and stimulatory neuronal factors regulating POMC biosynthesis have been identified, including dopamine, neuropeptide Y, thyrotropin-releasing hormone and sauvagine (Dotman *et al.* 1996, 1997). The *Xenopus* melanotrope cell may be an appropriate system to investigate whether PC2 gene expression is subject to the same complex regulations as the PC2 substrate POMC.

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