ERK-Regulated Double Cortin-Like Kinase (DCLK)-Short Phosphorylation and Nuclear Translocation Stimulate POMC Gene Expression in Endocrine Melanotrope Cells

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We tested whether double cortin-like kinase-short (DCLK-short), a microtubule-associated Ser/Thr kinase predominantly expressed in the brain, is downstream of the ERK signaling pathway and is involved in proopiomelanocortin gene (POMC) expression in endocrine pituitary melanotrope cells of *Xenopus laevis*. Melanotropes form a well-established model to study physiological aspects of neuroendocrine plasticity. The amphibian *X. laevis* adapts its skin color to the background light intensity by the release of α-MSH from the melanotrope cell. In frogs on a white background, melanotropes are inactive but they are activated during adaptation to a black background. Our results show that melanotrope activation is associated with an increase in DCLK-short mRNA and with phosphorylation of DCLK-short at serine at position 30 (Ser-30). Upon cell activation phosphorylated Ser-30-DCLK-short was translocated from the cytoplasm into the nucleus, and the ERK blocker U0126 inhibited this process. The mutation of Ser-30 to alanine also inhibited the translocation and reduced POMC expression, whereas overexpression stimulated *POMC* expression. This is the first demonstration of DCLK-short in a native endocrine cell. We conclude that DCLK-short is physiologically regulated at both the level of its gene expression and protein phosphorylation and that the kinase is effectively regulating POMC gene expression upon its ERK-mediated phosphorylation. (*Endocrinology* 152: 2321–2329, 2011)

Double cortin-like kinase-short (DCLK-short), one of the splice variants of the *DCLK1* gene, is abundantly expressed in the adult rodent brain (1, 2). *DCLK1* is a member of the double cortin (*DC*) gene family. Proteins encoded by this family are characterized by the presence of tubulin-binding domain, the so-called DC domain (for review see Ref. 3). DCLK1-derived proteins containing DC domains, like DCLK-long and DC-like, play well-established roles in neurogenesis and neuronal migration (4, 5). In contrast, the function of DCLK-short, a *DCLK1* splice variant that lacks DC domains and encodes a Ser/Thr kinase (6), is less well known. Physiological importance of DCLK is suggested by the fact that it is evolutionary well conserved, occurring in both fish and mammals (7, 8). It is hypothesized to play a role in neuronal plasticity (9) because it is abundant in the hippocampus, and its gene expression is enhanced in the dentate gyrus upon kainate treatment (2, 6). This hypothesis is supported by the recent demonstration that all short DCLK1 variants are up-regulated during brain-derived neurotrophic factor (BDNF)-

Abbreviations: BDNF, Brain-derived neurotrophic factor; Ct, cycle threshold; DC, double cortin; DCLK, double cortin-like kinase; EV, empty vector; GAPDH, glyceraldehyde 3-phosphate dehydrogenase gene; GFP, green fluorescent protein; MEK, MAPK kinase; NIL, neurointermediate lobe; p-DCLK, phosphorylated double-cortin-like kinase; POMC, proopiomelanocortin; Q-RT-PCR, quantitative RT-PCR; Rnase, ribonuclease; S/A, serine by alanine mutant; SDS, sodium dodecyl sulfate; Ser-30, serine at position 30; SSD, specific signal density; TBS, Tris-buffered saline; WT, wild type.

induced synapse consolidation (10). The N-terminus of DCLK-short possesses a serine- and proline-rich domain that contains the amino acid sequence PTSP, a target of the main component of growth factor signaling pathways, ERK (11, 12). Furthermore, the catalytic domain of DCLK-short contains the nuclear targeting motif RRRR at positions 393–396 (6), suggesting involvement of this kinase in the regulation of gene expression. When transfected into the COS-7 cell line, some DCLK is translocated from the cytoplasm into the nucleus after its cAMP-dependent phosphorylation (13), but whether its ERK phosphorylation site is involved in this process is not known.

DCLK-short is present in not only neurons but also the endocrine cell-derived PC12 cell line (14). However, up to now, the kinase has not been shown in a native endocrine cell. Therefore, in the present study, we investigated its presence in the endocrine melanotrope cells in the pituitary pars intermedia of the amphibian *Xenopus laevis*. We further studied the regulation and the action of DCLK-short in these cells in relation to the animal's physiology. Melanotrope cells promote pigment dispersion in dermal melanophores of *Xenopus* by releasing αMSH when animals are adapting to a black background. Melanotropes are inactive in toads on a white background but are strongly activated during adaptation to a black background. The melanotrope cell displays high plasticity because this activation involves a strong increase in cell size and an up-regulation of genes involved in the production, processing, and secretion of αMSH, including *POMC*, the gene of its precursor protein proopiomelanocortin (POMC) (15–17). These events are coordinated by several intracellular signaling cascades, including the ERK signaling pathway (17, 18). The simplicity of this physiological activation process together with the involvement of the ERK signaling pathway makes the *Xenopus* melanotropes an ideal experimental model to study the regulation and the function of DCLK-short in a native endocrine system.

In this study we tested our hypothesis that DCLK-short is present in endocrine melanotrope cells, is regulated during background adaptation of the animal, and is involved in the control of POMC mRNA expression as a downstream effector of ERK signaling. For this purpose, we determined the effect of cell activation (by black background adaptation) on the expression of DCLK-short mRNA and on the phosphorylation state and cellular distribution of DCLK-short protein. Subsequently the role of ERK in the phosphorylation of DCLK-short at position 30 (Ser-30) of the PTSP sequence has been demonstrated and the subcellular presence of the phosphorylated (p) kinase (p-DCLK-short) localized. Finally, the contribution of nuclear phosphorylated double-cortin-like kinase (p-

DCLK)-short in the regulation of POMC expression has been assessed.

Materials and Methods

Animals

Adult *X. laevis*, aged 6 months, were kept on a 12-h light, 12-h dark cycle (lights on at 0700 h) at 22 C and fed a diet of beef heart and Aquatic 3 (Dietex International, Witham, UK). Background adaptation was performed by placing animals for different periods in a black or white container under continuous illumination; full skin adaptation was achieved after 3 wk. Subsequently the animals were anesthetized by immersion in 0.1% MS222 (ICN Biomedicals, Aurora, OH), rapidly decapitated, and the pituitary neurointermediate lobe (NIL) or the whole pituitary gland together with the brain dissected. For cell transfection studies, before decapitation, animals were transcardially perfused with *Xenopus* Ringer's solution (112 mM NaCl; 2 mM KCl; 2 mM $CaCl_2$; 15 mM Ultra-HEPES; and 0.2% glucose, pH 7.4) containing 0.025% MS222 to remove blood cells. Animal treatment was in agreement with the Declaration of Helsinki and the Dutch law concerning animal welfare, as verified by the Committee for Animal Experimentation of Radboud University Nijmegen.

Quantitative RT-PCR (Q-RT-PCR)

NIL of 3-wk white-adapted animals, subsequently adapted for 0, 1, 3, 6, and 24 h to a black background, and animals adapted for 3 wk to a black background (19) were collected and homogenized in ice-cold Trizol (Life Technologies, Paisley, UK). Because the neural lobe contains mainly nerve terminals from neurons originating in the hypothalamus and the vast majority of the cells in the pars intermedia are melanotropes, the extracted mRNA will be mainly from the melanotropes located in the pars intermedia. RNA was extracted with chloroform and precipitated with isopropanol and glycogen. The RNA pellets were rinsed in ice-cold 75% ethanol, air dried, and resuspended in ribonuclease (Rnase)-free milli-Q water (Millipore, Amsterdam, Netherlands). The RNA concentration was measured by Nano-Drop 1000 (Thermo Fischer Scientific, Wilmington, DE). First-strand cDNA was synthesized at 70 C for 10 min with 0.1 μg RNA and 5 mU/μl random primers (Roche, Mannheim, Germany) at 37 C for 75 min in first-strand buffer (Life Technologies) with 10 mM dithiothreitol, 20 U Rnasin (Promega, Madison, WI), 0.5 mM deoxynucleotide triphosphates (Roche, Almere, The Netherlands), and 100 U Superscript II reverse transcriptase (Life Technologies) and finally in the same buffer at 95 C for 10 min.

For single-cell Q-RT-PCR, individual melanotrope cells exhibiting green fluorescent protein (GFP) fluorescence (for cell preparation, see below) were aspirated in Rnase-free milli Q water through a glass micropipette with a tip opening of 0.8–1 μm and transferred onto a 48 Ampligrid reaction slide (Advalytix, Munich, Germany). After air drying the sample, first-strand cDNA was synthesized on the slides using the Ampligrid cycler (Advalytix) in a mixture of 0.5 mU pd(N)6 random primer (Roche), 0.05 μM deoxynucleotide triphosphates (Roche), 1.0 mM dithiothreitol (Invitrogen, Carlsbad, CA), 10 U SuperScript II reverse transcriptase (Invitrogen), 1× first-strand buffer (Invitrogen), and 2.0 U Rnasin (Promega). The mixture was sealed

off with inert sealing oil (Advalytix). Incubation was at 70 C for 10 min, 37 C for 75 min, 95 C for 10 min, and 30 C for 1 min. Finally, Rnase-free milli Q water was added and the sample aspirated for use in the Q-RT-PCR.

cDNA amplification was carried out with power SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) and 0.6 μM of each primer. The primers for DCLK-short were based on *Silurana tropicalis DCLK1* (accession no. NM_001045640), and those for *POMC* and the gene of glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) were based on the respective *X. laevis* sequences (accession no. X03843 and U41753, respectively). The sequences were: DCLK-short forward, 5′-CAAGTGAACTC-TACCTTGTCATGGA-3′, reverse, 5′-GCTCAGTGTATTTGT-TGGTCGAAGT-3′; POMC forward, 5′-AGGGAACGATG-GAAGCAACA-3′, reverse, 5′-TGTCACCTGGAGCATTC-TGATT-3′, and GAPDH forward, 5′-GCTCCTCTCGCAA-AGGTCAT-3′, reverse, 5′-GGGCCATCCACTGTCTTCTG-3′. The temperature cycling protocol consisted of 10 min at 95 C and 40 cycles of, alternatingly, 15 sec at 95 C and 1 min at 60 C and was run on a 7500 real-time PCR system (Applied Biosystems).

For single-cell Q-RT-PCR, the same cycling protocol was followed but with 50 instead of 40 cycles. In these single-cell experiments cycle threshold (Ct) values of GAPDH, DCLK-short, and POMC were between 30 and 40 and were corrected by subtraction of the corresponding Ct values of the gene of the housekeeping enzyme GAPDH. Samples were included in the analysis only if the melting curve was at its maximum at the predicted melting temperature and a product with the correct molecular weight showed up on a 1% agarose gel.

DCLK-short alignment

The protein sequences, predicted on the basis of *S. tropicalis* DCLK-short transcripts (NP001039105.1) (20), were aligned with the known DCLK sequences of *Homo sapiens* (BAH12940.1), *Mus musculus* (NP001104521.1) and *Danio rerio* (AAI65597.1), using ClustalW software (European Bioinformatics Institute, Cambridge, UK) (21).

Melanotrope cell isolation

In some experiments the location of DCLK-short was studied in single melanotropes, which were isolated from NIL as described previously (22). In short, NIL were incubated in *Xenopus* Ringer's containing 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) but without CaCl$_2$, for 45 min at 22 C. Then NIL were gently triturated and the resulting cell suspension was centrifuged at $50 \times g$ for 10 min. The cell pellet was resuspended in *X. laevis* Leibovitz L15 medium consisting of XL-L15, 76% Leibovitz culture medium (Life Technologies), 1% kanamycin, 1% antibiotic/antimycotic (Life Technologies), 2 mM CaCl$_2$, and 10 mM glucose (pH 7.4) for immunocytochemistry and transfection studies. For Western blot analyses, incubation was in lysis buffer containing 50 mM HEPES (pH 7.2), 140 nM NaCl, 0.1% Triton X-100, 1% Tween 20, 1 mM EDTA, 0.1% deoxycholate, 0.0001% pepstatin, 0.0001% leupeptin, 0.0001% aprotinin, 0.0002% phenylmethanesulfonylfluoride, 0.01% benzamidine (Sigma-Aldrich), and 0.0008% calpain I and calpain II inhibitors (Boehringer, Mannheim, Germany). The pars nervosa of the pituitary gland, which remained undissociated in this protocol, was used as control.

Western blot analyses

To determine their DCLK contents, NIL were collected individually in ice-cold lysis buffer with 0.2% NaF to inhibit phosphatase activity. To establish the involvement of ERK signaling in DCLK-short phosphorylation before lysis, some NIL were incubated with 100 μM of MAPK kinase (MEK) inhibitor U0126 (Tocris Bioscience, Bristol, UK) in XL-L15 at 22 C for 1 d.

After 30 min incubation in lysis buffer, NIL and single melanotropes were homogenized and centrifuged at $16,000 \times g$ at 4 C for 30 min. The supernatant was incubated at 95 C for 5 min in sample buffer (62.5 mM Tris-HCl; 12.5% glycerol; 1.25% sodium dodecyl sulfate (SDS); 0.0125% bromophenol blue; 2.5% β-mercaptoethanol, pH 6.8). After running samples on a 12% SDS-polyacrylamide gel (Precise protein gels, Thermo Scientific, Rockford, IL) using BupH Tris-HEPES-SDS running buffer (Thermo Scientific), separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) using BupH Tris-glycine buffer (Thermo Scientific). Blots were rinsed in 0.1 M Tris-buffered saline (TBS; pH 7.6) and incubated in blocking buffer (TBS with 5% milk powder) and in blocking buffer containing either rabbit polyclonal p-DCLK antiserum raised against the phosphorylated Ser-30 residue in the PTSP domain [1:1000 (vol/vol); see below] or rabbit polyclonal DCLK antiserum [1:1000 (vol/vol)] (23). After rinsing with TBS with 0.2% Tween 20 blots were incubated in goat antirabbit peroxidase conjugate (1:1000; Nordic, Tilburg, The Netherlands) and rinsed again in TBS with 0.2% Tween 20 and TBS. Proteins were visualized by incubation in Supersignal West Pico chemiluminescent substrate solution (Thermo Scientific) using x-ray film (Fujifilm, Tokyo, Japan). After scanning the film (Scanjet 5590P; Hewlett Packard, Palo Alto, CA), the average immunostaining density of the protein bands was expressed as the specific signal density (SSD), using ImageJ software (version 1.37; National Institutes of Health, Bethesda, MD) (24).

Immunohistochemistry

The dissected brain and pituitary gland of white- and black-adapted animals were fixed in Bouin's fluid for 24 h and dehydrated in a graded series of ethanol. Paraffin sections of 7 μm thickness were mounted on glass slides coated with poly-L-lysine (Sigma-Aldrich), deparaffinized, rehydrated in a graded ethanol series, rinsed in 0.1 M sodium PBS (pH 7.6), treated with 0.5% Triton X-100 (Sigma Chemical, Zwijndrecht, The Netherlands) in PBS, rinsed, and preincubated in blocking buffer containing PBS, 2.5% normal horse serum (Vector Laboratories, Burlingame, CA), 2.5% normal goat serum (Jackson ImmunoResearch, Suffolk, UK), and skimmed milk. Then sections were incubated with first antiserum (1:1000 rabbit polyclonal p-DCLK) in blocking buffer. After rinsing in PBS, they were treated with biotin conjugate (1:200; Vector Laboratories) and rinsed in PBS. Subsequently sections were incubated with avidine-biotin complex (1:200; Vector Laboratories) and successively with 0.05% diaminobenzidine in the Tris buffer for 10 min and with 0.005% H$_2$O$_2$ for about 10 min. The latter reaction was controlled under a stereo microscope and stopped with Tris buffer. Then sections were rinsed in PBS, dehydrated in ethanol, coverslipped with Entellan (Merck, Darmstadt, Germany), and examined with a Nikon Microphot FXA microscope (Nikon, Tokyo, Japan).

To determine the degree of immunoreactivity of a NIL, the SSD of the immunoreactive signal was assessed using the ImageJ

software (National Institutes of Health) at five randomly taken places in the largest section profile of the pars intermedia. The SSD of the pars nervosa was determined in the same way.

To study the subcellular location of p-DCLK, melanotrope cells resuspended in XL-L15 were plated onto poly-L-lysine-coated, four-chamber-coverglass (Nalge Nunc, Rochester, NY), fixed in 4% paraformaldehyde for 15 min, rinsed with PBS, and stained for p-DCLK as described above. After rinsing three times in PBS, cells were stained for 15 min in 100 nM Hoechst 33258, washed five more times, and coverslipped with FluorSave and examined with an LSM2 confocal laser-scanning microscope (Leica, Wetzlar, Germany) using a ×63 oil immersion objective (numerical apperture 1.42) at 350 nm excitation wavelength. The pinhole size was set at two times the Airy disk, achieving an optical section resolution along the z-axis of approximately 2 μm.

Antiserum specificity

The high specificities of the rabbit polyclonal DCLK antiserum have been shown before (5). The rabbit polyclonal p-DCLK antiserum was generated against the peptide PSPTpSPGSLRKQ, corresponding to positions 24–38 of rat DCLK (Eurogentec, Rotterdam, The Netherlands). Specificity of the antiserum was demonstrated with ELISA against the native phosphorylated peptide and, moreover, as follows: point mutation was introduced into mouse wild-type (WT) DCLK-short cDNA in pcDNA3.1+ (Invitrogen) vector using a QuikChange site-directed mutagenesis kit according to the manufacturer's instruction (Stratagene, La Jolla, CA), disrupting the potential serine phosphorylation site of DCLK by replacing serine by alanine (S/A mutant). As can be seen in Supplemental Figure 1, both DCLK-short proteins were detected with the DCLK antiserum. Incubation with recombinant ERK slows down DCLK-short migration (Supplemental Fig. 1A, lanes 1 and 2, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) compared with control (without ERK; lanes 3 and 4), a result that can be partially explained when the protein is phosphorylated by ERK. Mutation of Ser-30 to alanine abolishes this particular ERK phosphorylation site so that p-DCLK cannot react with it (Supplemental Fig. 1B, lane 2). We therefore conclude that the p-DCLK antiserum recognizes only the Ser-30 phosphorylation site and is a reliable tool to detect p-DCLK. The epitopes used to generate the antibodies are 100% conserved between mammals and frogs (Supplemental Fig. 2). Thus, the antibodies can be used as a reliable tool to demonstrate DCLK and p-DCLK in frog tissue.

Transfection studies

Single melanotropes in XL-L15 were plated onto poly-L-lysine-coated, four-chamber coverglasses. After 24 h incubation in XL-L15 with 10% fetal calf serum (Life Technologies), cells were transfected with a DCLK-short-GFP construct placed in a pcDNA 3.1 plasmid (Invitrogen) behind a cyanomegalovirus promoter. Transfections were performed using 1 μg DNA per 5 μl lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Two DCLK-short-GFP constructs were used, one with the WT-DCLK-short sequence and the other with the serine residue at position 30 of the PTSP motif mutated to alanine, creating a nonphosphorylatable PTAP domain (S/A). Control cells were transfected with an empty vector (EV) only containing GFP. Fluorescence in these control cells was confined to the cytoplasm (data not shown). After transfection incubation

for 18 h, cells were rinsed, supplied with XL-L15 containing 10% fetal calf serum, and incubated for 4 d while medium was refreshed daily. Transfection efficiency (percentage of fluorescent cells) was between 15 and 25%. For localization of GFP constructs, cells were incubated with 100 μM U0126 (Tocris Bioscience) for 16 h, after which GFP fluorescence was examined by confocal laser-scanning microscopy (excitation wavelength 488 nm, emission wavelength 510–580 nm).

Statistics

All data were expressed as mean and SEM per experimental group. Random sampling procedures were used throughout the experiments. Data of Western blots and immunocytochemistry were tested with Student's *t* test and the Q-RT-PCR and GFP construct data with one-way ANOVA followed by Bonferroni's *post hoc* test, using Statistica (StatSoft, Tulsa, OK) ($\alpha = 5\%$), after appropriate transformation of the data on the basis of tests for normality (25) and the homogeneity of variance (Bartlett's χ^2 test) (26).

Results

To test our hypothesis that DCLK-short in *Xenopus* melanotropes is involved in the regulation of POMC expression in an ERK-dependent way, we performed four sets of experiments.

Experiment 1: analysis of the homology of *Xenopus* DCLK-short

DCLK-short is one of the splice variants of the *DCLK1* gene, together with DC-like and DCLK-long. In contrast to DCLK-long, it lacks the N-terminal DC domain (Supplemental Fig. 2A). Because the DNA sequence of DCLK-short of *X. laevis* is unknown, the amino acid sequence of frog DCLK-short was predicted on the basis of the DNA sequence of *S. tropicalis* and aligned with the sequence of DCLK-short of zebrafish, mouse, and human. This showed that all DCLK-short have strong similarities to each other (Supplemental Fig. 2B). Frog DCLK-short has an 89% amino acid sequence identity with mouse and human DCLK-short and a 71% sequence identity with its zebrafish orthologous gene. The N-terminal part of DCLK-short including the serine- and proline-rich domain is identical between frog, mouse, and human. Also, the nuclear retention sequence RRRR at positions 393–396 is identical in frog, mouse, and human, whereas in fish only one residue is different (Supplemental Fig. 2B).

Experiment 2: presence and regulation of DCLK-short

Using Q-RT-PCR, we found that DCLK-short mRNA is present in the NIL of both white- (inactive melanotropes) and black (active melanotropes)-adapted *X. laevis* (Fig. 1). In active melanotropes the amount of mRNA was
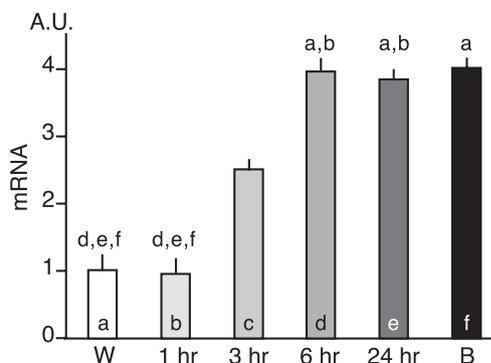
FIG. 1. Q-RT-PCR of temporal dynamics of DCLK-short mRNA amounts in the *Xenopus laevis* NIL during black adaptation. Increase in mRNA starts 3 h after placing white-adapted animals (W) on a black background and remains maximal from 6 h up to 24 h. NIL from animals adapted for 3 wk to a black background (B) serve as controls. Values represent means in arbitrary units (A.U.) + SEM (n = 5). *Letters on top of bars indicate groups that are statistically different (P < 0.05).*

about 4 times higher than in inactive ones ($P < 0.005$; n = 5). When animals were transferred from a white to a black background, the first indication of up-regulation was observed after 3 h, and maximum mRNA expression was reached within 6 h ($P < 0.005$; n = 5; Fig. 1).

To test for the presence of DCLK at the protein level, we used a polyclonal antiserum raised against DCLK. Because the epitopes are 100% conserved between mammals and frog (Supplemental Fig. 2B), we used this antiserum to specifically demonstrate *Xenopus* DCLK. Western blot analysis of NIL revealed a single band of 55 kDa (Fig. 2A), corresponding to the predicted molecular mass of DCLK-short (5, 27). Quantification of this product showed no difference between the black and white adaptation states ($P = 0.43$; n = 4; Fig. 2A).

With anti-p-DCLK serum, NIL from black-adapted animals displayed a strong 55-kDa signal. The SSD of this p-DCLK-short was more than 4 times higher in NIL of black-adapted animals than white ones ($P < 0.01$; n = 4; Fig. 2B). Incubating NIL of black-adapted animals for 1 d

in 100 μM MEK inhibitor U0126 did not affect the amount of the 55-kDa DCLK-short product ($P = 0.67$; n = 4; Fig. 2C) but reduced the amount of p-DCLK-short by 52% ($P < 0.05$; n = 4; Fig. 2D). Treatment of the samples with calf intestinal alkaline phosphatase (Promega Bioscience) for 30 min had no noticeable effect on the amount of the immunoreactive material as revealed with anti-DCLK and did not induce a shift in the molecular weight of the immunoreactive band but completely eliminated the immunoreactivity to anti-pDCLK (Supplemental Fig. 3).

To detect the source of p-DCLK-short in the NIL, we studied the presence of the p-DCLK by immunohistochemistry. In black-adapted animals, both the pars intermedia and the pars nervosa showed strong staining (Fig. 3A). In white-adapted animals, however, the staining in the pars intermedia was weaker (Fig. 3B,C; $P < 0.01$; n = 7), whereas the immunoreactivity of the pars nervosa did not differ from that in the black-adapted animals (Fig. 3B).

The presence of p-DCLK-immunoreactivity in single melanotropes was studied in more detail by Cy³ fluorescence. Hoechst 33258 was used as a nuclear marker. Confocal images, in which fluorescence intensity was optimized for a maximal Z-resolution, demonstrated that in active melanotropes p-DCLK fluorescence was present throughout the cytoplasm and the nucleus (Fig. 4A). However, in inactive melanotropes p-DCLK-fluorescence was not seen in the nucleus but only in the cytoplasm (Fig. 4B). This indicates that upon cell activation p-DCLK gets translocated to the nucleus. We also observed that active melanotropes were larger than inactive ones (Fig. 4), which is consistent with earlier morphometry and ultrastructural studies on intact NILs showing that the melanotropes in black-adapted animals are larger and have a higher secretory activity than the melanotropes in white-adapted animals (16).
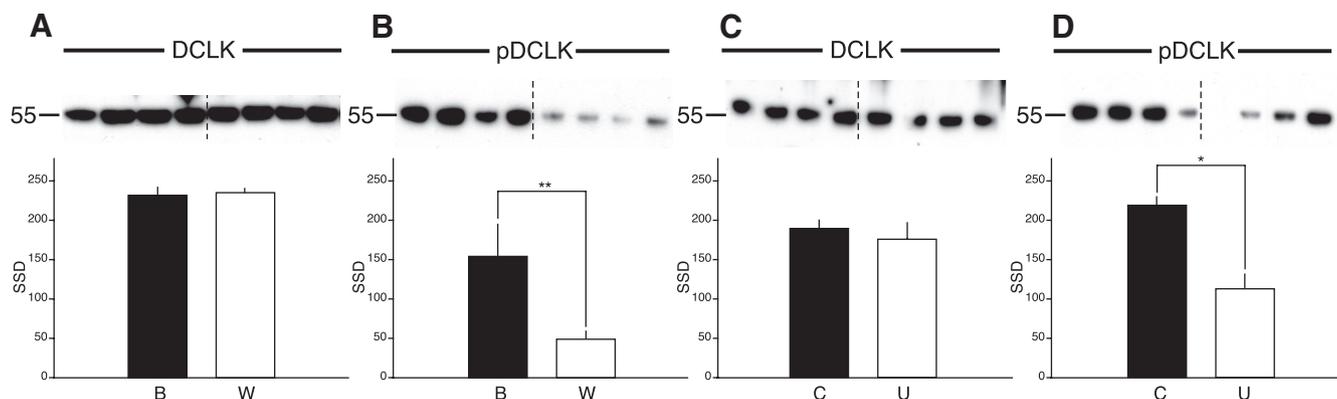

FIG. 2. Western blots of DCLK-short (A and C) and p-DCLK-short (B and D). A and B, NIL from black- (B) and white (W)-adapted *Xenopus* (*top panels*) and SSD of the 55-kDa (p-DCLK-short immunoreactive band) (*lower panels*). C and D, One day *in vitro* treatment of NIL from black-adapted animals with control XL-L15 medium (C) or 100 μM ERK inhibitor, U0126 (U). Values are means in arbitrary units (A.U.) + SEM (n = 4). Statistical difference was compared with black-adapted or control animals, respectively. *, $P < 0.05$; **, $P < 0.01$.
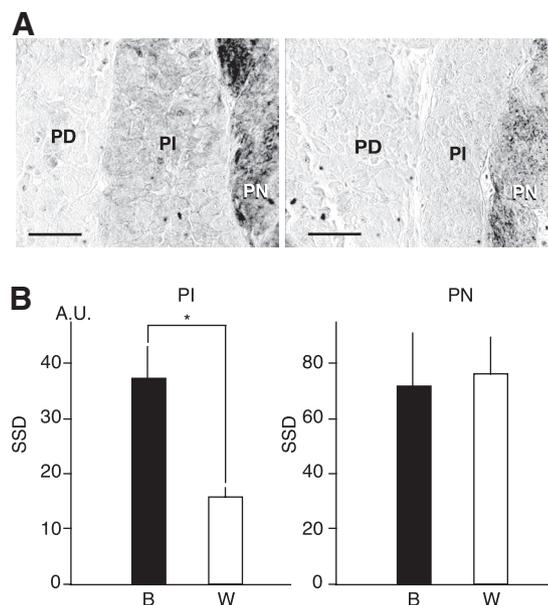
FIG. 3. Immunohistochemistry of p-DCLK in the *Xenopus* pituitary gland. A, The pars intermedia (PI) is uniformly stained after black adaptation (*left panel*) but only weakly stained after white adaptation (*right panel*). PD, Pars distalis; PN, pars nervosa. *Bars*, 50 μm. B, SSD of p-DCLK-immunoreactive signal in pars intermedia (*left panel*) and pars nervosa (*right panel*). Means are in arbitrary units (A.U.) + SEM (n = 7). *, Statistical difference compared with control ($P < 0.01$).

Experiment 3: the role of the PTSP sequence in the subcellular compartmentalization of p-DCLK-short

WT-DCLK-short transfected cells (n = 80, from four animals) displayed clear cytoplasmic GFP fluorescence (Fig. 5A), and in about 50% of these cells, strong fluorescence was also seen in the nucleus (Fig. 5B). In contrast, all S/A-DCLK-short cells revealed only cytoplasmic GFP fluorescence (n = 78 cells from four animals; Fig. 5B). After treatment for 16 h with 100 μM U0126, the percentage of cells displaying nuclear fluorescence was only 24% of the total number of transfected cells ($P < 0.001$; n = 73 cells from four animals; Fig. 5B).

Experiment 4: the role of p-DCLK-short in POMC mRNA expression

The effect of DCLK-short phosphorylation on POMC mRNA transcription was assessed by single-cell Q-RT-PCR, comparing control melanotropes transfected with an EV with melanotropes in which either the WT- or the S/A-DCLK-short construct was overexpressed. The Ct values for GAPDH were 35.3 ± 1.8, 35.2 ± 1.4, and 35.4 ± 1.9 for EV and WT- and S/A-DCLK-short transfected cells, respectively. For POMC these values were 31.5 ± 0.6, 29.1 ± 0.8, and 33.0 ± 1.1, respectively. WT-DCLK-short-expressing melanotropes (n = 11 cells, three animals) revealed 60% more POMC mRNA expression than control EV cells (n = 12 cells, three animals; $P < 0.05$; Fig. 6). POMC mRNA expression in S/A-DCLK-
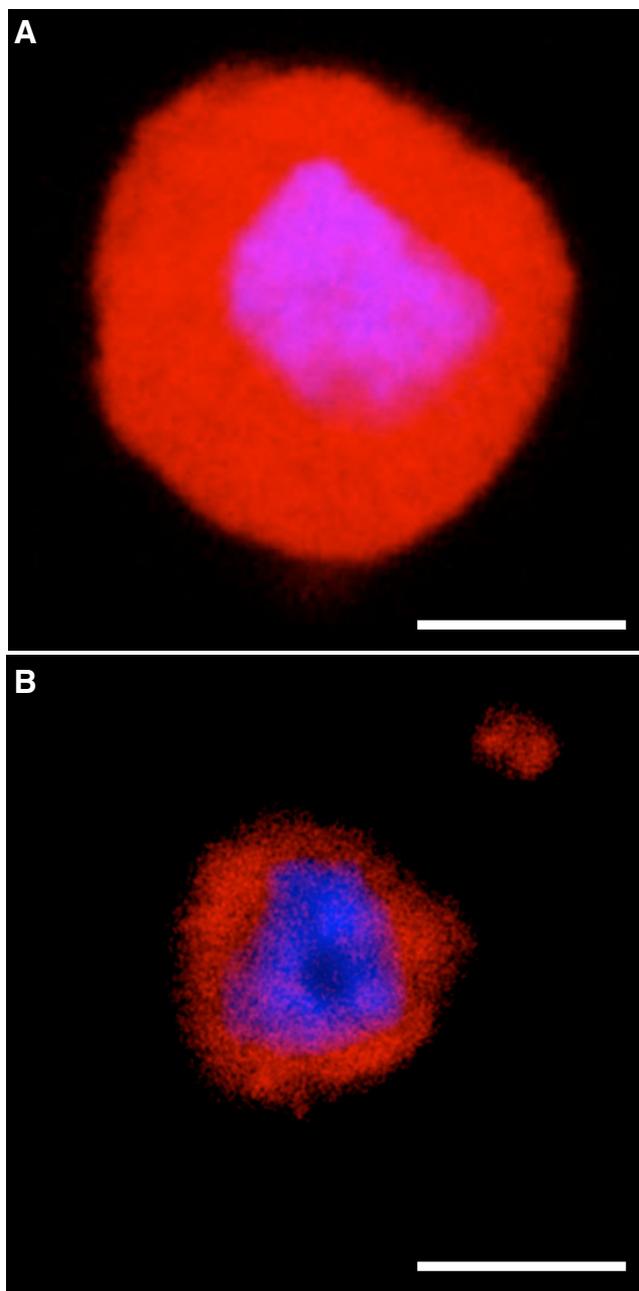

FIG. 4. p-DCLK-short location in isolated melanotropes. Confocal laser-scanning immunofluorescence microscopy of a single melanotrope cell stained for p-DCLK (*red*) and Hoechst (*blue*) from a black-adapted *Xenopus* (A) and from a white-adapted *Xenopus* (B). *Bars*, 5 μm.

short-expressing melanotropes did not significantly differ from the controls (n = 10 cells, three animals; $P = 0.07$). Expression of POMC mRNA in S/A melanotropes was 2.2 times lower than in WT melanotropes ($P < 0.01$; Fig. 6).

Discussion

We have investigated the possibility that DCLK-short is involved in the control of POMC mRNA expression and
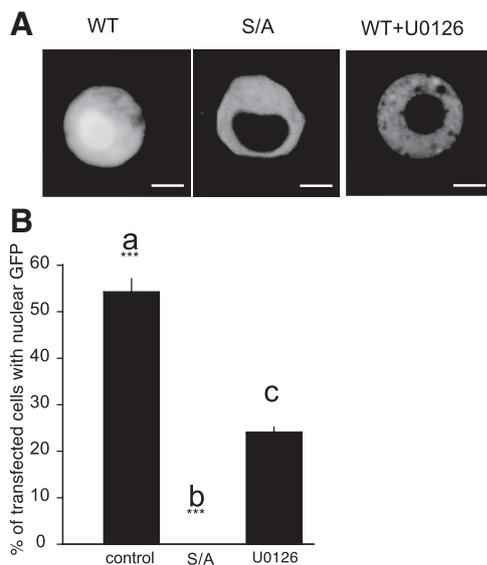
FIG. 5. Effect of S/A mutation and U0126 incubation on presence of DCLK-short in nucleus of melanotrope cells from black-adapted animals. A, Confocal laser-scanning fluorescence microscopy of GFP-labeled WT-DCLK-short in both cytoplasm and nucleus and in cytoplasm only of S/A mutant DCLK-short and WT-DCLK-short treated for 3 d with 10 μM U0126 (U). *Bars*, 5 μm. B, Percentage of melanotrope cells displaying GFP fluorescence in the nucleus for WT-DCLK-short (WT; n = 80 cells), S/A-DCLK-short (S/A; n = 78 cells), and WT-DCLK-short after treatment with 10 μM U0126 (U; n = 73 cells) (mean + SEM). All three groups differ statistically significantly from each other ($P < 0.001$).

acts downstream of ERK, using the *Xenopus* melanotrope cell. This protein has been described in neurons and in the endocrine-derived PC12 cell line (14), but our study is the first showing the presence and *in vivo* regulation of DCLK-short mRNA expression and DCLK-short protein phosphorylation in a pituitary cell. Moreover, we show for the first time that DCLK-short phosphorylation is associated with regulation of gene expression, in our case with the stimulation of POMC. Alignment of the frog DCLK-short amino acid sequence with that of mouse, human, and zebrafish reveals that DCLK-short is highly conserved among vertebrate classes. In particular, the N-ter-
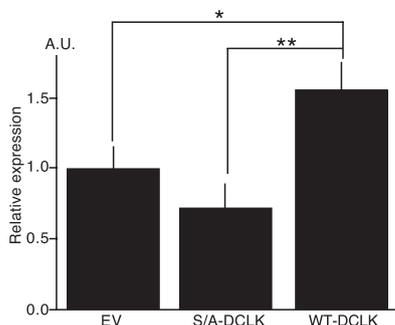

FIG. 6. Q-RT-PCR reveals stimulatory effect of DCLK-short on POMC mRNA expression. WT-DCLK-short expressing melanotropes (WT; n = 11) reveal more expression than control EV-transfected cells (n = 12) and S/A-DCLK-short expressing cells (n = 10). *, $P < 0.05$; **, $P < 0.01$.

minal region, containing the ERK phosphorylation domain PTSP with a serine at position 30, and large parts of the C-terminal catalytic domain, including the nuclear retention sequence at residues 393–396, are conserved between the frog and the mammalian species. This may indicate that DCLK-short has an important function in cellular signaling throughout vertebrate evolution. We have exploited the well-described *Xenopus* melanotrope cell to gain insight into the function of DCLK-short. Below we discuss the outcomes of our experiments with particular reference to the possibility that p-DCLK-short acts as a downstream intranuclear effector of ERK signaling and plays a role in POMC expression.

DCLK-short is expressed in melanotrope cells

Our Q-RT-PCR analysis revealed the presence of DCLK-short mRNA in the *Xenopus* NIL. Because this mRNA was very rapidly up-regulated after placing animals from a white to a black background (within 3 h), DCLK-short may be an important factor in early melanotrope cell activation, possibly acting as an immediate effector gene. Furthermore, the fact that the transcription of DCLK-short is still activated after 3 wk of black adaptation strongly suggests that the gene plays a role in the maintenance of the active state of the melanotropes. Previous *in vitro* studies with mammalian cell lines link DCLK-short expression to neurotrophins (10, 14). Possibly, also in *Xenopus* melanotropes, BDNF signaling is involved in DCLK-short expression because melanotrope cells produce endogenous BDNF and express its receptors (19, 28, 29).

Our data reveal that phosphorylation at the Ser-30 position of DCLK-short in melanotrope cells is coupled to physiological adaptation of the animal to a black background. As the Western blot analysis demonstrates, Ser-30 p-DCLK-short is strongly up-regulated in black-adapted animals, whereas the total amount of (unphosphorylated plus phosphorylated) DCLK-short is not affected by background adaptation.

These results differ from the strong up-regulation of DCLK-short mRNA during background adaptation. Apparently transcription and translation are not one to one coupled. A possible explanation for this discrepancy is protein turnover, whereby increased expression of the mRNA is not paralleled by an increase in total protein content. The fact that the strong phosphorylation of DCLK-short seen in melanotropes of black-adapted animals was inhibited by the MEK inhibitor U0126 indicates that DCLK-short is a downstream signaling molecule of the ERK signal cascade. This notion is supported by our *in vitro* study of purified recombinant DCLK-short, showing that WT-DCLK-short is strongly phosphorylated upon

recombinant ERK application. Quantification of the degree of p-DCLK immunoreactivity shows that in contrast to the situation in the melanotrope cells in the pars intermedia, DCLK phosphorylation in the adjacent neural lobe does not depend on the background light condition. This observation supports the conclusion that p-DCLK-short has a specific role in the melanotrope cell during black background adaptation.

Studies on mammalian cell lines have shown the intranuclear presence of DCLK-short (7, 13, 27). Our immunocytochemistry of single melanotropes places this observation in a physiological context. Whereas in active melanotropes p-DCLK-short is present in both the cytoplasm and nucleus, in inactive melanotropes p-DCLK-short is present only in the cytoplasm. This observation indicates that cell activation is associated with transport of p-DCLK-short from the cytoplasm into nucleus and suggests that the phosphorylated kinase is involved in the regulation of gene expression.

Phosphorylation of Ser-30 is required for nuclear DCLK-short translocation

We examined whether the predicted Ser-30 ERK phosphorylation site of DCLK-short is involved in the protein's nuclear translocation. For this purpose, we expressed two GFP-DCLK-short constructs in active melanotropes, namely a WT-DCLK-short and an S/A-point mutant DCLK-short in which Ser-30 of the PTSP sequence had been replaced by an alanine residue. Because GFP fluorescence microscopy showed that WT-DCLK-short occurred in both the melanotrope cytoplasm and nucleus, whereas S/A-DCLK-short was present only in the cytoplasm, we conclude that phosphorylation of Ser-30 in the PTSP sequence is required for the nuclear translocation of DCLK-short. The results also imply that DCLK-short phosphorylation occurs in the cytoplasm, after which the phosphorylated kinase translocates to the nucleus. Because treatment of WT-DCLK-short melanotropes with the MEK inhibitor U0126 largely prevents the occurrence of p-DCLK in the nucleus, p-ERK-mediated phosphorylation of Ser-30 apparently is required for nuclear translocation of p-DCLK-short. Upon U0126 treatment, some nuclear GFP fluorescence was still visible in a minority (24%) of cells. This fluorescence may be, at least partly, due to incomplete inhibition of ERK-dependent phosphorylation, but it may also be due to the known capability of DCLK to autophosphorylate in an ERK-independent way (1, 30). Such autophosphorylated DCLK would then translocate into the nucleus. The fact that only 50% of the WT-DCLK-short cells displayed nuclear translocation may be related to the notion that amphibian pituitary melanotrope cells exhibit heterogeneity as to their secretory activity (16, 31, 32).

p-DCLK-short stimulates POMC transcription

Because ERK has been implicated in expression of POMC (33), and in melanotropes this expression is inhibited by the MEK inhibitor U0126 (34), we entertained the idea that p-DCLK-short is a downstream effector of the ERK pathway, for the purpose of regulating the expression of POMC. We thus investigated a possible role of p-DCLK-short in POMC expression. To relate the effect of transfection, visualized by GFP fluorescence, directly to cellular gene expression activity, we applied Q-RT-PCR to single melanotropes. In cells expressing the WT-DCLK-short construct POMC mRNA expression was markedly increased compared with controls, whereas in S/A-DCLK-short expressing cells an (nonsignificant) inhibitory trend in POMC expression was found. Overexpression of the S/A mutant in theory should have worked as a competitive inhibitor of endogenous DCLK-short. Possibly, the inclusion of GFP in the protein reduces its biological activity. Irrespective of this, the results of our experiments with the WT-DCLK construct indicate that phosphorylation of DCLK-short at the Ser-30 is necessary for the kinase to translocate to the nucleus.

Conclusions

Our studies show that p-DCLK-short acts as a downstream intranuclear effector of ERK signaling in *Xenopus* melanotrope cells and demonstrate that, in this role, the kinase is involved in the regulation of POMC expression. Although several proteins have been shown to be phosphorylated by DCLK-short (6, 30), our report is the first to show that nuclear localized p-DCLK-short is involved in the regulation of a functional secretory gene, *POMC*.

References

1. **Edelman AM, Kim WY, Higgins D, Goldstein EG, Oberdoerster M, Sigurdson W** 2005 Doublecortin kinase-2, a novel doublecortin-

related protein kinase associated with terminal segments of axons and dendrites. J Biol Chem 280:8531–8543

2. **Ohmae S, Takemoto-Kimura S, Okamura M, Adachi-Morishima A, Nonaka M, Fuse T, Kida S, Tanji M, Furuyashiki T, Arakawa Y, Narumiya S, Okuno H, Bito H** 2006 Molecular identification and characterization of a family of kinases with homology to Ca²⁺/calmodulin-dependent protein kinases I/IV. J Biol Chem 281:20427–20439

3. **Dijkmans TF, van Hooijdonk LW, Fitzsimons CP, Vreugdenhil E** 2010 The doublecortin gene family and disorders of neuronal structure. Cent Nerv Syst Agents Med Chem 10:32–46

4. **Shu T, Tseng HC, Sapir T, Stern P, Zhou Y, Sanada K, Fischer A, Coquelle FM, Reiner O, Tsai LH** 2006 Doublecortin-like kinase controls neurogenesis by regulating mitotic spindles and M phase progression. Neuron 49:25–39

5. **Vreugdenhil E, Kolk SM, Boekhoorn K, Fitzsimons CP, Schaaf M, Schouten T, Sarabdjitsingh A, Sibug R, Lucassen PJ** 2007 Doublecortin-like, a microtubule-associated protein expressed in radial glia, is crucial for neuronal precursor division and radial process stability. Eur J Neurosci 25:635–648

6. **Engels BM, Schouten TG, van Dullemen J, Gosens I, Vreugdenhil E** 2004 Functional differences between two DCLK splice variants. Brain Res Mol Brain Res 120:103–114

7. **Reiner O, Coquelle FM, Peter B, Levy T, Kaplan A, Sapir T, Orr I, Barkai N, Eichele G, Bergmann S** 2006 The evolving doublecortin (DCX) superfamily. BMC Genomics 7:188

8. **Shimomura S, Nagamine T, Nimura T, Sueyoshi N, Shigeri Y, Kameshita I** 2007 Expression, characterization, and gene knockdown of zebrafish doublecortin-like protein kinase. Arch Biochem Biophys 463:218–230

9. **Hevroni D, Rattner A, Bundman M, Lederfein D, Gabarah A, Mangelus M, Silverman MA, Kedar H, Naor C, Kornuc M, Hanoch T, Seger R, Theill LE, Nedivi E, Richter-Levin G, Citri Y** 1998 Hippocampal plasticity involves extensive gene induction and multiple cellular mechanisms. J Mol Neurosci 10:75–98

10. **Le Hellard S, Håvik B, Espeseth T, Breilid H, Løvlie R, Luciano M, Gow AJ, Harris SE, Starr JM, Wibrand K, Lundervold AJ, Porteous DJ, Bramham CR, Deary IJ, Reinvang I, Steen VM** 2009 Variants in doublecortin- and calmodulin kinase like 1, a gene up-regulated by BDNF, are associated with memory and general cognitive abilities. PLoS One 4:e7534

11. **Sturgill TW, Ray LB, Erikson E, Maller JL** 1988 Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature 334:715–718

12. **Bibel M, Barde YA** 2000 Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 14:2919–2937

13. **Silverman MA, Benard O, Jaaro H, Rattner A, Citri Y, Seger R** 1999 CPG16, a novel protein serine/threonine kinase downstream of cAMP-dependent protein kinase. J Biol Chem 274:2631–2636

14. **Dijkmans TF, van Hooijdonk LW, Schouten TG, Kamphorst JT, Vellinga AC, Meerman JH, Fitzsimons CP, de Kloet ER, Vreugdenhil E** 2008 Temporal and functional dynamics of the transcriptome during nerve growth factor-induced differentiation. J Neurochem 105:2388–2403

15. **Martens GJ, Weterings KA, van Zoest ID, Jenks BG** 1987 Physiologically-induced changes in proopiomelanocortin mRNA levels in the pituitary gland of the amphibian Xenopus laevis. Biochem Biophys Res Commun 143:678–684

16. **de Rijk EP, Jenks BG, Wendelaar Bonga SE** 1990 Morphology of the pars intermedia and the melanophore-stimulating cells in Xenopus laevis in relation to background adaptation. Gen Comp Endocrinol 79:74–82

17. **Jenks BG, Kidane AH, Scheenen WJ, Roubos EW** 2007 Plasticity in the melanotrope neuroendocrine interface of Xenopus laevis. Neuroendocrinology 85:177–185

18. **van den Hurk MJ, Cruijsen PM, Schoeber JP, Scheenen WJ, Roubos EW, Jenks BG** 2008 Intracellular signal transduction by the extracellular calcium-sensing receptor of Xenopus melanotrope cells. Gen Comp Endocrinol 157:156–164

19. **Kidane AH, Heinrich G, Dirks RP, de Ruyck BA, Lubsen NH, Roubos EW, Jenks BG** 2009 Differential neuroendocrine expression of multiple brain-derived neurotrophic factor transcripts. Endocrinology 150:1361–1368

20. **Klein SL, Strausberg RL, Wagner L, Pontius J, Clifton SW, Richardson P** 2002 Genetic and genomic tools for Xenopus research: The NIH Xenopus initiative. Dev Dyn 225:384–391

21. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG** 2007 Clustal W and Clustal X, version 2.0. Bioinformatics 23:2947–2948

22. **Zhang H, Langeslag M, Breukels V, Jenks BG, Roubos EW, Scheenen WJ** 2008 Calcium channel kinetics of melanotrope cells in Xenopus laevis depend on environmental stimulation. Gen Comp Endocrinol 156:104–112

23. **Boekhoorn K, Sarabdjitsingh A, Kommerie H, de Punder K, Schouten T, Lucassen PJ, Vreugdenhil E** 2008 Doublecortin (DCX) and doublecortin-like (DCL) are differentially expressed in the early but not late stages of murine neocortical development. J Comp Neurol 507:1639–1652

24. **Abramoff M, Magelhaes PJ, Ram RJ** 2004 Image processing with ImageJ. Biophotonics Int 11:36–42

25. **Shapiro SS, Wilk MB** 1965 An analysis of variance test for normality (complete samples). Biometrika 52:591–611

26. **Snedecor GW, Cochran WG, eds.** 1989 Statistical methods. Ames, IA: Iowa State University Press

27. **Burgess HA, Reiner O** 2001 Cleavage of doublecortin-like kinase by calpain releases an active kinase fragment from a microtubule anchorage domain. J Biol Chem 276:36397–36403

28. **Kramer BM, Cruijsen PM, Ouwens DT, Coolen MW, Martens GJ, Roubos EW, Jenks BG** 2002 Evidence that brain-derived neurotrophic factor acts as an autocrine factor on pituitary melanotrope cells of Xenopus laevis. Endocrinology 143:1337–1345

29. **Wang LC, Meijer HK, Humbel BM, Jenks BG, Roubos EW** 2004 Activity-dependent dynamics of coexisting brain-derived neurotrophic factor, pro-opiomelanocortin and α-melanophore-stimulating hormone in melanotrope cells of Xenopus laevis. J Neuroendocrinol 16:19–25

30. **Shang L, Kwon YG, Nandy S, Lawrence DS, Edelman AM** 2003 Catalytic and regulatory domains of doublecortin kinase-1. Biochemistry 42:2185–2194

31. **Gracia-Navarro F, González de Aguilar JL, Vázquez-Martínez RM, Tonon MC, Vaudry H, Malagón MM** 1998 Melanotrope cell heterogeneity in the pars intermedia of amphibians. Ann NY Acad Sci 839:223–228

32. **Vazquez-Martinez R, Peinado JR, Gonzalez De Aguilar JL, Desrues L, Tonon MC, Vaudry H, Gracia-Navarro F, Malagon MM** 2001 Melanotrope cell plasticity: a key mechanism for the physiological adaptation to background color changes. Endocrinology 142:3060–3067

33. **Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E** 2002 Activation and Induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 16:1638–1651

34. **Kuribara M, Kidane AH, Vos GA, de Gouw D, Roubos EW, Scheenen WJ, Jenks BG** 2011 Extracellular-signal regulated kinase regulates production of proopiomelanocortin in pituitary melanotroph cells. J Neuroendocrinol 23:261–268