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Physiological Control of Xunc18 Expression in Neuroendocrine Melanotrope Cells of *Xenopus laevis**

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ABSTRACT

In mammals, the brain-specific protein munc18–1 regulates synaptic vesicle exocytosis at the synaptic junction, in a step before vesicle fusion. We hypothesize that the rate of biosynthesis of munc18–1 messenger RNA (mRNA) and the amount of munc18–1 present in neurons and neuroendocrine cells are related to the physiologically controlled state of activity. To test this hypothesis, the homolog of munc18–1 in the clawed toad *Xenopus laevis*, xunc18, was studied in the brain and in the neuroendocrine melanotrope cells in the intermediate lobe of the pituitary gland, at both the mRNA and the protein level. In toads adapted to a black background, the melanotropes release the peptide α-melanophore-stimulating hormone (α-MSH), which induces darkening of the skin, whereas in animals adapted to a white background the cells hardly release but store α-MSH, making the animal's skin look pale. The intermediate pituitary lobe of black-adapted animals revealed a strong hybridization reaction with the xunc18 mRNA probe, whereas a much weaker hybridization was observed in the intermediate lobe of white-adapted animals (optical density black: 3.4 ± 0.2 *vs.* white: 0.8 ± 0.1; $P < 0.02$). Immunocytochemically, *Xenopus* munc18-like protein has been detected throughout the brain, in identified neuronal perikarya as well as in axon tracts. Western blot analysis and immunocytochemistry further demonstrated the presence of xunc18 in the neural, intermediate and distal lobe of the pituitary gland. Xunc18 protein was furthermore determined in immunoblots of homogenates of melanotropes dissociated from the pituitary gland. In melanotropes of toads adapted to a black background, the integrated optical density of the xunc18 immunosignal was 2.7 ± 0.5 times higher than in cells of white-adapted toads ($P < 0.0001$). It is concluded that, in *Xenopus* melanotrope cells, the amounts of both xunc18 mRNA and xunc18 protein are up-regulated in conjunction with the induction of exocytosis of α-MSH as a result of a physiological stimulation (environmental light condition). We propose that xunc18 is involved in physiologically controlled exocytotic secretion of neuroendocrine messengers. (*Endocrinology* **142:** 1950–1957, 2001)

THE MECHANISM of secretion by exocytosis is conserved throughout evolution, from yeast to mammalian neurons (1–3), but the regulatory aspects of this complicated secretory process are far from understood. Currently, the exocytosis of classical neurotransmitters from synaptic vesicles is being studied extensively, and many proteins have been identified that may play a role in the different steps of the synaptic vesicle exocytosis cycle (4, 5). Among these, the protein munc18, originally cloned from rat brain (6), is of particular interest. *In vitro*, munc18 interacts with the plasma membrane protein syntaxin I (6–8), the vesicle-associated protein DOC2 (9) and X11/MINT (10). It is assumed that munc18 acts upstream of the ultimate exocytotic fusion of the vesicle membrane with the plasma membrane (4, 8, 9). Experiments with null mutants of the brain-specific munc18–1 in mouse (12) and the munc18 homologs SEC1 in yeast (13), UNC-18 in *C. elegans* (14), rop in *Drosophila* (15, 16), support the idea that munc18–1 plays an essential role in neuronal secretion by exocytosis.

Up to now, studies on the dynamics of munc18 and munc18 isoforms during exocytosis were carried out in genetic (mutants; 11, 14–16) or biochemical (*in vitro*; 7, 8) approaches. Here, we study munc18–1 in a physiological neuroendocrine cell model, addressing the question if the degrees of expression of munc18–1 messenger RNA (mRNA) and of munc18–1 protein can be under physiological control. For this purpose, the neuroendocrine melanotrope cell in the pituitary gland of the South-African clawed toad *Xenopus laevis* has been chosen, as the secretory activity of this cell type can be physiologically manipulated *in vivo* by changing the background light intensity of the animal's environment. The melanotrope cells are situated in the intermediate pituitary lobe and produce the POMC-derived peptide α-melanophore-stimulating hormone (α-MSH), which is contained in secretory granules (17–19). When *Xenopus* is placed on a black background, α-MSH is released from the granules by exocytosis (20), whereas on a white background the melanotropes hardly secrete α-MSH (20, 21) because α-MSH secretion is synaptically inhibited by neurons from the suprachiasmatic nucleus (SCN), the suprachiasmatic melanotrope-inhibiting neurons (SMINs; 22–24). In the present study, we investigated by quantitative *in situ* hybridization the mRNA expression of the *Xenopus* munc18–1 isoform, xunc18 (25) in melanotrope cells in the pars intermedia of toads that had

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been adapted to either a black or a white background. In addition, the degree of expression of xunc18 protein was studied by quantitative immunoblotting. The results were compared with data on the presence of xunc18 mRNA in the brain and in other parts of the pituitary gland. Moreover, xunc18 expression was related to the expression of the α-MSH precursor POMC in both the intermediate and distal pituitary lobes.

It is concluded that the light intensity of the background specifically determines the degree of expression of both xunc18 mRNA and xunc18 protein in the melanotropes of *X. laevis*, and that xunc18 plays a role in the exocytosis of POMC-derived peptides from endocrine cells.

Materials and Methods

Animals

Young-adult (aged 6 months) specimens of *Xenopus laevis*, with a body weight of 28–32 g, were raised under standard laboratory conditions, and fed on beef heart and Trouvit trout pellets (Trouw, Putten, The Netherlands) weekly. They were kept under constant illumination at a water temperature of 22 ± 1 C, and on a black or a white background for 3 weeks, to reach full skin color adaptation. All experiments were carried out under the guidelines of the Dutch law concerning animal welfare.

In situ hybridization

Black- and white-adapted animals were anesthetized with 0.1% tricaine methane sulfonate (MS222, Sandoz Pharmaceuticals Corp., Basel, Switzerland) and transcardially perfused for 5 min with ice-cold 0.6% sodium chloride solution, to remove blood cells. Subsequently, they were perfused with 250 ml ice-cold Bouin's fixative. Dissected brains and pituitary glands were postfixed in the same fixative, for 16 h, dehydrated through a graded series of ethanol, and embedded in paraffin. Tissue sections (6 μm) were mounted on poly-L-lysine-coated slides and, after deparaffination, treated with 0.1% pepsin in 0.2 N HCl (15 min, 37 C), 2% paraformaldehyde (5 min), and 1% hydroxylammonium chloride (15 min). Hybridization was performed for 16 h at 50 C, in 4× SSC, 50% formamide, 1× Denhardt's, 10% dextran sulfate, 25 mM sodium phosphate (pH 7.4), and 0.2 mg/ml yeast transfer RNA, using a 724-bp xunc18 mRNA probe (25). After washing in 2 × SSC, 1 × SSC, 0.5 × SSC, and 0.1 × SSC at 20 C, and in 0.1 × SSC at 37 C, color reaction was performed with nitroblue tetrazolium X-phosphate according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Treatment with 0.1 M triethanolamine was carried out to prevent aspecific binding of the probe. Specificity of the hybridization signal was assessed in control experiments with sense RNA probe, and by treatment with RNase A (20 μg/ml).

To determine relative differences in the strength of hybridization signals of animals adapted to either a white or a black background, quantitative *in situ* hybridization was performed. For each animal (n = 3) three sections were studied, cut in a vertical, sagittal plane through the brain and the pituitary gland. Throughout the experiment, a random sampling procedure was maintained. Three areas of 12,500 μm^2 each were sampled: the pituitary intermediate and neural lobes, and the ventral hypothalamic nucleus (VH). Staining intensities were determined by densitometry of digitized images.

Cell isolation and immunoblotting

To show the presence of xunc18 protein in melanotropes, these cells were dissociated from the pituitary gland as previously described (26) with some modifications. Briefly, animals were perfused with 10 ml Ringer's solution (112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM HEPES, pH 7.4) to remove blood cells. Then, intermediate lobes were dissected and 10 lobes from black-adapted animals and 10 from white-adapted animals were pooled for each experiment. Lobes were incubated in Ringer's solution containing 0.25% trypsin (Life Technologies, Inc., Renfrewshore, UK) and cells were dissociated by gentle trituration of the lobes with a siliconized Pasteur's pipette in Leibowitz's L15 medium to which 10% FCS (Life Technologies, Inc.) had been added. The suspension was transferred to a syringe and filtered through a nylon gauze (pore size 150 μm) by air pressure, to remove undissociated tissue. The dispersed cells were washed with XL15, collected by centrifugation at 500 rpm, and resuspended in SDS sample buffer (62.5 mM Tris/HCl, 12.5% glycerol, 1.25% SDS, 0.0125% bromephenol blue and 2.5% β-mercaptoethanol). Following the same procedure, cells of 10 pituitary distal lobes from white- and 10 from black-adapted animals were obtained. Total brain fractions were made by homogenizing the brains from 3 white- and 3 black-adapted animals in sample buffer. Protein contents on the gel were the same for white- and for black-background-adapted animals, namely 0.13 μg per lane for total brain fractions, 62 ng per lane for melanotrope cells and 65 ng per lane for distal lobe cells. A rat brain lysate with known protein content (10 $\mu g/\mu l$; Transduction Laboratories, Inc., Lexington, KY) was used as a standard.

Equal amounts of protein were loaded on a 12.5% resolving SDS-polyacrylamide gel and transferred to nitrocellulose membranes (0.45 μm, Schleicher & Schuell, Inc., Dassel, Germany) in 192 mM glycine, 50 mM Tris and 20% methanol, using the mini-protean II cell system (Bio-Rad Laboratories, Inc., Hemel Hampstead, UK). Then, the nitrocellulose membranes were washed in TBS containing 0.2% Tween 20 (TBST) and incubated in block buffer (5% BSA in TBST) for 2 h. Xunc18 and POMC proteins were studied by incubating corresponding blot parts with a polyclonal munc18–1 antiserum raised in rabbit (type 6.12; 27) and the ST-62 POMC antiserum (both 1:1000) for 16 h, both in block buffer. The specificity of the xunc18 antiserum was tested by the use of preimmune serum and by preabsorbing it with 5 times excess of purified munc18 protein. The high specificity of the ST-62 POMC antiserum was previously described (21). After rinsing, immunodetection was carried out with 0.04% 3,3′ diaminobenzidine tetrahydrochloride as described above. The staining intensities of xunc18 and POMC bands were determined by densitometry of digitized images of paired lanes (5 blots of black- and 5 blots of white-adapted animals).

Immunocytochemistry

After anesthetization, perfusion and dissection (see above), brains and pituitary glands were postfixed in ice-cold Bouin's fixative for 2 h, and cryoprotected by immersion in 20% sucrose in sodium phosphate buffer (PBS, pH 7.4) for 16 h. Horizontal, transversal and sagittal 20 μm serial sections were cut in a cryostat, mounted on poly-L-lysine-coated slides, and allowed to air dry for 16 h at 37 C. Rinsing and incubation steps were carried out in 50 mM Tris-buffered saline (pH 7.6) containing 150 mM sodium chloride and 0.3% Triton X-100 (TBS-TX; Sigma, St. Louise, MO) at 20 C. Then, sections were rinsed in TBS-TX for 30 min and, to prevent aspecific binding, preincubated in block buffer containing 20% normal goat serum in TBS-TX, for 20 min. As primary antiserum, munc18–1 antiserum (1:1000; 27) was used, in block buffer for 16 h at 20 C. After several rinses in TBS-TX, sections were immunostained using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The reaction product was visualized with the peroxidase-antiperoxidase method using 0.04% 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.015% H_2O_2 in TBS-TX for 15 min. The reaction was terminated by several rinses in Tris-HCl. Finally, sections were dehydrated in a graded series of ethanol, cleared in xylene and mounted in Entellan (Merck & Co., Inc., Darmstadt, Germany). The neuroanatomical nomenclature was adopted from Neary and Northcutt (28).

Densitometry and statistics of Western blots and digitized images

DAB-peroxidase-stained xunc18-immunoblots of dissociated melanotropes of black- and white-adapted animals, as well as microscope images of brains and pituitary glands processed for either *in situ* hybridization or immunocytochemistry, were captured with a CCD camera, digitized with a VIDAS system (Kontron Instruments Ltd., München, Germany), converted to TIFF format, and analyzed with Image Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD). *In situ* staining intensities were expressed as means of OD ± SEM and tested with a one-way ANOVA (α = 5%), which was preceded by tests for the joint assessment of normality (29) and the homogeneity of variance (30). Immunoreactivity of the bands was expressed as means

of integrated OD (IOD) ± SEM and analyzed with Student's paired two-sample *t* test for means. This parameter was chosen assuming that the strength of the signal depends on both the intensity and the width of the stained band.

Results

Xunc18 mRNA

In situ hybridization with the 724-bp digoxigenin-labeled antisense xunc18 mRNA probe shows high expression of xunc18 mRNA throughout the *Xenopus* central nervous system (Fig. 1A). The following main neuronal centers were found to be well stained (from rostral to caudal): olfactory bulb, medial and lateral pallium, nucleus accumbens, septum, various thalamic and hypothalamic nuclei including the anterior and ventromedial thalamic nuclei, magnocellular nucleus, suprachiasmatic nucleus, ventral hypothalamic nucleus and posterior tubercle, optic tectum, tegmentum, cerebellum, locus coeruleus, raphe nucleus and hindbrain somatosensory nuclei. When *in situ* hybridizations of the brains of white- and black-adapted animals were compared, no obvious differences were seen either in the pattern or in the intensity of the signals. No labeling was obtained with the sense probe, whereas incubations with RNase A after hybridization did not have any effect on the pattern or the intensity of the staining.

In contrast to the brain, the pituitary intermediate lobe demonstrates a remarkable difference among the two adaptation states with respect to the strength of the expression of xunc18 mRNA. In white-adapted animals, the lobe reveals a weak hybridization signal, but in black-adapted animals the signal is strongly stained (Fig. 1, B and C). The endocrine cells of the distal lobe show a moderate to strong expression of xunc18 mRNA, which does not differ in intensity with respect to the two adaptation conditions. No clear hybridization signal was seen in the pituitary neural lobe, nor in sections incubated with the sense xunc18 mRNA probe or treated with RNase A.

To extend the qualitative observations described above with respect to the effect of background light intensity on the expression of xunc18 mRNA, OD measurements were made of the strength of the hybridization signal in the pituitary intermediate lobe and in two control areas, the VH and the pituitary neural lobe. Whereas the controls do not exhibit any

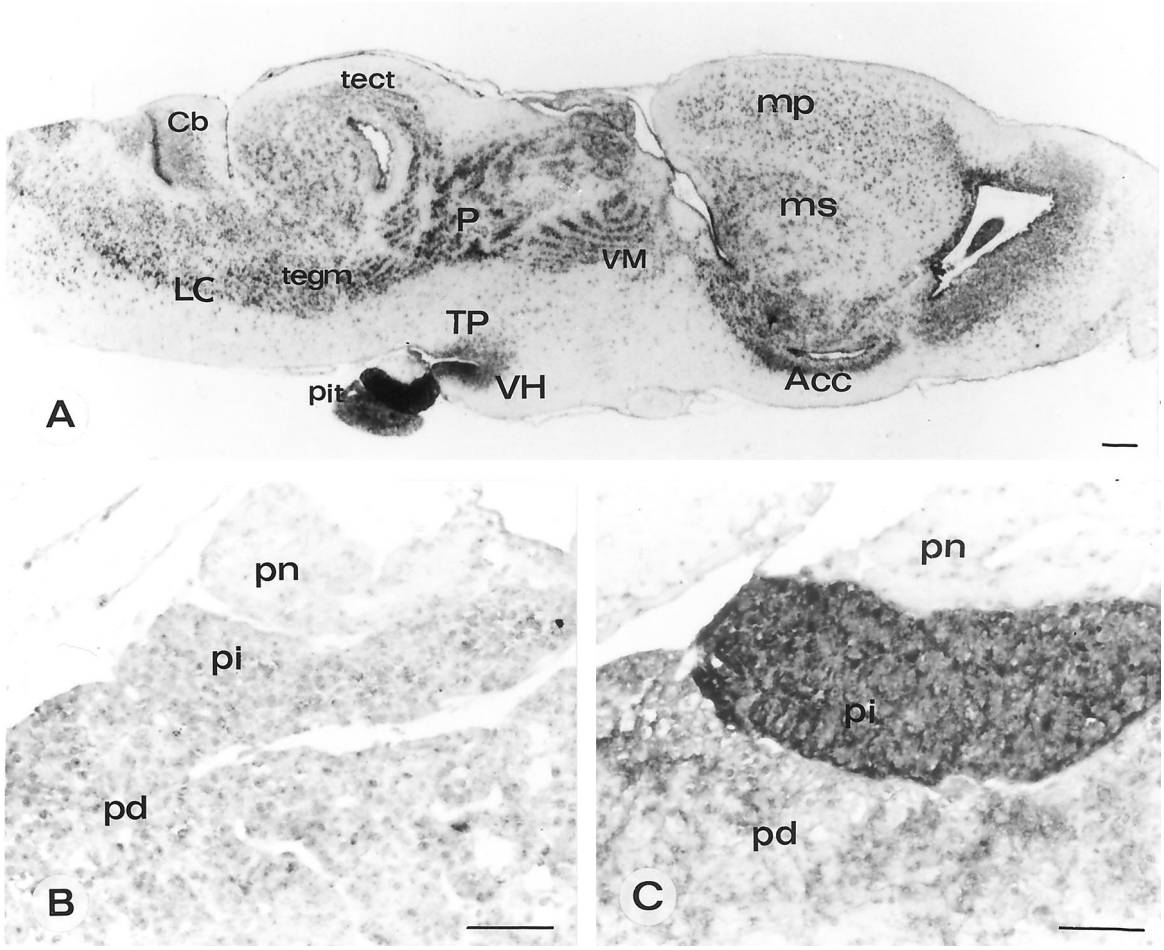

FIG. 1. *In situ* hybridization showing the expression of xunc18 in the *Xenopus* brain and pituitary gland. A, Overview of a sagittal section of brain and pituitary gland of a black-adapted toad. Acc, nc. accumbens; Cb, cerebellum; LC, locus coeruleus; mp, medial pallium; ms, medial septum; *P*, posterior thalamic nucleus; pit, pituitary; tect, tectum; tegm, tegmentum; TP, posterior tubercle; VH, ventral hypothalamic nucleus; VM, ventromedial thalamic nucleus. *Bar*, 150 μm. B and C, Sagittal sections of pituitary gland of white-adapted (B) and black-adapted (C) *Xenopus*. pd, pars distalis; pi, pars intermedia; pn, pars nervosa. *Bar*, 35 μm.

significant difference in OD of the hybridization signal between the two adaptation states, the OD in the pituitary intermediate lobe of black-adapted toads is 4.2 times higher than in white-adapted ones (OD black: 3.4 ± 0.2 *vs.* OD white: 0.8 ± 0.1; $P < 0.02$) (Fig. 2).

Xunc18 protein

Staining of Western blots of rat brain as well as of *Xenopus* brain and pituitary gland with the 6.12 antiserum revealed one band with a molecular mass of 67 kDa, which is the same as the molecular mass of rat munc18–1 (6) (Fig. 3). Both the rat band and the *Xenopus* band were absent when the antiserum had been preabsorbed with excess (10 μg/ml) purified munc18–1 protein (data not shown).

Using this antiserum, immunocytochemistry showed various brain centers to be xunc18-immunoreactive (Fig. 4, A and B), all of which revealed a xunc18 mRNA signal as well. Moreover, throughout the brain various immunoreactive fibers were seen (Fig. 4C), some of which have many varicosities that appear to be in close contact with neuronal cell bodies, suggesting the presence of axo-somatic synaptic contacts (Fig. 4D). In the neural lobe of the pituitary gland, the numerous axon terminals are strongly stained, whereas the endocrine cells in the distal lobe reveal various degrees of immunoreactivity (Fig. 4, E and F). As to the intermediate lobe, the melanotrope cells are clearly positive and are contacted by the immunoreactive varicosities of the intermediate lobe fiber network originating in the SCN (Fig. 4F).

To study the effect of the background light condition on the amount of xunc18 protein, the IOD of the xunc18 band in Western blots of the *Xenopus* brain and pituitary gland was examined in animals adapted to different background conditions. The IOD does not differ when homogenates of brains of white- *vs.* black-adapted animals are compared (Fig. 3A). To be able to study the xunc18 contents of melanotrope cells without interference by xunc18-containing axons and varicosities of the intermediate lobe fiber network, dispersed melanotrope cells were analyzed. Homogenates containing such single cells show the 67-kDa band (Fig. 3B; *top*) indicating the presence of xunc18. For dissociated cells from black-adapted animals this band is clearly more intense than

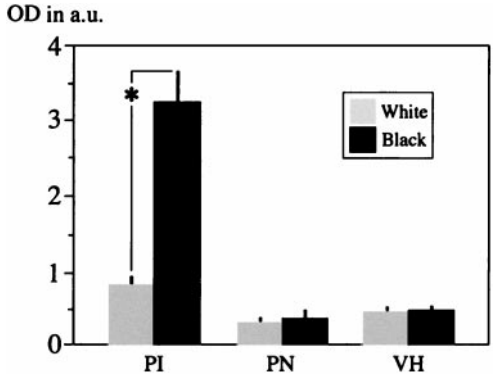

FIG. 2. Quantitative *in situ* hybridization data of xunc18 mRNA levels in white-adapted and black-adapted *X. laevis*. PI, pars intermedia; PN, pars nervosa; VH, ventral hypothalamic nucleus. Optical density (OD) expressed in arbitrary units (a.u.). *Vertical bars* represent SEM. *Asterisk* indicates statistically significant difference ($P < 0.02$; n = 3).

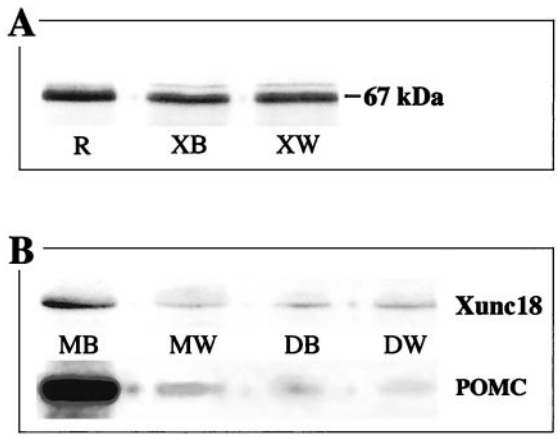

FIG. 3. A, Western blot of munc18–1 in rat brain (R) and its homolog xunc18 in brain of black-adapted (XB) and white-adapted (XW) *X. laevis*. The immunoreactive band corresponds to a molecular mass of 67 kDa. B, Xunc18- and POMC-immunoreactive bands in Western blot of homogenates of dissociated melanotrope cells (M) and dissociated distal pituitary endocrine cells (D), of black-adapted (B) and white-adapted (W) *X. laevis*.

for cells from white-adapted ones, as appears from densitometry (mean ratio IOD black/white ± SEM = 2.7 ± 0.5; n = 5; $P < 0.0001$; Fig. 5). This up-regulation of the xunc18 protein under black-adaptation condition is surpassed by the strong up-regulation of POMC (mean ratio IOD black/white ± SEM = 6.6 ± 1.3; n = 5; $P < 0.0001$; Fig. 3B *bottom*; Fig. 5). Blots of homogenates of endocrine cells dissociated from the distal lobe also reveal the 67-kDa band, but the IOD of this band does not show a significant difference when the two adaptation conditions are compared (Fig. 3B; *top*).

Discussion

The aim of this study was to investigate whether the rate of biosynthesis and storage of the homolog of the mammalian unc18–1 exocytosis-associated protein in the amphibian *Xenopus laevis*, xunc18, is related to the physiologically controlled state of activity of the neuroendocrine melanotrope cells in the intermediate pituitary lobe. More generally, this might shed light on the physiological regulation of such proteins in peptidergic neuroendocrine cells.

Melanotrope cells secrete by exocytosis of secretory granule contents

Exocytosis of secretory granule contents is the common and main mechanism of secretion by secretory cells in general and endocrine and neuroendocrine cells in particular (*e.g.* 21, 31–33). Activation of α-MSH secretion from the *Xenopus* melanotrope cell (by placing animals on a black background) results in a strong depletion of secretory granules from the cytoplasm, whereas inhibition of secretion (by placing animals on a white background) leads to a strong accumulation of secretory granules in the cytoplasm. This clearly proves the involvement of the secretory granule pathway in activated α-MSH secretion (34). This pathway was recently revealed in detail, by studying the vacuolar H^+-ATPase (V-ATPase), which is able to establish and maintain the secretory granule acidic microenvironment essential for proper transport, sorting, processing and release of

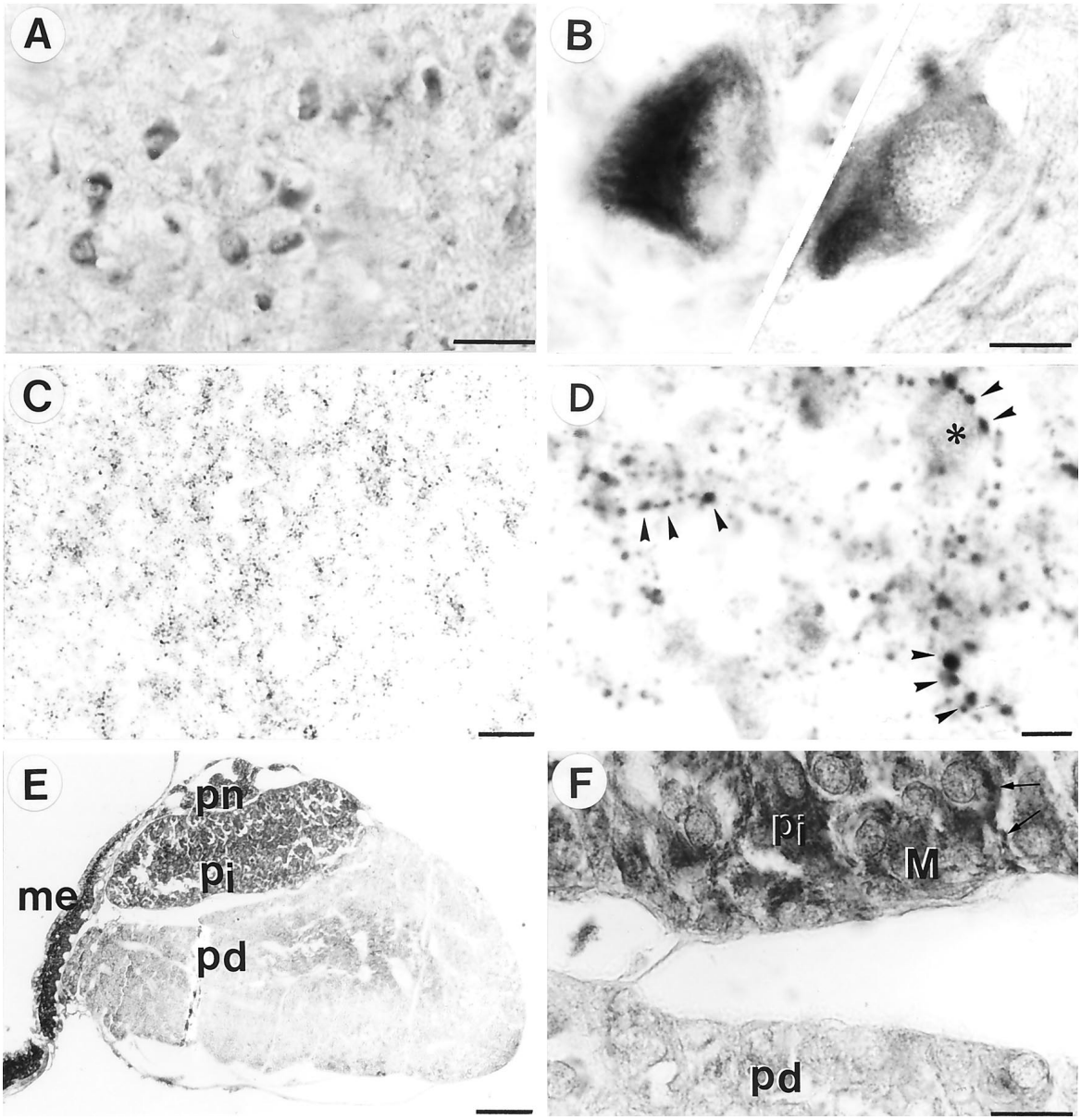

FIG. 4. Sagittal sections through the brain (A–D) and pituitary gland (E, F) of black-adapted *Xenopus laevis*, showing xunc18-immunoreactivity. A, Positive neuronal perikarya in somatosensory nuclei of the hindbrain. B, Details of immunoreactive perikarya in the hindbrain. C, High density of immunoreactive fibers in the thalamic area. D, Detail of immunoreactive fibers in thalamic area with varicosities (*arrowheads*) some of which seem to contact a neuronal cell body (*asterisk*). E, Pituitary gland with strongly immunoreactive median eminence (me) and pars nervosa (pn), moderately stained pars intermedia (pi) and heterogeneously immunoreactive pars distalis (pd). F, Detail of pars intermedia (pi) with immunoreactive melanotropes (M) and pars distalis (pd). *Arrows* indicate immunoreactive fiber of axonal network innervating melanotrope cells. *Bars* (E), 100 μm; (A), 50 μm; (C, F), 25 μm; (B, D), 10 μm.

Xenopus regulated secretory proteins. When melanotrope cells are treated with a specific V-ATPase inhibitor (bafilomycin A1), intracellular accumulation of POMC and its cleavage products (including α-MSH) occurs (35).

Immunoelectron microscopy has shown that α-MSH exclusively exists within the secretory granules, confirming that α-MSH secretion must occur by release of the granule contents (20, 21, 34). The activated *Xenopus* melanotrope cell secretes its secretory material by increased exocytosis from secretory granules, as has been demonstrated by 1) electron microscopy, which has shown that exocytosis figures occur only rarely or are even completely absent in melanotropes in white-adapted *Xenopus*, but are very numerous in melanotropes from black-adapted animals (36; and Roubos, E. W., unpublished data) and by 2) membrane capacitance measurements (Scheenen, W. J. J. M., unpublished data) that demonstrate that activation of secretion leads to discrete, stepwise increases in plasma membrane capacitance as a result of fusion of individual granule membranes during exocytosis.

This strong increase of exocytotic secretion in activated melanotrope cells makes these cells highly suitable objects to study our hypothesis that the expression of exocytosis proteins such as xunc18 is physiologically regulated.

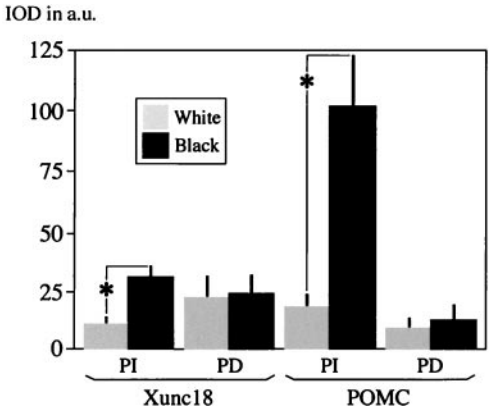

FIG. 5. Quantitative densitometry data of Western blots of xunc18 and POMC proteins in dissociated melanotrope cells from the pars intermedia (PI) and in endocrine cells dissociated from the pars distalis (PD), from white-adapted and black-adapted *X. laevis*. IOD expressed in arbitrary units (a.u.). *Vertical bars* represent SEM. *Asterisks* indicate statistically significant difference ($P < 0.0001$; n = 5).

Expression of xunc18 mRNA and xunc18 protein

In this study, we first strengthen our previous assumption (25) that xunc18 mRNA occurs throughout the brain and the pituitary intermediate and distal lobe of *Xenopus laevis*. Furthermore, we show that neurons and endocrine cells are immunoreactive to an antiserum raised against munc18–1 that recognizes a brain and pituitary protein with the calculated molecular mass of munc18–1 (67 kDa; 6). Therefore, we conclude that these positive neurons and endocrine cells synthesize and store xunc18 mRNA and xunc18 protein. The widespread presence of xunc18 mRNA and xunc18 protein in the intermediate and anterior pituitary gland of *X. laevis* indicates that xunc18 is not only involved in the secretion of classical (nonpeptidergic) neurotransmitters from a synapse but has an important function in the secretion of protein hormones from endocrine cells. Moreover, the occurrence of xunc18 protein in the neural lobe of the pituitary suggests that xunc18 also plays a role in the secretion of peptidergic neurohormones into the circulation.

Physiological regulation of xunc18 mRNA and xunc18 protein

The endocrine melanotrope cells of black-adapted *X. laevis* are actively secreting cells, whereas melanotropes of white-adapted animals hardly secrete α-MSH but store the peptide by accumulating secretory granules in the cell interior (*e.g.* 19, 34). The degree of expression of xunc18 mRNA in the endocrine melanotrope cells of *X. laevis* is also related to the state of adaptation of the animal to the environmental light condition: whereas melanotropes of white-adapted toads show a low expression of xunc18 mRNA, melanotrope cells of black-adapted animals exhibit a clearly higher xunc18 mRNA expression, as appears from the qualitative and quantitative *in situ* hybridization study. Similarly, the expression of xunc18 protein is higher in melanotropes from black-adapted toads than in cells from white-adapted ones, as demonstrated by quantitative immunoblotting of homogenates of dissociated melanotropes. These results permit the conclusion that the physiological stimulus of background light intensity controls both the cellular levels of xunc18 mRNA and xunc18 protein. Although the distal pituitary lobe also expresses xunc18 mRNA, this expression is not influenced by background light intensity, indicating that the physiological regulation of xunc18 biosynthesis and storage by environmental light conditions specifically concerns the intermediate lobe of the pituitary gland.

Possible role of xunc18 in the secretory pathway

Apparently, the action of munc18–1 is not restricted to the release of classical neurotransmitters at a specialized site of the presynaptic membrane of the neuron, as munc18–1 has also been demonstrated in endocrine cells (37, 38). However, up to now, the role of munc18–1 in endocrine cells has not been defined. Here we present evidence that the biosynthesis as well as the storage of the *Xenopus* homolog of munc18–1, xunc18, depends on the state of secretory activity of the endocrine melanotrope cell. In this secretory process, POMC undergoes various steps to yield peptide end products, including cleavage, sorting, packaging, posttranslational modifications, and exocytosis. Indications for the role of xunc18 in this complicated process may be obtained from comparing the degree of its expression under different adaptation conditions with that of POMC. Melanotrope cells are highly specialized in the biosynthesis of POMC, as about 75% of all mRNA in active melanotropes encodes for this precursor protein (37) and the expression of POMC mRNA is stimulated about 30-fold upon transferring animals from a white to a black background (17, 39, 40). This strong stimulation is explained by the high demand for the POMC end product α-MSH under black-background condition. Furthermore, the 30-fold increase in POMC mRNA is similar to the increase in mRNAs encoding for other proteins involved in the biosynthesis and processing of POMC in *X. laevis* melanotropes, such as the prohormone convertase PC2, its molecular chaperone 7B2, the secretogranins II and III (SGII and SGIII) and carboxypeptidase E, which are increased upon black-background adaptation up to 35-fold (41, 42). As we show here, xunc18 mRNA is also expressed at a clearly higher degree under black- than under white-adaptation condition, but this increase is less pronounced, being only 4-fold. Moreover, also at the protein level xunc18 shows expression dynamics different from POMC, as it is about 2 times less strongly enhanced (2.7-fold) by black-background adaptation than the POMC protein (about 6-fold; 40; present study). Therefore, we propose that xunc18 does not have a function in the biosynthesis or processing of POMC but rather in a step more downstream in the secretory process. However, it also differs from proteins that are released from secretory granules (*e.g.* α-MSH, PC2, 7B2 and S6II), as these proteins show similar amounts in melanotrope cells of black- and white-adapted animals, which is due to the fact that they are released under black-adaptation condition and accumulate during white adaptation (40–42; Kuiper and Martens, personal communication). Therefore, we assume that xunc18 in *Xenopus* melanotrope cells is not secreted via secretory granules, but rather is a component of the exocytotic machinery controlling a late step of the secretory process, just preceding secretory granule exocytosis. In this respect, xunc18 may collaborate with

DOC2 and SNAP-25 with which it is coexisting in *X. laevis* in both the brain and the three lobes of the pituitary gland (25, 43).

Xunc18 and peptide secretion mechanisms

In neurons, munc18–1 is thought to be involved in the exocytosis of classical neurotransmitters from synaptic vesicles, in a step upstream of vesicle fusion with the presynaptic membrane (6, 8, 12). On the basis of the present study and because of the apparent molecular homology of *Xenopus* xunc18 with mammalian munc18–1 (25), we propose that this role also holds for munc18 in mammalian (neuro)endocrine cells. The up-regulation of xunc18 in melanotrope cells of black-adapted animals likely reflects an activation of the proteinergic machinery permitting increased exocytotic peptide hormone release. Although at the ultrastructural level, specialized release sites such as those present in neuronal synapses (active zones) have not been identified in endocrine cells including melanotropes, it is known that peptide-containing granules release their contents at docking sites (44) likely representing endocrine release sites (hotspot). Such spots might be the sites of action of xunc18 and munc18–1. Besides munc18–1, various other exocytosis proteins possibly involved in exocytosis, have been found in endocrine cells (38, 43–48). This fact supports the idea that the protein aspect of the mechanism controlling the exocytotic release of classical neurotransmitters has much in common with that controlling the release of (endocrine) peptides.

Furthermore, the background light condition also controls the level of the exocytosis protein SNAP-25 in both the neuronal network contacting the melanotropes as well as the neuroendocrine melanotropes themselves (43). This indicates that different exocytosis proteins are physiologically regulated in a co-ordinated way, revealing a picture of a multicomponent exocytosis protein machinery that acts and is regulated as one plastic entity. Still, the regulation of the expression of these proteins (and of their isoforms) might be accomplished via different pathways, as SNAP-25 is a (plasma) membrane-bound protein whereas munc18 resides in the cytoplasm (43).

On the basis of the present data on the physiologically induced expressions of xunc18 mRNA and xunc18 protein in *Xenopus* melanotropes, we propose that in general the expressions of exocytosis proteins are under physiological control, enabling neuronal and endocrine cells to tune their exocytosis activity more effectively to changes in the demand for neurotransmitter, neurohormone and hormone release.

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