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DIFFERENTIAL DISTRIBUTION AND REGULATION OF EXPRESSION OF SYNAPTOSOMAL-ASSOCIATED PROTEIN OF 25 kDa ISOFORMS IN THE *XENOPUS* PITUITARY GLAND AND BRAIN

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Abstract—Synaptosomal-associated protein of 25 kDa (SNAP-25) regulates various membrane fusion processes including exocytosis by endocrine and neural cells. To increase our understanding of the occurrence and regulation of SNAP-25 isoforms, we identified and characterized SNAP-25a and SNAP-25b mRNAs in the pituitary gland and brain of the amphibian *Xenopus laevis*. The proteins are strongly conserved and are resistant to botulinum neurotoxin A but not to botulinum neurotoxin E, as shown by Western blotting. The spatial distribution of the two SNAP-25 isoforms was assessed with *in situ* hybridization. Both SNAP-25a mRNA and SNAP-25b mRNA reside in cells in the pituitary distal lobe and, particularly, in the endocrine melanotrope cells in the pituitary intermediate lobe. The melanotrope cells are involved in the background adaptation process of the skin by releasing α-melanophore-stimulating hormone. Quantitation of the respective *in situ* hybridization signals in the *Xenopus* pars intermedia indicated a differential response, SNAP-25b mRNA being more highly expressed in black-adapted animals than SNAP-25a mRNA, and more than in white-adapted toads. This differential upregulation was also studied by real-time reverse transcriptase polymerase chain reaction, showing that in the intermediate pituitary lobe, both isoforms are physiologically controlled by the background light intensity stimulus, but with different intensities; in black-adapted animals SNAP-25b mRNA is upregulated by 3.33 times compared with white-adapted animals, but SNAP-25a only by 1.96 times. As to neural tissue, *in situ* hybridization showed that both isoforms coexist throughout the brain, sometimes with similar strengths, but in various areas either SNAP-25a mRNA or SNAP-25b mRNA expression is prevalent.

It is speculated that each of the SNAP-25 isoforms in the *Xenopus* pituitary and brain has a distinct function in cellular fusion processes including secretion, and that their occurrence and regulation depend on the type of secreted neurotransmitter/hormone and/or the activity state of the cell.

Key words: docking/fusion complex, exocytosis proteins, neurotransmitter release, real-time RT-PCR, *in situ* hybridization.

A set of highly conserved proteins plays a role during formation, transport and exocytosis of synaptic vesicles and secretory granules (e.g. Bennett and Scheller, 1993; Martin, 1994; Südhof, 1995; Jahn et al., 2003). One of the key players in these processes is the synaptosomal-associated protein of 25 kDa, SNAP-25, first identified as a neuron-specific mRNA in the murine brain (Branks and Wilson, 1986; Oyler et al., 1989). In the pituitary gland and the brain, SNAP-25 is ubiquitously expressed by endocrine cells and neurons, albeit at varying levels (Oyler et al., 1989; Jacobsson et al., 1994; Roth and Burgoyne, 1994; Sadoul et al., 1995; Jacobsson and Meister, 1996; Kolk et al., 2000). Together with syntaxin I and synaptobrevin, it forms the core complex, also called ternary complex or SNAP-receptor (SNARE) complex, which is essential for membrane fusion (e.g. Bennett and Scheller, 1993; Söllner et al., 1993; Südhof, 1995). The fact that the core complex proteins appear to have been highly conserved throughout evolution and are a target for clostridial neurotoxins emphasizes their functional importance (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994). SNAP-25 can be specifically cleaved by botulinum neurotoxins A and E (BoNT/A and BoNT/E), which inhibit hormone release (Ahnert-Hilger and Weller, 1993; Sadoul et al., 1995; Höhne-Zell and Gratzl, 1996) and neurotransmission (Binz et al., 1994; Xu et al., 1998; Keller and Neale, 2002). Furthermore, SNAP-25 is implicated in vesicle fusion events during e.g. neurite extension and axon elongation (Catsicas et al., 1991; Osen-Sand et al., 1993, 1996; Shirasu et al., 2000). Experiments with heterozygous coloboma mutant mice indicate that SNAP-25 is essential for the control of locomotor activities and for learning abilities (Hess et al., 1992, 1994, 1996; Raber et al., 1997),

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Abbreviations: BoNT, botulinum neurotoxin; Ct, cycle threshold; DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; α-MSH, α-melanophore-stimulating hormone; OD, optical density; RT-PCR, reverse transcriptase polymerase chain reaction; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble NSF attachment protein receptor.

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while the majority of the early thalamocortical connections develop normally in null mutants (Molnár et al., 2002).

The expression of multiple isoforms of SNARE proteins may underlie the specificity of regulated membrane fusion events (Linial, 1997). SNAP-25 isoforms may originate from gene and/or genome duplication, as shown for bony fish (Risinger and Larhammar, 1993; Risinger et al., 1998), or from alternative splicing, as was found in mammals and chicken (Oyler et al., 1989; Catsicas et al., 1991; Bark, 1993; Bark and Wilson, 1994a). In these latter cases, alternative splicing of exon 5 of a single gene generates two SNAP-25 isoforms, SNAP-25a and SNAP-25b, which mainly differ in the cysteine-rich area that undergoes post-translational palmitoylation (Bark, 1993; Bark and Wilson, 1994b). Differences in the fatty acid acylation sites imply that these isoforms are targeted to different membranes (Hess et al., 1992; Veit, 2000).

In the rat, two SNAP-25 variants appear to be differentially expressed through development. *In situ* hybridization shows that SNAP-25b is most abundantly expressed in the adult brain, whereas SNAP-25a predominates during embryonic and early postnatal life (Bark et al., 1995; Boschert et al., 1996). However, in adrenal and pituitary cells and in some areas of the adult brain, SNAP-25a is the prevalent isoform (Jacobsson et al., 1994; Bark et al., 1995; Boschert et al., 1996; Jacobsson and Meister, 1996). The subcellular locations and trafficking of the two SNAP-25 isoforms differ considerably in cranial nerve nuclei and when co-expressed within the same neuroblastoma cell (Jacobsson et al., 1999; Andersson et al., 2000). Interestingly, the isoform mRNA levels appear to be affected by nerve injury, induction of long-term potentiation, neurotrophic factors and neurotoxic (kainate) agents (Boschert et al., 1996; Jacobsson and Meister, 1996; Roberts et al., 1998; Marti et al., 1999; Hepp et al., 2000). These findings raise the idea that SNAP-25a and SNAP-25b mRNAs are differentially regulated in their expression.

Recently, we showed that in the pituitary gland of the amphibian *Xenopus laevis*, fusion-related proteins including SNAP-25, munc-18 and DOC2 are under regulatory control of a physiological stimulus, namely, a change in the background light condition that controls α-melanophore-stimulating hormone (α-MSH) secretion from melanotrope cells in the intermediate pituitary lobe (e.g. Roubos, 1997; Kolk et al., 2000, 2001; Kramer et al., 2001; Roubos et al., 2002; Jenks et al., 2003). In these studies on *Xenopus* no distinction was made between potential SNAP-25 isoforms, as they concerned the total SNAP-25 pool. To add to the understanding of the physiological regulation of SNARE protein isoforms, we here describe the identification and characterization of *Xenopus* SNAP-25a and SNAP-25b, their distribution in the pituitary gland, and the regulation of their expression in the neuroendocrine melanotrope cells during the physiological process of skin adaptation to background light intensity. Furthermore, attention has been paid to their expressions in the brain. The occurrence and expression of SNAP-25a and SNAP-25b mRNAs were studied using *in situ* hybridization. When *X. laevis* is placed on a black background, the neuroendocrine melanotrope cells secrete α-MSH, which disperses pigment in dermal melanophores leading to a black appearance of the animal (Jenks et al., 1993; Roubos, 1997). On a white background, α-MSH secretion is synaptically inhibited by neurons in the suprachiasmatic nucleus (Ubink et al., 1998). To support our hypothesis that *Xenopus* SNAP-25 isoforms are physiologically regulated independently from each other, we compared the expression of each SNAP-25 mRNA isoform in melanotropes of black-adapted animals with the mRNA expression in white-adapted ones, using quantitative *in situ* hybridization and real-time reverse transcriptase polymerase chain reaction (RT-PCR).

EXPERIMENTAL PROCEDURES

Animals

Adult *X. laevis*, aged 6 months, were obtained from our laboratory stock. The animals were fed trout pellets (Trouvit; Trouw, Putten, The Netherlands) in combination with ground beef heart, and kept under constant illumination and temperature (21±1 °C). Before the experiments, the animals had been adapted to either a black or a white background for 3 weeks. Before decapitation, toads were anesthetized with 0.1% tricaine methane sulfonate (MS222; Novartis, Basel, Switzerland). The minimum number of animals required to produce reliable data was used, and all efforts were made to minimize suffering. All experiments were carried out under the guidelines of the European Communities Council Directive 86/609/EEC and the Dutch law concerning animal welfare.

Incubation with BoNTs, and Western blotting

Toxins were activated by preincubation with 20 mM dithiothreitol (DTT), for 30 min at 37 °C. *Xenopus* brain homogenates (10 μg) were incubated in a volume of 20 μl, with 40, 100 or 200 nM of DTT-reduced BoNT/A or BoNT/E holotoxin, for 1 h at 37 °C. Homogenate preparation and Western blotting were performed as described previously (Kolk et al., 2000), with minor modifications. In brief, samples were boiled in SDS-sample buffer (62.5 mM Tris–HCl, 12.5% glycerol, 1.25% SDS, 0.0125% Bromophenol Blue and 2.5% β-mercaptoethanol) for 5 min, to inactivate the toxin, and centrifuged for 5 min. The total concentration of proteins in a homogenate was determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Equal amounts (10 μg/lane) of total protein were loaded on a 12.5% resolving SDS–polyacrylamide gel, and blotted onto polyvinyl difluoride membranes (Hybond-P; Amersham Pharmacia Biotech, Buckinghamshire, UK), using the Bio-Rad mini-protean II cell system (Bio-Rad, Hemel Hampstead, UK). Membranes were blocked using 2.5% non-fat dry milk and 2.5% NGS in Tris-buffered saline containing 0.2% Tween 20 (Sigma, St. Louis, MO, USA). Immunodetection was carried out using affinity-purified polyclonal anti-SNAP-25 raised against the whole coding region of rat SNAP-25 (Alomone Laboratories, Jerusalem, Israel; diluted 1:100), for 16 h at 4 °C in blocking buffer. The specificity of the antiserum was previously confirmed (Kolk et al., 2000). SNAP-25 was visualized with the enhanced chemifluorescence and/or peroxidase–antiperoxidase staining method, according to the manufacturer's instructions (Roche, Mannheim, Germany). Scanned images of blots were digitally analyzed using a GS 700 densitometer (Bio-Rad) and Molecular Analyst software (Bio-Rad). Parallel incubation of rat brain homogenate with BoNT/A shows that the rat isoform is cleaved (not shown), which demonstrates that the toxin used in our experiments was active.

Cloning and sequencing

A *X. laevis* cDNA library was screened with a rat SNAP-25a fragment as a probe. The recombinant library was prepared from *Xenopus* intermediate lobe tissue (Kuiper et al., 2000). Positive clones were isolated by three consecutive rounds of plating, and genomic DNA was analyzed by dye terminator cycle sequencing on a CEQ2000 automated DNA analyser (Beckman Instruments, Fullerton, CA, USA). To distinguish between RNA levels of SNAP-25a and SNAP-25b, a 1009 bp *Hin*dIII fragment from clone X7 (encoding SNAP-25a) and a 961 bp *Xba*I fragment from clone X19 (encoding SNAP-25b) were subcloned into pBluescript II SK^+ at the corresponding sites. The resulting plasmids were designated pBlue-SNAP-25a and pBlue-SNAP-25b.

RNA extraction and cDNA synthesis

Freshly dissected neurointermediate lobes were individually collected in 500 μl ice-cold Trizol (Life Technologies, Paisley, UK) and homogenized by sonification. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 30 μl RNase-free H_2O. Total RNA was measured with an Eppendorf Biophotometer (Vaudaux-Eppendorf AG, Basel, Switzerland). First strand cDNA synthesis was performed using 1 μg RNA dissolved in 11 μl RNase-free H_2O containing 0.25 mU pd(N)6 (random primers; Roche) at 70 °C for 10 min, followed by double strand synthesis in 1× strand buffer (Life Technologies) with 10 mM DTT, 20 U Rnasin (Promega, Madison, WI, USA), 0.5 mM dNTPs (Roche) and 100 U reverse transcriptase (Superscript II; Life Technologies) at 37 °C for 75 min and at 95 °C for 10 min.

PCR

PCR was performed in a total volume of 25 μl in a buffer solution containing 5 μl of template cDNA, 3 mM $MgCl_2$, 0.625 U FastStart *Taq*DNA polymerase, 0.25 mM dNTPs (Roche) and 0.6 mM of each primer. The following primers for SNAP-25a and SNAP-25b were designed based on the *Xenopus* sequence (see Results). SNAP-25a, forward primer: 5′-AGGCATGAACCATATCAACCAA-3′, SNAP-25b, forward primer: 5′-GGGAATGGAACAAATCAATAAG-3′. The common reverse primer, fitting both transcripts with 100% identity, was 5′-TCAATCTCATTGCCCATATC-3′. The optimum temperature cycling protocol was determined to be 95 °C for 10 min followed by 40 reaction cycles at 95 °C for 30 sec, at 56 °C for 30 s and at 72 °C for 2 min, using a programmable thermocycler (Eppendorf; Mastercycler Gradient, Hamburg, Germany). After PCR, the reaction products were run on a 2% agarose gel and visualized with ethidium bromide to check the lengths of the amplified DNAs.

Real-time RT-PCR

Real-time RT-PCR was performed in a total volume of 25 μl buffer solution containing 5 μl of template cDNA, 1× SYBR Green buffer (Applied Biosystems, Foster City, CA, USA), 3 mM $MgCl_2$, 0.625 U AmpliTaq Gold (Applied Biosystems), 0.25 mM dNTPs (Applied Biosystems) and 0.6 μM of each primer. For SNAP-25a and SNAP-25b the same primer sets as for RT-PCR were used. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using Vector NTI Suite (InforMax, Bethesda, MA, USA) and PrimerExpress software (Applied Biosystems) based on the *Xenopus* cDNA sequence (acc. number U41753) for GAPDH. The following primer pair was used: 5′-GCCGTGTATGTGGTGGAATCT-3′ and 5′-AAGTTGTCGTTGATGACCTTTGC-3′ (product size: 230 bp). The optimum temperature cycling protocol was determined to be 95 °C for 10 min followed by 40 reaction cycles at 95 °C for 15 s and at 60 °C for 1 min, using a 5700 GeneAmp PCR system (Applied Biosystems). For each reaction, the cycle threshold (Ct) was determined, i.e. the number of cycles needed to detect fluorescence above an arbitrary threshold (0.8). At this threshold Ct-values are within the exponential phase of the amplification. To estimate the relative amounts of SNAP-25a and SNAP-25b mRNAs in neurointermediate lobes of white versus black-adapted animals, the Ct-values for SNAP-25a and SNAP-25b were normalized to Ct values (dCt), by subtracting them from the Ct values for GAPDH.

In situ hybridization

RNA probe synthesis. Sense and antisense RNA probes were generated using the T3/T7 RNA transcription system (Roche) according to the manufacturer's specifications, with recombinant pBlue-SNAP-25a and pBlue-SNAP-25b vectors as templates. In brief, 1 μg of linearized transcription vector containing either the SNAP-25a or the SNAP-25b insert was transcribed in the presence of digoxigenin-RNA labeling mix, followed by precipitation in the presence of 10 μg tRNA and 4 M LiCl, thereby producing a fragment of approximately 1000 bp in length. RNA was collected by ethanol precipitation followed by centrifugation (10,000×*g*; 30 min) at 4 °C, the pellet was resuspended in 40 ml of TE buffer (10 mM Tris–HCl/0.1 mM EDTA; pH 8.0), and stored until use at −80 °C.

Tissue preparation and in situ hybridization. After anaesthetization, toads were transcardially perfused with ice-cold 0.6% sodium chloride solution, for 5 min, to eliminate blood cells, and subsequently fixed with 250 ml ice-cold Bouin's fluid. Dissected brains with pituitary glands attached, were postfixed in the same solution, for 16 h at 4 °C, dehydrated in a graded ethanol series, and embedded in paraffin. Sections (7 μm) were mounted on poly-L-lysine-coated slides, and air-dried for 16 h at 37 °C. After deparaffination, sections were sequentially treated with 0.1% pepsin in 0.2 N HCl for 15 min at 37 °C, 2% paraformaldehyde for 5 min and 1% hydroxyl ammonium chloride for 15 min, at room temperature. For *in situ* hybridization, paraffin sections were air-dried and covered with 100 μl of hybridization solution (4× SSC, 50% formamide, 1× Denhardt's, 10% dextran sulfate, 25 mM sodium phosphate and 0.2 mg/ml yeast tRNA; pH 7.4) containing digoxigenin-labeled antisense RNA. Hybridization was performed for 16 h at 55 °C, in a humidified chamber. After washing in 2× SSC, 1× SSC, 0.5× SSC and 0.1× SSC at room temperature and in 0.1× SSC at 37 °C, sections were pre-incubated in blocking buffer (2% normal goat serum and 1% bovine serum albumin in Tris-buffered saline; pH 7.6) for 1 h and then incubated in alkaline phosphatase-conjugated anti-digoxigenin (1:500 in blocking buffer; Roche). Hybridization was visualized using nitro-blue tetrazolium (Roche) and X-phosphate (4-toluidine salt; Roche) as substrates. Sections were mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) and examined under a Zeiss photomicroscope. All solutions were prepared with 1% diethyl pyrocarbonate-treated water (Sigma). SNAP-25a and SNAP-25b expressions were studied in sagittal and transversal, consecutive sections. The nomenclature of the brain areas is according to Northcutt and Kicliter (1980), Neary and Northcutt (1983), Nikundiwe and Nieuwenhuys (1983) and Tuinhof et al. (1993).

In control experiments, sense RNA was transcribed and hybridized to tissue sections as described for the antisense probe. Alternatively, sections were treated with RNase A (20 μg/ml in Tris-buffered saline; pH 7.5) for 30 min at 37 °C prior to *in situ* hybridization. A 30-fold excess of unlabelled antisense probe specific for SNAP-25a was used prior to hybridization with digoxigenin-labeled SNAP-25a. The same procedure was carried out for SNAP-25b. In none of these cases a hybridization signal was found.

Image analysis and statistical analysis

Digital images were obtained with a CoolSNAP color CCD-camera (Roper Scientific, Tucson, AZ, USA) and analyzed with Image Pro

Plus version 3.0 software (Media Cybernetics, Silver Spring, MD, USA). To quantify the *in situ* signals of each SNAP-25 mRNA isoform, the pituitary pars intermedia of four white- and of four black-adapted animals was studied. Per animal, staining intensity was determined in three sections, each with a sampling area of 5000 μm^2. A random sampling procedure was maintained throughout the study. In the blot experiment, intensities of immunoreactive bands were digitized and measured as described for *in situ* hybridization.

Intensities of *in situ* hybridizations and immunoblots were expressed as means of optical density (OD)±S.E.M. Means were tested with two-way analysis of variance, as well as with one-way analysis of variance (Bliss, 1967) preceded by tests for the joint assessment of normality (Shapiro and Wilk, 1965) and for the homogeneity of variance (Bartlett's test; Bliss, 1967) and followed by post hoc comparison of means using Duncan's multiple range test (Steel and Torrie, 1960; $\alpha = 5\%$).

RESULTS

Xenopus SNAP-25 isoforms are highly homologous to SNAP-25 proteins of other species

cDNAs encoding SNAP-25 isoforms, we screened a cDNA library prepared from *X. laevis* intermediate pituitary lobes. DNA sequencing revealed open reading frames encoding

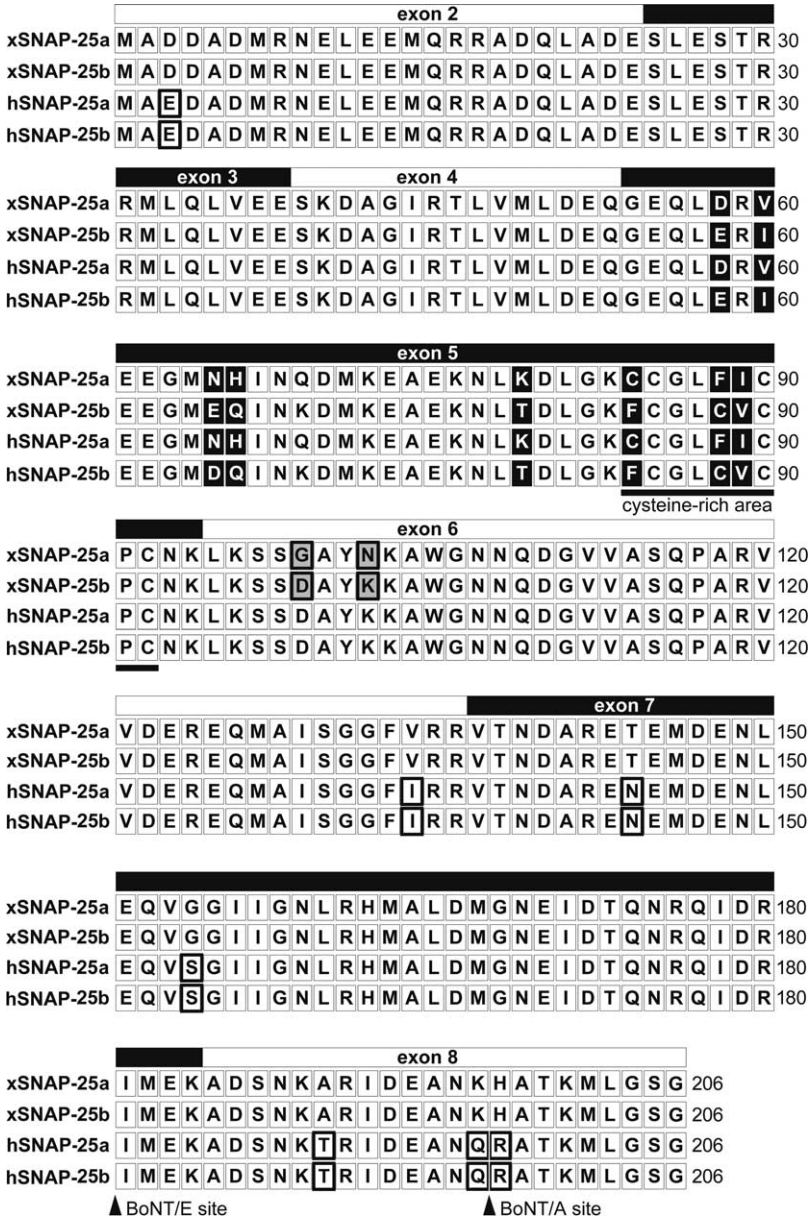

Fig. 1. Alignment of the predicted amino acid sequences of *Xenopus* SNAP-25a and SNAP-25b, with SNAP-25a and SNAP-25b proteins of the human (Bark and Wilson, 1994a). The exon organization of the human gene is indicated above the alignments. The BoNT/E cleavage site (180–181) and the BoNT/A cleavage site (197–198) are marked by arrowheads, and the cysteine-rich area in exon 5 involved in palmitoylation is underlined. Black boxes indicate differences in exon 5 between a- and b-proteins, for *Xenopus* and for human, gray boxes indicate the differences between *Xenopus* a- and b-proteins in exon 6, and white boxes show differences between human and *Xenopus* for both a- and b-proteins.

	Drosophila	*Torpedo*	Goldfish	*Xenopus*	Chicken	Mouse	Human
Drosophila		60%	56%	57%	61%	63%	60%
Torpedo	60%		78-81%	81%	81%	80%	74%
Goldfish	56%	78-81%		86%	91-94%	89%	84%
Xenopus	57%	81%	86%		97%	95%	95%
Chicken	61%	81%	91-94%	97%		100%	97%
Mouse	63%	80%	89%	95%	100%		91%
Human	60%	74%	84%	95%	91%	97%	

Fig. 2. Amino acid identities of *Xenopus* SNAP-25a and SNAP-25b with SNAP-25 proteins of other species: yeast SEC9 (Brennwald et al., 1994), *Drosophila* and *Torpedo* (Risinger et al., 1993), goldfish (Risinger and Larhammar, 1993), chicken (Catsicas et al., 1991; Bark, 1993), mouse (Oyler et al., 1989) and human (Bark and Wilson, 1994a).

two proteins of 196 amino acids each. Fig. 1 shows the predicted amino acid sequences for these proteins, together with the amino acid sequences of human SNAP-25a and b. The exons coding for the sequences within the human SNAP-25 proteins are also indicated in Fig. 1. On the basis of the alignment of the *Xenopus* proteins with SNAP-25 sequences of the human (and other species; not shown) we designated the isoforms *Xenopus* SNAP-25a and *Xenopus* SNAP-25b. Comparison of the SNAP-25a with the SNAP-25b isoform revealed a 94.7% amino acid sequence identity between the two proteins. Major differences were found in exon 5, similar to the situation in mammals and chicken (Oyler et al., 1989; Catsicas et al., 1991; Bark, 1993; Bark and Wilson, 1994a).

Comparison of *Xenopus* SNAP-25a and SNAP-25b with SNAP-25 of other species shows that the proteins are extremely well conserved (Fig. 2). From a phylogenetic point of view, they lie between the goldfish (bony fish) and the chicken (birds), which is mainly based on minor sequence differences in the central region. The regions of highest conservation reside in the amino- and the carboxy-terminal of the proteins, where coiled-coil structures are likely present to bind syntaxin I (residues 21–100) and synaptobrevin (residues 180–206; Chapman et al., 1994; Hayashi et al., 1994). Like SNAP-25 proteins of other species, *Xenopus* SNAP-25a and SNAP-25b do not possess a hydrophobic stretch of amino acids forming the transmembrane domain. Instead, they show a cysteine-rich area in their central region (Fig. 1). In *Xenopus* SNAP-25a, the cysteine residues occur at positions 84, 85, 90 and 92, whereas in SNAP-25b they are present at positions 85, 88, 90 and 92, which possibly are palmitoylated and participate in the association of SNAP-25 with cellular membranes (Hess et al., 1992; Veit, 2000).

Xenopus SNAP-25 is resistant to cleavage by BoNT/A

To demonstrate the presence of SNAP-25 proteins unequivocally, we incubated *Xenopus* brain homogenate (10 μg) with increasing concentrations (40, 100 and

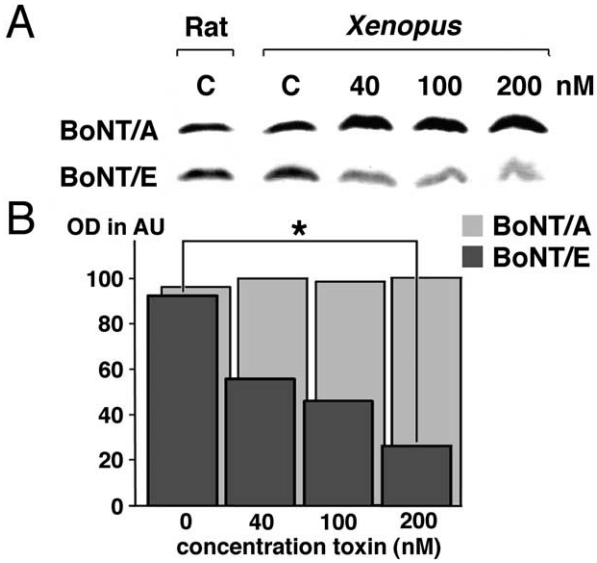

Fig. 3. *Xenopus* SNAP-25 is resistant to BoNT/A cleavage. Rat brain homogenate served as a control (c). (A) Immunoblot showing SNAP-25 in *Xenopus* brain either in the absence (c; 5 μg/lane) or the presence of increasing concentrations (40, 100, 200 nM; 10 μg/lane) of BoNT/A or BoNT/E. (B) Decline in the immunoreactive signal (OD, in arbitrary units, AU) when 40 and 200 nM BoNT/E are compared, but not with increasing concentrations of BoNT/A. Asterisk indicates significant difference ($P<0.01$). (The experiment was repeated twice with essentially the same results.)

200 nM) of DTT-reduced BoNT/A or BoNT/E holotoxin. As shown in Fig. 3, no obvious change in the optical density of the SNAP-25-immunoreactive band was found at any BoNT/A concentration. However, when homogenates were incubated with BoNT/E, cleavage clearly took place, being lowest at 40 nM and highest at 200 nM. These results indicate that *Xenopus* SNAP-25 is insensitive to BoNT/A proteolysis. The BoNT/A cleavage site of SNAP-25 resides between residues 197 and 198, coding for glutamine and arginine, respectively. As in SNAP-25-like genes of yeast and rays (Risinger et al., 1993), this site is not present in *Xenopus* SNAP-25a and SNAP-25b, as glutamine and arginine have been substituted by lysine and histamine, respectively (Fig. 1). The BoNT/E cleavage site at position 180–181 does not occur in yeast but is conserved in all animal species studied up to now, including *Xenopus* (Fig. 1).

Background adaptation and SNAP-25 isoforms in the pituitary gland

The spatial distribution of SNAP-25a and SNAP-25b mRNAs was examined by *in situ* hybridization using digoxigenin-labeled antisense probes. Both mRNAs were detected in the pituitary gland. No hybridization signals were found with the sense probe or after pretreatment of the tissue with RNase A.

Occurrence of SNAP-25 mRNAs. Whereas the neural lobe is completely devoid of a positive *in situ* hybridization signal for each of the mRNA probes, the various endocrine cell types of the anterior lobe demonstrate variable labeling intensities with both probes, the signal for SNAP-25b mRNA being generally somewhat stronger than for SNAP-25a mRNA (Fig. 4). Attention was focused on the neuroendocrine melanotrope cells in the intermediate lobe. Upon qualitative observation, in the white-adaptation

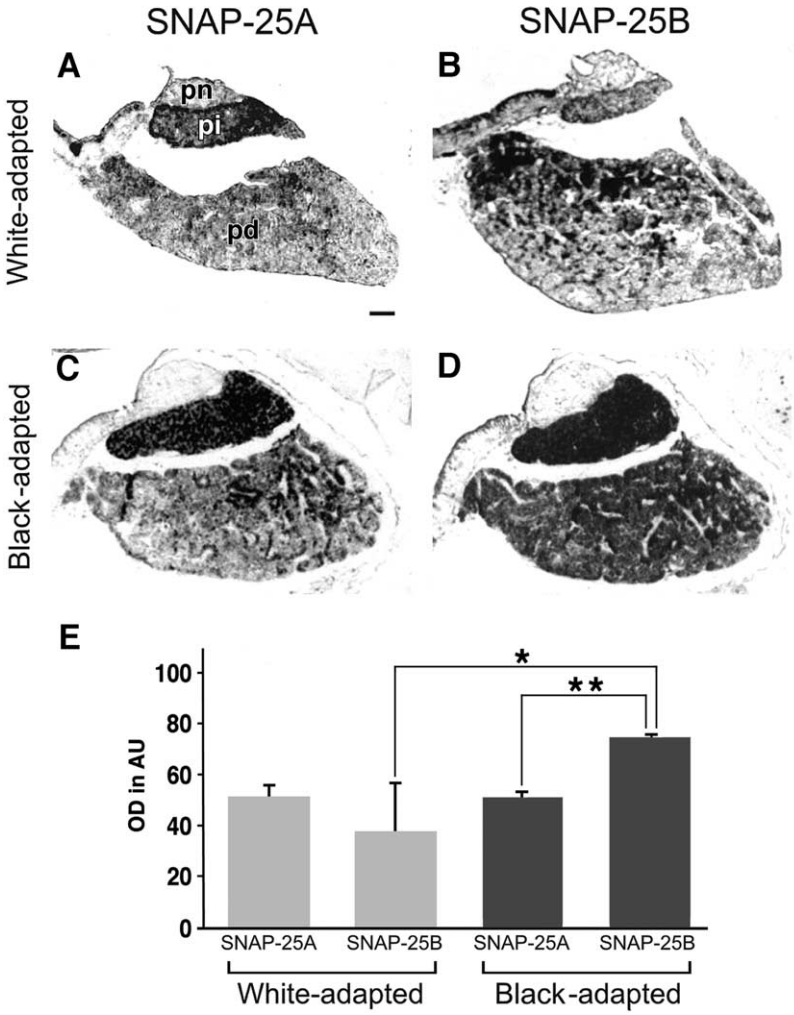

Fig. 4. *In situ* hybridization of sagittal sections of the pituitary gland, stained for SNAP-25a (A, C) and SNAP-25b (B, D). Compared with white-adapted animals (A, B), in black-adapted animals expression of SNAP-25a in melanotrope cells in the pars intermedia (pi) appears the same or only slightly higher (C) but expression of SNAP-25b is much higher (D). (E) Quantitation of *in situ* hybridization signals of SNAP-25a and SNAP-25b in the pars intermedia, in white- and black-adapted animals ($n=4$), expressed as OD in arbitrary units (AU). In black-adapted animals, SNAP-25b expression is clearly higher than in white ones and also higher than SNAP-25b expression. * $P<0.05$, ** $P<0.001$. pd, pars distalis; pn, pars neuralis. Scale bar=100 μm (A–D).

Table 1. Real-time RT-PCR of expressions of mRNAs of SNAP-25a (a) and SNAP-25b (b) in the neurointermediate pituitary lobe of white- (W) and black- (B) adapted Xenopus[a]

Factor	Isoform	Parameter	*n*	Mean±S.E.M.	Sign
(1)	a	dCt (W)	3	9.27±0.02	(5)***
(2)	a	dCt (B)	3	8.35±0.07	(6)***
(3)	a	ddCt (W-B)	3	0.92±0.07	>0*
(4)	a	E (B/W)	3	1.96±0.08	>1*
(5)	b	dCt (W)	4	6.75±0.05	(1)***
(6)	b	dCt (B)	4	5.11±0.08	(2)***
(7)	b	ddCt (W-B)	4	1.62±0.08	>0**
(8)	b	E (B/W)	4	3.33±0.11	>1***
(9)	b/a	ddCt (W)	3	2.67±0.03	>0*** (10)**
(10)	b/a	ddCt (B)	3	3.31±0.06	>0*** (9)**
(11)	b/a	E (W)	3	6.37±0.06	>1*** (12)**
(12)	b/a	E (B)	3	10.05±0.15	>1** (11)**

[a] Each PCR run included determination of mRNA of the housekeeping gene GAPDH. The SNAP-25 values (Ct; not shown) were subtracted from the GAPDH values to give the dCt. For each isoform, ddCt (W-B) is the difference between the dCt of white and black animals [dCt(W)−dCt(B)], and has been translated into the relative mRNA expression E (B/W) according to $E=2^{Ct}$. Similarly, ddCt (W) and ddCt (B) are the differences between the dCt of SNAP-25a and of SNAP-25b in white and black animals, respectively, and E (W) and E (B) are their relative (b/a) mRNA expressions. All factors statistically analyzed demonstrate a significant difference, shown as follows: each factor is numbered in the first column, and the factor from which it differs is indicated in the last column (Sign). Parameter E is always significantly higher than 1 (>1), indicating that each isoform occurs more in black than in white animals [E (B/W)] and that isoform b is more expressed than isoform a in both light adaptation conditions [E (W) and E (B), respectively]. Moreover, the light condition has a stronger effect on the expression of isoform b than of isoform a, because factor 12 is significantly higher (+58%) than factor 11. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

condition, the melanotropes appear to be moderately stained for both SNAP-25a and SNAP-25b mRNAs (Fig. 4A, B). In black-adapted animals, however, SNAP-25b mRNA is much stronger expressed (Fig. 4D) whereas the expression of SNAP-25a seems to be similar or only moderately higher (Fig. 4C).

Differential expressions of SNAP-25 isoforms. The qualitative impression that the two isoform mRNAs are differentially expressed in the *Xenopus* pars intermedia as a function of the light adaptation condition, was confirmed by quantitative *in situ* hybridization and by real-time RT-PCR. First, quantitation of the *in situ* hybridization signals revealed that in black-adapted animals SNAP-25b mRNA is more highly expressed than the mRNA of SNAP-25a (Fig. 4E; +48%; $P<0.05$) and, moreover, is more highly expressed than in white-adapted animals (Fig. 4E; +96%; $P<0.05$). The existence of a statistically significant ($P<0.05$; two-ANOVA) interaction between the factors 'isoform type' and 'adaptation state' indicates the differential reaction of the two isoforms to the changed background condition.

Taking into account that quantitation of *in situ* hybridization signals may lead to an underestimation of differences in mRNA amounts, which is due to the non-linear relationship between staining intensity and amount of mRNA, we subsequently tested reliability of the differences observed with quantitative *in situ* hybridization with real-time RT-PCR of the neurointermediate lobe. Values for the respective SNAP-25 isoforms were corrected for GAPDH household gene mRNA expression. The PCR data (Table 1) show that the two isoforms differ from each other in their expression, both in relative and absolute amounts, when the two adaptation conditions are considered. So, assuming that the dCt value is an indication for the absolute amount of mRNA of a particular isoform in the *Xenopus* pars intermedia, the SNAP-25b is more strongly expressed than SNAP-25a, in both white- and black-adapted animals. This appears from comparing the respective dCt values (Table 1; white: factor 5 is lower than factor 1; $P<0.001$; black: factor 6 is lower than factor 2; $P<0.001$). As a consequence, the corresponding E values, indicating the relative (b versus a) amounts of mRNA, are both significantly higher than 1: factor 11, E(W)=6.37 ($P<0.001$) and factor 12, E(B)=10.05 ($P<0.01$). Interestingly, the prevalence of SNAP-25b is significantly stronger in black than in white animals. In fact, the E (B/W), based on the dCt values of black and white animals, respectively, indicates that isoform b is expressed 3.33 times as high in black as in white animals (Table 1, factor 8), whereas SNAP-25a is expressed only 1.96 times higher in black animals, as E (B/W)=1.96 (Table 1, factor 4). This indicates that the adaptation condition differentially stimulates the expression of each isoform, a difference that is statistically significant as appears from comparing the respective E values (Table 1; factor 12 is significantly higher, viz. +58%, than factor 11; $P<0.001$).

Expression of SNAP-25 isoforms in the brain

In the brain, hybridization signals with each probe occur in neuronal perikarya including their axon hillocks, whereas the cell nucleus, axons and axon terminals (as far as discernable) are devoid of staining. As in the pituitary gland, no hybridization was seen with the sense probe or after pretreatment with RNase A.

Occurrence of SNAP-25 mRNAs. The following qualitative anatomical description equally applies to each of the SNAP-25 isoform mRNAs, and is illustrated in Fig. 5 for

SNAP-25b. In the telencephalon, the most rostral perikarya expressing SNAP-25 mRNA are situated in the olfactory bulb, where the internal granular and external plexiform cell layers and the mitral cell layers are stained. More caudally, a mediolateral band of SNAP-25-positive perikarya was found in the medial and lateral septum. Expression was furthermore observed in the medial and lateral parts of the pallium (Fig. 5A). Caudally in the ventral telencephalon, a few SNAP-25-positive neurons are present in the striatum but a much larger population of positive neurons was encountered in the nucleus accumbens. These numerous cells, which occur in two subgroups, decrease in number in a caudal direction. The largest subgroup is present in the dorsal part of the nucleus, whereas cells of the other subgroup occur rather scattered in the ventral part of the nucleus. Dorsal to the anterior commissure, positive signals were seen in the medial and lateral amygdala.

In the diencephalon, groups of positive neurons were found in the preoptic part of the hypothalamus. The most rostral group is present in the anterior preoptic nucleus and in both the parvo- and the magnocellular neurons of the magnocellular nucleus (Fig. 5C). Another group occurs in the caudal part of the preoptic area, namely, in the suprachiasmatic nucleus (Fig. 5B), which contains scattered and small cells arranged in a 'butterfly' configuration. Labeling was also observed in the outer cell layers of the dorsal and ventral habenular nuclei and in the pinealocytes of the pineal gland. A cluster of parallel arranged, caudal, positive neurons is situated in the posterior tubercle and more dorsally in the ventromedial thalamic nucleus. In the infundibular area, numerous positive neurons are present in the

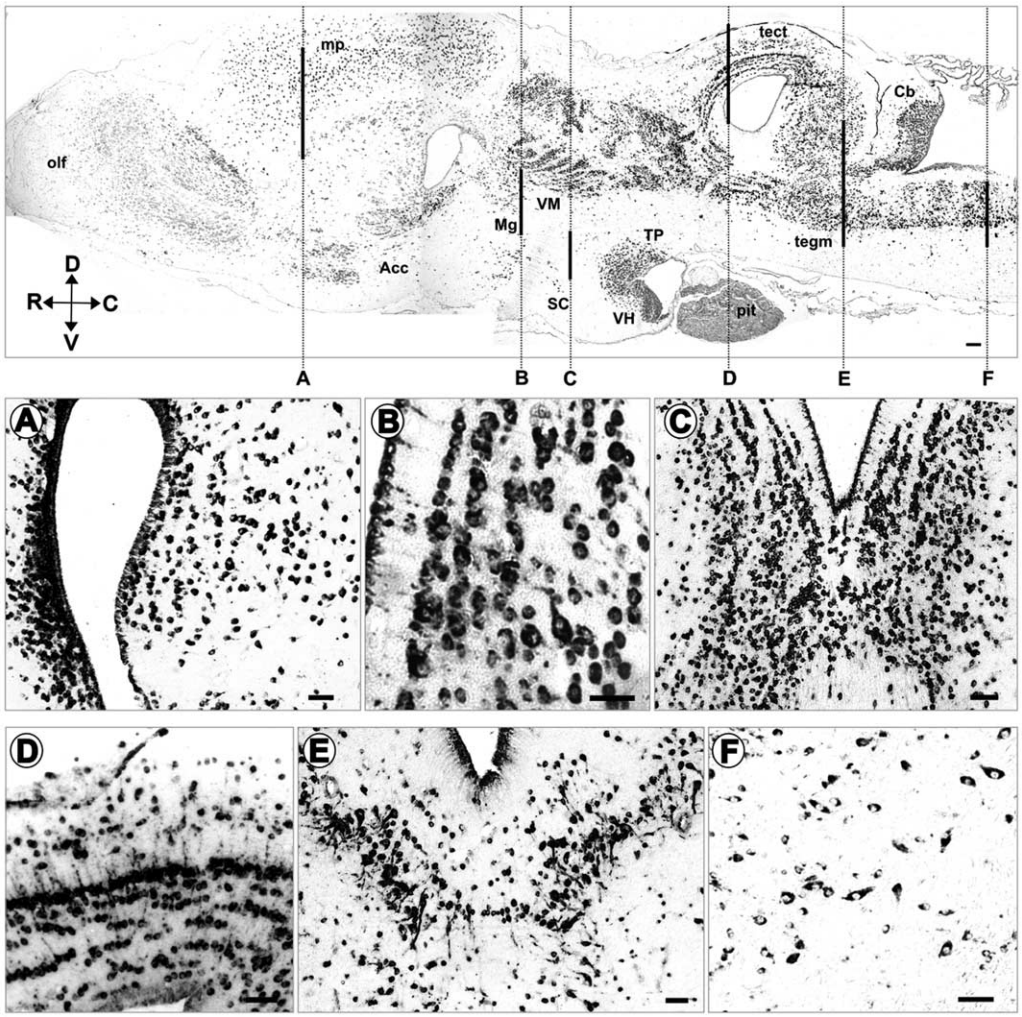

Fig. 5. Spatial distribution of SNAP-25b mRNA in the *Xenopus* brain. Upper panel: sagittal section of brain and pituitary gland. Anatomical coordinates in upper panel are indicated in the bottom left corner (C, caudal; D, dorsal; R, rostral; V, ventral). Vertical lines represent the planes of the transversal sections A–F. (A) medial pallium; (B) suprachiasmatic nucleus; (C) magnocellular nucleus; (D) several layers of the optic tectum; (E) omnipolar magnocellular neurons of the oculomotor nucleus (III); (F) motoneurons of the hindbrain. Thickenings of the lines indicate sites of photomicrographs in middle and lower panel. Acc, nucleus accumbens; Cb, cerebellum; Mg, magnocellular nucleus; mp, medial pallium; olf, olfactory bulb; pit, pituitary; SC, suprachiasmatic nucleus; tect, optic tectum; tegm, tegmentum; TP, tubercle posterior; VH, ventral hypothalamic nucleus; VM, ventromedial hypothalamic nucleus. Scale bars=50 μm.

ventral hypothalamic nucleus and in the paraventricular organ.

In the dorsal mesencephalon, the several cell layers of the optic tectum show hybridization signals (Fig. 5D). Also the torus semicircularis reveals stained neurons. In the tegmentum, the omnipolar magnocellular neurons of the oculomotor nucleus (III) are positive (Fig. 5E), as are the anterior nuclei.

In the rhombencephalon, the cerebellum contains stained pyramidal neurons and small cerebellar neurons. Staining was furthermore observed in the hindbrain including the locus coeruleus and the raphe nucleus, and in several motoneurons (Fig. 5F).

Differential expression of SNAP-25 isoforms. Distributions of both mRNAs in main regions of the *Xenopus* brain are given in Table 2, and some are illustrated in Fig. 6. Although throughout the brain the two mRNAs of the SNAP-25 isoforms coexist, many regions reveal clear differences in the strengths of the respective hybridization signals. Thus, although in some brain regions both mRNAs occur with the same or similar hybridization strengths (e.g. in the striatum, medial amygdala, habenular nuclei, pineal gland and hindbrain motoneurons), in most areas either one of the mRNAs prevails. SNAP-25a mRNA is more strongly expressed than SNAP-25b in, particularly, the rostral part of the brain (e.g. in the mitral cells and external plexiform layer of the olfactory bulb, in the dorsal, lateral and medial septum, and in the pallium) and in a few other brain areas such as the anterior and posterior thalamic nuclei and the cerebellum. SNAP-25b mRNA is the main isoform in the rest of the brain including the nucleus accumbens, the lateral amygdala, layers III and IV of the optic tectum, the locus coeruleus and the raphe nucleus, and most of the thalamic part of the diencephalon, including the ventromedial thalamic nucleus, the ventral hypothalamic nucleus and the paraventricular nucleus.

Table 2. Distribution of SNAP-25a and SNAP-25b mRNAs in the *Xenopus* brain and pituitary gland, as visible with *in situ* hybridization[a]

Brain region	SNAP-25a	SNAP-25b
Telencephalon		
Olfactory bulb		
Mitral cells (Obml)	+++	+
Internal granule cells (OBigl)	++	++
External plexiform cells (Obepl)	++	+
Septum		
Medial (ms)	+++	+
Lateral (ls)	+++	++
Pallium		
Medial (mp)	+++	++
Lateral (lp)	+++	++
Dorsal (dp)	+++	+
Striatum (Str)	++	++
Accumbens (Acc)	++	+++
Amygdala		
Pars medialis (Apm)	++	++
Pars lateralis (Apl)	++	+++
Diencephalon		
Habenular nuclei (Hv, Hd)	+++	+++
Pineal gland (E)	+++	+++
Ventromedial thalamic nucleus (VM)	++	+++
Anterior thalamic nucleus (A)	+++	++
Posterior thalamic nucleus (P)	+++	++
Magnocellular nucleus (Mg)	++	+++
Anterior preoptic nucleus (Poa)	++	+++
Suprachiasmatic nucleus (SC)	++	+++
Paraventricular nucleus (NPv)	++	+++
Posterior tubercle (TP)	++	+++
Ventral hypothalamic nucleus (VH)	++	+++
Pituitary gland		
Pars nervosa (pn)	–	–
Pars intermedia (pi)	s.d.	s.d.
Pars distalis (pd)	++	++
Mesencephalon		
Optic tectum (tect)		
Layer I	+	+
Layer II	+	++
Layer III	+	++
Layer IV	+	++
Layer V	+	++
Layer VI	+++	+++
Torus semicircularis (Tor)	++	+++
Nucleus oculomotorius (III)	+++	+++
Anterior tegmental nucleus (Av, Ad)	++	+++
Rhombencephalon		
Cerebellum (Cb)	+++	++
Raphe nucleus (Ra)	++	+++
Locus coeruleus (LC)	++	+++
Hindbrain motoneurons	+++	+++

[a] Four degrees of staining intensity of neurons in the various regions have been indicated: +, low but distinct signal; ++, moderate signal; +++, strong signal; −, no detectable signal; s.d., stimulus-dependent.

DISCUSSION

For proper intracellular membrane trafficking and chemical intercellular communication, fusion of a (message-containing) vesicle or granule with the appropriate target membrane is essential. Many fusion-related proteins and their isoforms are involved in specific aspects of these processes, such as vesicle transport, docking, priming and exocytosis. In this study, we present and discuss the characterization, distribution and (differential) regulation of SNAP-25a and SNAP-25b isoforms in the pituitary gland and the brain of the amphibian *X. laevis*.

Conserved SNAP-25 splice variants differ in the cysteine-rich area

The present cDNA cloning and sequence analysis reveals that *X. laevis* contains at least two SNAP-25 isoforms, which contain similar 3′ and 5′ sequences but clearly differ in the positions of the four cysteine residues in the central part of the protein (exon 5). These central cysteines are most likely post-translationally palmitoylated, allowing the protein to form a stable membrane association (Hess et al., 1992; Lane and Liu, 1997; Vogel and Roche, 1999). Differences in spacing of the cysteine residues between the SNAP-25a and SNAP-25b isoforms may result in binding of the proteins to different membranes (Lane and Liu, 1997; Veit, 2000). In this way, SNAP-25 isoforms could

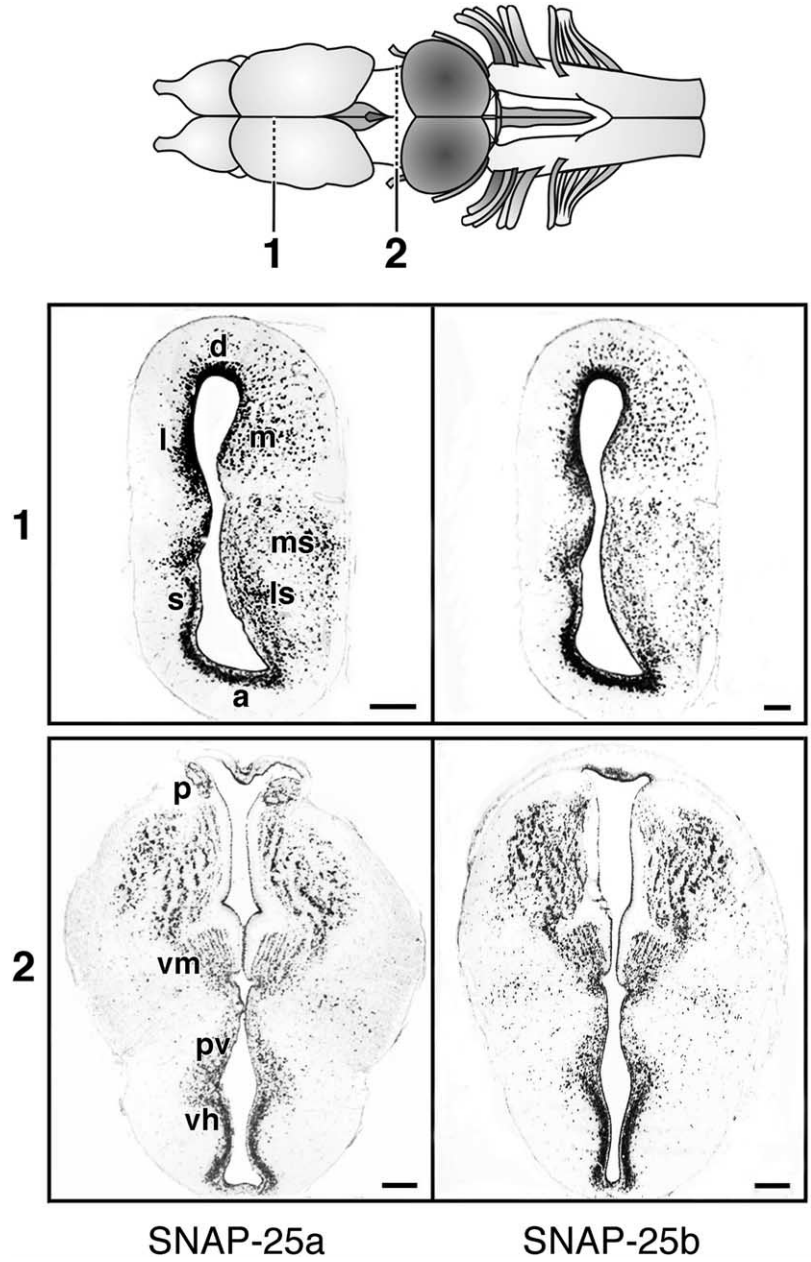

Fig. 6. Occurrence of SNAP-25a and SNAP-25b mRNAs in the *X. laevis* brain, as shown by *in situ* hybridizations. Schematic dorsal view of the brain at the top indicates the two transverse levels (1, left part, and 2) where photomicrographs were taken. a, nucleus accumbens; d, dorsal pallium; l, lateral pallium; ls, lateral septum; m, medial pallium; ms, medial septum; p, posterior thalamic nucleus; pv, paraventricular nucleus; s, striatum; vh, ventral hypothalamic nucleus; vm, ventromedial thalamic nucleus. Scale bars=100 μm.

exert their function by acting at different targets, for instance at the plasma membrane versus the synaptic vesicle/secretory granule membrane. This might be reflected by the differences in spatial distribution of SNAP-25a and SNAP-25b in the developing versus the adult rat brain (Bark et al., 1995; Boschert et al., 1996). Moreover, we previously showed that SNAP-25 is not only present on the plasma membrane but also in the perinuclear/Golgi area and on the membrane of hormone-containing secretory granules in *Xenopus* melanotropes (Kolk et al., 2000). This demonstration of the SNAP-25 protein at multiple sites can possibly be explained from different subcellular functions of the respective SNAP-25 isoforms (Sorensen et al., 2003).

SNAP-25 isoforms may originate in two ways: 1) from multiple genes that are derived from either gene or genome duplication, or 2) from differential splicing of one gene transcript. In bony fish, SNAP-25a and SNAP-25b isoforms are derived from two genes (Risinger and Larhammar, 1993; Risinger et al., 1998) whereas in mammals and chicken, type a and type b isoforms are splice variants that originate from alternative splicing of one SNAP-25

gene product (Oyler et al., 1989; Catsicas et al., 1991; Bark, 1993; Bark and Wilson, 1994a). However, only one SNAP-25 protein without isoforms is found in cartilaginous fish (Risinger et al., 1993). Apparently, no simple phylogenetic line can be drawn as to the formation of SNAP-25 isoforms.

Xenopus SNAP-25 isoforms can be cleaved by BoNT/E but not by BoNT/A

Whereas our studies mainly concern the detection of mRNAs encoding for SNAP25, we have used BoNTs to show specifically the presence of SNAP25 protein. Generally, BoNTs inhibit the exocytotic release of many messengers by cleaving specific proteins involved in secretion (McMahon et al., 1992; Blasi et al., 1993). However, these toxins are not equally effective (MacKenzie et al., 1982; Binz et al., 1994; Washbourne et al., 1998; Xu et al., 1998; Keller and Neale, 2002), which might be related to different functions of these proteins in different cell types due to differences in three-dimensional protein structure. We show that *Xenopus* SNAP-25 is susceptible to proteolysis by BoNT/E in a concentration-dependent manner but is resistant to BoNT/A. This result is explained by the fact that *Xenopus* SNAP-25 possesses the BoNT/E cleavage site at position 180–181, which is conserved in all species examined (except yeast) but lacks the BoNT/A cleavage site at position 197–198. Apparently, the latter toxin cleavage site in the C-terminal region is not fully conserved during animal evolution as it is also lacking in SNAP-25 of *Torpedo*. It has been assumed that binding of SNAP-25 with syntaxin involves the interaction of C-terminal α-helical domains to form coiled-coil structures (Chapman et al., 1994; Hayashi et al., 1994). *Xenopus* SNAP-25 can bind syntaxin in immunoprecipitation studies. Obviously, the biochemical properties of the *Xenopus* SNAP-25 isoforms are preserved, suggesting that amino acid substitutions have not significantly affected the function(s) of these proteins.

The expressions of *Xenopus* SNAP-25 isoforms in melanotrope cells are differentially regulated

We have considered the possibility that the presence of a particular SNAP-25 isoform depends on the activity state of the neuron or endocrine cell, and is physiologically regulated. To test this hypothesis, we used an animal model in which the activity of a readily identifiable secretory cell type can be controlled *in vivo*, in a physiological way. For this purpose, we have studied the well-described background adaptation reflex of the amphibian *X. laevis*.

The neuroendocrine pituitary melanotropes in a black-adapted *Xenopus* are in a higher activity state than melanotropes in a white-adapted animal (de Rijk et al., 1990; Berghs et al., 1997) and secrete much more α-MSH into the circulation (Jenks et al., 1993). Many melanotrope secretory products and proteins involved in the secretory process, including SNAP-25, are controlled in their expressions by the background light condition stimulus (Martens et al., 1987; Kolk et al., 2000, 2001; Kuiper et al., 2000; Kramer et al., 2001; Roubos et al., 2002; Jenks et al., 2003). The quantitative *in situ* hybridization data indicate that the black background stimulus differentially affects the expressions of the two SNAP-25 mRNAs isoforms: in contrast to SNAP-25a mRNA, the SNAP-25a mRNA signal is more highly expressed than under the white-adaptation condition. Our PCR data support this conclusion: in animals on a black background, SNAP-25A mRNA is much more highly expressed than in white-adapted ones. The finding with RT PCR that black-background adaptation causes an increase in SNAP-25a mRNA that is significantly lower than that of SNAP-25b mRNA, again supports the idea that the two isoforms react in a differential way to the physiological stimulus of background light intensity. The fact that no upregulation of SNAP-25a was seen with *in situ* hybridization, may well be due to the lower sensitivity of this method compared with RT PCR.

The finding of differential upregulation of SNAP-25 isoforms is the first demonstration of a physiological, and differential, regulation of SNAP-25 isoforms. We speculate that the two proteins play different, perhaps complementary, roles in the complex control mechanism of hormone secretion.

Different degrees of expression, different functions?

The differences in the expression of SNAP-25a and SNAP-25b mRNAs in the *Xenopus* brain raise the possibility that the expression of an isoform is related to a particular neuron property, such as the presence of a special type of neurochemical messenger. Although SNAP-25b is most abundant in the brain, there seems to be a correlation between SNAP-25 isoform expression and the occurrence of a specific class of neurotransmitters. For example, in the olfactory bulb, NPY-positive neurons are present in the internal granule cell layer (Lázár et al., 1993), correlating with a relatively high expression of SNAP-25b and a lower expression of SNAP-25a. In contrast, dopaminergic fibers in the olfactory bulb are mainly found in the external plexiform and mitral cell layers (González et al., 1993), correlating with dominant expression of SNAP-25a. Furthermore, there is a strong expression of SNAP-25b in thalamic and hypothalamic nuclei, which have an overall peptidergic phenotype (Tuinhof et al., 1993). These findings suggest a role of SNAP-25b in neuropeptide secretion, whereas SNAP-25a could be particularly involved in the secretion of catecholamines. The fact that the SNAP-25 isoforms are differentially expressed in rat spinal motoneurons and cranial nerves (Jacobsson et al., 1996, 1999) supports this speculative idea.

To conclude, we speculate that the SNAP-25a and SNAP-25b isoforms have distinct functions in neuronal and endocrine fusion processes, such as occur in the secretory process, and that their occurrence and regulation depend on the secreted neurotransmitter/hormone phenotype and/or the activity state of the cell.

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