Expression and thyroid hormone regulation of annexins in the anterior pituitary

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Abstract

Due to their property to bind to phospholipids in a Ca^{2+} -dependent manner, proteins of the annexin superfamily are involved in many membrane-related events and thus in various forms of physiological and pathological processes. We were therefore interested in analyzing the mRNA expression of the annexins in the severely disorganized pituitaries of the athyroid $\text{Pax8}^{-/-}$ mice in comparison with that of control animals. In neither condition was mRNA expression of the annexins A3, A7, A8, A9, A11, and A13 detectable. The annexins A2, A4, and A6 were equally expressed in wild-type and $\text{Pax8}^{-/-}$ mice. Transcript levels of A1 and A10 were highly increased and those of A5 were significantly decreased in the athyroid mutants compared with controls. Treatment of $\text{Pax8}^{-/-}$ mice with

physiological doses of thyroxine for 3 days normalized the mRNA expression of A1, A5, and A10 indicating that the expression of these annexins is directly regulated by thyroid hormone (TH). Since A5 exhibits by far the highest transcript levels of all annexins in the pituitary and its regulation by TH could be also confirmed at the protein level, we analyzed the mRNA expression of pituitary hormones in $\text{A5}^{-/-}$ mice. In these mutants, only the β -FSH mRNA expression was found to be significantly reduced, while the mRNA expression levels of the other pituitary hormones were not altered. These results support the concept that annexins might serve important albeit redundant functions as modulators of pituitary hormone secretion.

Journal of Endocrinology (2007) **195**, 385–392

Introduction

The annexins represent an ancient superfamily of structurally related proteins with the common property to bind to phospholipids in the presence of Ca^{2+} . The N-terminal sequence of each annexin is unique and appears to represent the specificity of the individual protein, whereas the highly conserved C-terminal domain, termed the core, comprises four (in annexin 6 eight) repeated sequences of 70 amino acids representing the Ca^{2+} -binding sites (Gerke & Moss 2002). In the Ca^{2+} -bound conformation, the unique architecture of the core enables the proteins to dock onto negatively charged membrane surfaces. This property links annexins to many membrane-related events such as the organization of membrane domains, membrane cytoskeleton linkages, the regulation of ion fluxes across membrane and certain exo- and endocytotic transport steps (Hayes *et al.* 2004, Rescher & Gerke 2004, Gerke *et al.* 2005).

The precise function of the individual annexins in physiological processes, however, remains widely enigmatic, although the biochemical and structural features of the different annexins have been studied extensively and a plethora of effects has been assigned to these proteins. Several annexins, such as A1, A2, A3, A6, A7, and A11, have been linked to exocytotic processes such as chromaffin granule exocytosis of adrenergic cells, and many annexins have also been identified in endocrine organs (Creutz 1992, Hayes *et al.* 2004, Gerke *et al.* 2005), including annexins A1 and A5 in the anterior pituitary (Traverso *et al.* 1999, Kawaminami *et al.* 2002), the master gland of the endocrine system.

We were therefore interested in studying the expression of the annexins in the anterior pituitary not only in wild-type animals but also in the pituitary of the athyroid $\text{Pax8}^{-/-}$ mouse. These mice lack the thyroid hormone (TH) producing follicular cells of the thyroid gland and are thus completely athyroid in postnatal life (Mansouri *et al.* 1998). These mutants can therefore be

considered as an ideal animal model to study the consequences of congenital hypothyroidism (CH) which is a common disorder mostly caused by thyroid dysgenesis or agenesis affecting 1 in 3600 newborns (Kopp 2002, De Felice & Di Lauro 2004). If not treated immediately after birth by TH replacement therapy, severe forms of CH lead to cretinism, a syndrome characterized by many metabolic disturbances, growth and mental retardation as well as many neurological deficits (Larsen *et al.* 2003, Roberts & Ladenson 2004, Bernal 2005). Consequently, untreated Pax8-deficient animals exhibit deafness, ataxia, early death around weaning and a completely distorted appearance of the anterior pituitary such as hypertrophy and hyperplasia of thyrotropes and an almost complete absence of lactotropes (Friedrichsen *et al.* 2004). Due to the properties of the annexins mentioned above and the findings that in the thyroid several members of the annexin family are regulated by TH (el Btaouri *et al.* 1996), we also expected major changes in annexin metabolism in the pituitaries of the athyroid Pax8^{-/-} mice.

Materials and Methods

Animals

Animal procedures were approved by the Animal Welfare Committee of the Medizinische Hochschule Hannover. Mice were kept at a constant temperature (22 °C) and light cycle (12 h light:12 h darkness) and were provided with standard laboratory chow and tap water *ad libitum*. Male mice were used for the experiments and wild-type controls were littermates of Pax8^{-/-} and annexin A5^{-/-} mice respectively. If required, Pax8^{-/-} animals were injected for 3 days with physiological doses of thyroxine (T₄; P18–P21, 18 ng/g body weight (BW) subcutaneously) to restore a euthyroid status in these mice (Friedrichsen *et al.* 2004). Genotyping of Pax8 and annexin A5 was performed as described elsewhere (Flamant *et al.* 2002, Brachvogel *et al.* 2003). Animals were killed by decapitation at postnatal day 21 (P21), tissues were isolated quickly, frozen on dry ice, and stored at -80 °C until further processing.

Real-time PCR

Total RNA was isolated from pooled mouse pituitaries ($n \geq 4$ per gender and genotype, at least three pools) using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA). cDNA was generated with the Invitrogen Thermo-script RT-PCR System (Invitrogen) and digested with RNase as suggested by the supplier. Samples without reverse transcriptase were used as negative controls to confirm the absence of genomic DNA. Quantitative real-time PCR was performed using the iCycler iQ Multi-Color Real-time PCR Detection System and the iQ SYBR Green Supermix (Bio-Rad). Cyclophilin was used as housekeeping gene for normalization. Samples were analyzed in triplicate and a dilution series was used in each run to determine the PCR

efficiency for each pair of primers. Student's *t*-test was applied to test for significance. *P* values below 0.05 were considered as significant and marked as indicated in the figure legends. The following primers were chosen to generate the PCR-fragments:

Cyclophilin: GCAAGGATGGCAAGGATTGA and AGCAATTCTGCCTGGATAGC
Thyroid-stimulating hormone- β (TSH- β): CCGCAC-CATGTTACTCCTTA and GTTCTGACAGCCT-CGTGTAT
Prolactin (PRL): GCAGTCACCATGACCATGAA and AGATTGGCAGAGGCTGAACA
Growth hormone (GH): CGTTTCTCGCTGCTGCTCAT and GTCCGAGGTGCCGAACATCA
Follicle-stimulating hormone- β (FSH- β): CGCA-CACCAAGTGGTATTGA and AGAGCAATCTGCT-GCCATAG
Luteinizing hormone- β (LH- β): AGTACTCGGAC-CATGCTAGG and CAACTCTGGCCGACAGAGAAT
Annexin A1 (ANXA1): TTCCTCAAGCAGGCCCGTTT and TAAGTACGCGGCCCTTGATCT
ANXA2: GTCTACTGTCCACGAAATCC and TCTC-CAGGTGGCCAGATAAG
ANXA3: TCACCTTCGGCTGAGCTTCTG and CC-TGTTTCATACGCTGCTTGG
ANXA4: TCTCCGCACCAGAGGAACCTT and TT-GCTGCTCAGCTCCGACTT
ANXA5: TTGGCGTGTGCATCGGTCCT and TGG-CGCTGAGCATTGCTTCG
ANXA6: TCTGCTGCAGAACCTGAGAC and TCAC-TATCAGCCGCTCGAAC
ANXA7: CTGCAGGTCAGGAGTCATCT and TGT-GCAGGTGGCTGTGGATA
ANXA8: CACTGAGCAGAGGCCAACTA and CCTC-TTGGTGAGCACGTCTA
ANXA9: GTGCTTCACGACGCTGAACC and CCA-CACCTTGGCCTGCGATA
ANXA10: TACCCTGGTTACTTTCCTCTC and GG-CCATACATGCTCTGATAG
ANXA11: GCTGGATAACGCTAGCCAACT and GGC-GGATACATTCCATAAGG
ANXA13: GTCTTATCCAGCAGGACATC and TGTTGCTTCGTGTGCATAGG

In vitro transcription

Radioactive- or digoxigenin-labeled RNA probes were generated from cDNA subclones in Bluescript SKII + plasmids or pGEM plasmids (Promega). *In vitro* transcription was carried out according to the standard protocols with [³⁵S]UTP and [³⁵S]CTP as labeled nucleotides or using a DIG RNA Labeling Kit (Roche). Probes were generated from cDNA fragments corresponding to nt 632–962 of annexin A1 (accession no. NM010730), nt 529–958 of annexin A5 (accession no.

D63423), and nt 575–1023 of annexin A10 (accession no. NM011922). Probe generation from cDNAs of pituitary hormones was carried out as described previously (Friedrichsen *et al.* 2004, Mittag *et al.* 2005). cRNA probes were diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 0.05% tRNA, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.4), 1× Denhardt's solution, 100 µg/ml sonicated salmon sperm DNA, 1 mM EDTA, and 10 mM dithiothreitol) to a final concentration of 5×10^4 c.p.m./µl for radioactive-labeled probes and 5 ng/µl for digoxigenin-labeled probes.

In situ hybridization (ISH)

After the animals were decapitated, pituitaries were removed rapidly, embedded in Tissue-Tek medium (Sakura Finetek, Torrance, CA, USA) and frozen on dry ice. Sections (16 µm) were cut on a cryostat (Leica, Benthelm, Germany), thaw-mounted on silane-treated slides, and stored at -80°C until further processing. ISH was carried out as described previously (Schäfer & Day 1995, Friedrichsen *et al.* 2003). Briefly, frozen sections were fixed in a 4% phosphate-buffered paraformaldehyde solution (pH 7.4) for 1 h at room temperature (RT), rinsed with PBS, and treated with 0.4% phosphate-buffered Triton X-100 solution for 10 min. After washing with PBS and water, tissue sections were incubated in 0.1 M triethanolamine (pH 8) containing 0.25% (v/v) acetic anhydride for 10 min. Following acetylation, sections were rinsed several times with PBS, dehydrated by successive washing with increasing ethanol concentrations, and air-dried.

Following application of the labeled cRNA probes, sections were coverslipped and incubated in a humid chamber at 58°C for 16 h. Thereafter, cover slips were removed in $2 \times \text{SSC}$ (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). The sections were then treated with RNase A (20 µg/ml) and RNase T₁ (1 U/ml) at 37°C for 30 min. Successive washes followed at RT in $1 \times$, $0.5 \times$, and $0.2 \times \text{SSC}$ for 20 min each and in $0.2 \times \text{SSC}$ at 65°C for 1 h. For digoxigenin-labeled probes, sections were rinsed with P1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and then incubated for 2 h in blocking solution provided by the manufacturer of the kit. After incubation overnight with an anti-digoxigenin antibody conjugated with alkaline phosphatase (1:1000 dilution; Roche), the tissue sections were washed with P1. Staining proceeded for 2–6 h in substrate solution containing nitroblue tetrazolium chloride (340 µg/ml NBT; Biomol, Hamburg, Germany), X-phosphate (175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate; Biomol), 100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl₂ (pH 9.5). For radioactive probes, the tissue was dehydrated and exposed to Biomax MR Film (Kodak, Sigma-Aldrich) for 48 h. For microscopic analysis, the sections were dipped in NTB2 (Kodak, Integra Biosciences) nuclear emulsion and stored at 4°C . After exposure for 14 days, autoradiograms were developed in D19 (Kodak, Sigma-Aldrich) for 4 min and fixed in Rapid Fix (Kodak, Sigma-Aldrich) for 4 min. If required, sections were counterstained with Cresyl violet and then photographed

under dark- or brightfield illumination. Sense probes that were used to confirm the specificity of the hybridization reaction did not show any signal (data not shown).

Western blot of annexin A5

Three pituitaries of each genotype were pooled and homogenized in 50 µl of 100 mM HEPES (pH 7.0). After centrifugation for 3 min at 8000 g, 10 µl supernatant were applied on a 7% SDS-polyacrylamide gel. Proteins were subsequently transferred to a nitrocellulose membrane (Optitrans BA-S 83; Schleicher and Schuell, Dassel, Germany) which was blocked by 3% BSA in PBS. Annexin A5 was detected with a polyclonal rabbit anti-annexin A5 antibody (1:3000; Hyphen, Biomed, Neuville, France) and a rabbit polyclonal anti-β-actin antibody (1:5000; Abcam, Cambridge, UK) was used for normalization. Primary antibodies were visualized using a horseradish peroxidase-coupled secondary antibody (1:30 000; Promega) and the ECL detection system (Pierce, Rockford, IL, USA).

Determination of serum LH/FSH

Serum levels of FSH and LH were determined as described elsewhere (Haavisto *et al.* 1993, van Casteren *et al.* 2000).

Results

Expression of annexin mRNA in pituitaries of wild-type and Pax8^{-/-} animals

Quantitative real-time PCR was used to analyze in wild-type and athyroid Pax8^{-/-} mice the transcript levels of all 12 annexins (Fig. 1). mRNA expression of annexins A3, A7, A8, A9, A11, and A13 was in all analyzed pituitaries below detection limits; and annexins A2, A4, and A6 were found to be equally expressed in the pituitaries of wild-type and

Figure 1 mRNA expression of all 12 annexins in the pituitaries of wild-type and Pax8^{-/-} mice at 3 weeks of age as analyzed by real-time PCR. Values (mean $\pm$ S.E.M.) were normalized against cyclophilin (* $P < 0.05$; ** $P < 0.005$).

Pax8^{-/-} mice. The mRNA levels of annexins A1 and A10 were significantly upregulated under athyroid conditions by a factor of 5 and 30 respectively. In comparison, annexin A5 transcript levels were found to be decreased by half.

Analysis of annexins A1, A5, and A10 mRNA expression in the pituitary

In order to validate the quantitative real-time PCR data, we determined the mRNA expression pattern of annexins A1, A5, and A10 in pituitary sections of wild-type and Pax8^{-/-} by ISH histochemistry. In agreement with the real-time PCR results, A1-specific labeling in wild-type pituitary sections was close to background, whereas in Pax8^{-/-} mice a scattered expression pattern was observed throughout the anterior lobe (Fig. 2). In contrast, A5 was found to be ubiquitously expressed throughout the anterior pituitary of wild-type mice. These signal intensities were visibly reduced

in Pax8^{-/-} animals; however, higher mRNA expression was still found in areas bordering the intermediate lobe. The downregulation of A5 expression in pituitaries of Pax8^{-/-} mice was not only restricted to the mRNA levels, but could also be reproduced by western blot analysis (Fig. 3). With an antibody against A5 (Brachvogel *et al.* 2003), a prominent band of 36 was clearly visible in pituitary homogenates of wild-type animals, whereas in Pax8^{-/-} pituitaries the A5-specific band was very faint. Finally, in agreement with our real-time PCR data, ISH analysis also revealed that A10 transcripts are not detectable in the pituitaries of wild-type mice, whereas in the pituitaries of Pax8^{-/-} animals very intense and highly clustered signals of A10 mRNA expression were observed (Fig. 2). These signals colocalized with TSH mRNA indicating that A10 is mainly expressed in thyrotropic cells (Supplementary Fig. 1, which can be viewed online at <http://joe.endocrinology-journals.org/content/vol195/issue3/>).

Figure 2 mRNA expression of annexins A1, A5, and A10 in the pituitaries of 3-week-old wild-type and Pax8^{-/-} mice as analyzed by *in situ* hybridization histochemistry. Slides were viewed under darkfield illumination. Hybridization signals were not observed with the corresponding sense probes (not shown). Scale bar, 500 μ m.

Figure 3 Analysis of annexin A5 protein in pituitaries of wild-type and Pax8^{-/-} mice at 3 weeks of age by western blot analysis. Antibodies against β -actin were used to confirm adequate protein loading.

Regulation of Adenohypophyseal annexins A1, A5, and A10 mRNA expression by TH

Changes in transcript levels of A1, A5, and A10 as observed by real-time PCR and ISH could be either due to a direct regulation of these annexins by TH or indirectly due to the altered cellular composition of the pituitary in Pax8^{-/-} mice. In order to differentiate between these two options, we treated Pax8^{-/-} mice with physiological doses of T₄ for 3 days, a time period too short to restore normal cell composition. Quantitative real-time PCR revealed that this treatment rapidly normalized the mRNA expression of all three annexins (Fig. 4), strongly indicating that the transcript levels of these annexins are directly regulated by TH.

Pituitary hormone expression in annexin A5-deficient mice

The high expression of annexin A5 in pituitaries of euthyroid control animals prompted us to analyze whether deletion of A5 would affect pituitary hormone expression. Our ISH analysis of hypophyseal hormone expression in A5-deficient mice revealed that compared with controls, the number of FSH- β expressing cells was reduced and, for LH, a tendency of lower expression was noted. The mRNA expression patterns of TSH- β , GH, and PRL were not changed (Fig. 5). These results were validated by quantitative real-time PCR (Fig. 6). Compared with wild-type controls, only the β -FSH mRNA levels were significantly reduced by half in pituitaries of A5^{-/-} mice. β -LH mRNA levels also showed a tendency towards a decrease in A5-deficient animals, but the difference did not reach statistical significance. When the serum levels of the gonadotropins were analyzed by RIA, we also did not find significant differences between the two genotypes (LH: wt 0.18 ± 0.08 ng/ml versus A5^{-/-} 0.26 ± 0.12 ng/ml; FSH: wt 39.61 ± 2.51 ng/ml versus A5^{-/-} 43.63 ± 2.48 ng/ml; values in mean $\pm$ S.E.M., $P > 0.5$). The mRNA transcript levels of all other hormones were found to be unaltered.

Discussion

As in athyroid Pax8^{-/-} mice, the cellular composition of the anterior pituitary is dramatically distorted and various forms of

Figure 4 mRNA expression of annexins A1, A5, and A10 in the pituitaries of 3-week-old wild-type, untreated Pax8^{-/-}, and thyroxine (T₄)-treated Pax8^{-/-} mice as assessed by real-time PCR (** $P < 0.005$; *** $P < 0.001$).

pathological processes alter the expression of most annexins (Gerke & Moss 2002). We were interested in analyzing the expression of the annexins in pituitaries of athyroid Pax8^{-/-} mice and control animals. Of the 12 annexins known, expression of only six members of the annexin family could be reliably detected using real-time PCR. Using this method, mRNA expression of the annexins A3, A7, A8, A9, A11, and A13 was neither detected in the pituitaries of Pax8^{-/-} mice nor in those of control animals. While transcripts of the annexins A2, A4, and A6 were clearly detectable but not significantly different in the mutants compared with wild-type mice, mRNA expression of A1 and A10 was found to be highly upregulated and A5 expression significantly downregulated in the athyroid animals compared with controls. The reciprocal regulation of A1 and A5 corresponds nicely with their opposite roles in the PKC-dependent release of pituitary peptides after glucocorticoid stimulation of rat pituitary cells (John *et al.* 2002). Interestingly, the expression levels of A1, A5, and A10 rapidly

Figure 5 *In situ* hybridization analysis of pituitary hormone expression in typical sections of wild-type and annexin A5-deficient mice at 3 weeks of age. TSH, β -thyroid-stimulating hormone; GH, growth hormone; PRL, prolactin; LH, β -luteinizing hormone; FSH, β -follicle-stimulating hormone; POMC, proopiomelanocortin. Scale bar, 500 μ m.

normalized when the athyroid mutants were treated for 3 days with physiological doses of T_4 (18 ng/g body weight daily), thus indicating that the gene transcription of these annexins is directly regulated by TH. Our finding fits well with the data of a previous study describing a direct action of TH on the expression of the annexins A1, A2, and A5 in the thyroid gland of rats and thyroid cells in culture (el Btaouri *et al.* 1996).

Figure 6 Analysis of pituitary hormone mRNA expression in wild-type and annexin A5-deficient animals using quantitative real-time PCR. Values (mean $\pm$ S.E.M.) were normalized against cyclophilin. Wild-type mRNA levels were set to 1 for all hormones. For the abbreviations used, please see legend to Fig. 5 (* $P < 0.05$).

In the pituitary, our studies complement previous reports describing the influence of peripheral hormones on A1 expression such as an augmentation by glucocorticoids and an inhibition by estrogen (Philip *et al.* 1997, Morris *et al.* 2002, John *et al.* 2004). Together with the fact that annexin A1 protein is found abundantly in folliculo-stellate cells (Traverso *et al.* 1999) but not in the endocrine cells of the pituitary, these data fit well with the concept that annexin A1 acts as a paracrine or juxtacrine mediator of pituitary hormone secretion (John *et al.* 2004). This interpretation is further supported by an apparently low turnover of the A1 protein. As shown here, A1 mRNA in the pituitary is expressed at very low levels while A1 protein is known to be present in large amounts and easy to detect immunohistochemically in folliculo-stellate cells (Traverso *et al.* 1999). Via the intrapituitary communication system established by these cells (Fauquier *et al.* 2001, 2002), the multihormonal regulation of annexin A1 expression might thus provide an integrative mechanism to balance pituitary hormone secretion according to the overall hormonal condition of the organism. This hypothesis fits well with the observation that annexin 1 is required for glucocorticoids to exert an inhibitory effect on the release of TSH (Taylor *et al.* 1995).

Annexin A5 exhibits the highest transcript levels of all annexins expressed in the anterior pituitary and our analysis by *in situ* hybridization histochemistry revealed a rather ubiquitous distribution of A5 transcripts among all cells of the anterior pituitary. Compared with control animals, the athyroid Pax8^{-/-} mice exhibited significantly decreased A5 mRNA levels which rapidly normalized after treatment with physiological doses of T_4 . Together with the fact that ovariectomy increases the expression of A5 in rat pituitary gonadotropes (Kawaminami *et al.* 1998b), this demonstrates again that the expression of annexin A5 is also controlled by more than one peripheral hormone and thereby indicates that A5 might also serve modulatory functions.

Immunohistochemical studies previously suggested that A5 is expressed preferentially but not exclusively in pituitary

gonadotropes (Kawaminami *et al.* 1998a, 2004) and *in vitro* studies indicated that A5 not only enhances gonadotropin secretion following stimulation by gonadotrophin-releasing hormone (GnRH; Kawaminami *et al.* 2002), but also stimulates basal PRL secretion while counteracting thyrotrophin-releasing hormone (TRH) induced PRL release albeit at exceedingly high concentrations (Kawaminami *et al.* 2004). However, *in vivo* studies deciphering a biological function of A5 in the pituitary or other tissues are still missing and mice with a targeted disruption of the A5 gene have not revealed any obvious abnormalities (Brachvogel *et al.* 2003), presumably due to the redundant function of other family members. In agreement with the inconspicuous phenotype of the A5-deficient animals, our analysis of the pituitaries from A5-deficient mice revealed only some reduction in the expression of β -FSH mRNA, while the transcript levels of the other pituitary hormones were not significantly altered. As β -FSH and β -LH are both produced in the gonadotropes, one would also expect the levels of β -LH to be lowered. Although such a tendency could be observed in A5-deficient mice, this difference did not reach statistical significance. This could be either due to the high biological variance in LH levels or to a greater influence of A5 on β -FSH than on β -LH expression. The predominant regulation of one gonadotropin is remarkable, but not unusual as it also occurs for other modulating factors such as steroidogenic factor 1 (Brown & McNeilly 1999). Although we cannot exclude at present the possibility that A5 may be also involved in secretory processes, the difference in β -FSH mRNA expression does not seem to be of major biological relevance, since the gonadotropin serum levels are not altered and the reproductive capacity of A5-deficient mice is not impaired (Brachvogel *et al.* 2003).

Among all annexins expressed in the pituitary, A10 mRNA expression, almost undetectable in control animals and T₄-treated Pax8-deficient mice, is most strongly influenced by the athyroidism of the mutants. Unfortunately, very little is known about the role of annexins A10 and A10 knockout mice have not been generated to date to elucidate the biological function of this annexin; therefore, there is no basis for any speculation.

In agreement with previous studies in the field of annexin research, our analysis of TH regulated annexin expression in the pituitary supports the concept that these molecules might function as modulators rather than as essential factors of different endocrine processes. In the belief that the functions of specific annexins are concealed by possible redundancies in the family, there is hope that the generation of animals deficient in multiple annexins might provide a clue as to the biological role of these intriguing molecules in pituitary physiology.

Acknowledgements

We thank Melanie Kraus and Petra Affeldt for excellent technical assistance, Ahmed Mansouri and Peter Gruss for kindly providing Pax8^{+/−} animals, and Valerie Ashe for linguistic help. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 11 September 2007

Accepted 13 September 2007

Made available online as an Accepted Preprint

13 September 2007