Exocytotic protein components in rat pituitary gland after long-term estrogen administration

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Abstract

Recently, a set of proteins involved in the docking and fusion machinery of secretory organelles has been identified in anterior pituitary cells. In this study we analyzed, by Western blotting and immunocytochemistry, the expression of several proteins involved in exocytosis after long-term administration of 17β-estradiol (E_2) in Fischer 344 rats. No differences were observed in the amount of synaptosomal-associated protein of 25 kDa, synaptobrevin 2, syntaxin 1, synaptotagmin I and Rab3a in total brain homogenates from treated rats after E_2 administration. In striking contrast, the levels of all of these exocytotic proteins, including cellubrevin, were notably decreased in pituitary glands of E_2-treated rats. In addition, no differences were observed in the *in vitro* basal and 8-Br-cAMP-induced prolactin (PRL) release between pituitary cells from control and E_2-treated rats, whereas TRH-induced PRL release in anterior pituitary cells from E_2-treated animals was higher than in control donors. In conclusion, this study shows that protein components of the exocytotic machinery are specifically down-regulated in the pituitary gland of E_2-treated Fischer 344 rats.

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Introduction

In regulated exocytosis, secretory vesicles fuse with the plasma membrane in response to a physiological stimulus. Neuronal and endocrine cells are highly specialized cells that release their chemical signals for intercellular communication by regulated exocytosis. Membrane depolarization or the action of a secretagogue usually induces a transient rise in cytoplasmic calcium concentration, which triggers exocytosis (Burgoyne & Morgan 1993).

Insights have recently been made into the molecular mechanisms underlying exocytosis in neuronal cells. A set of proteins involved in the docking and fusion of the two neuronal secretory organelles (synaptic vesicles and large dense-cored vesicles) has been identified. Some of these synaptic proteins, like synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin, are located at the plasma membrane, whereas others, such as synaptobrevin, synaptophysin, Rab3A, synapsin and synaptotagmin, are located on synaptic and large dense-cored vesicle membranes (for review see Südhof 1995, Linial & Parnas 1996). In addition, the soluble proteins N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment protein (SNAPs) are essential for membrane trafficking (Rothman 1994). According to the SNAP receptors (SNARE) hypothesis, a suitable interaction between plasma membrane proteins (t-SNAREs: SNAP-25 and syntaxin) and vesicular membrane proteins (v-SNAREs: synaptobrevin) with the soluble factors (NSF and SNAPs) is essential for the initiation of the fusion process (Söllner *et al.* 1993*a*,*b*, Rothman 1994). Moreover, the identification of isoform families of most synaptic proteins, including non-neuronal isoforms (e.g. cellubrevin as a synaptobrevin isoform), provides a further level of complexity (Linial 1997).

Many of the above proteins were originally identified as neuronal-specific components due to their abundant expression in nervous tissues. However, a functional role for these proteins and their non-neuronal isoforms has been proposed in different endocrine cell types (Lang *et al.* 1997, Morgan & Burgoyne 1997). These molecular components of the exocytotic machinery have also been identified in anterior pituitary cells (Wendland & Scheller 1994, Marquéze *et al.* 1995, Oho *et al.* 1995, Redecker *et al.* 1995, Aguado *et al.* 1996, Jacobsson & Meister 1996, Majó *et al.* 1998). In adenohypophyseal cells, t-SNAREs are located at the plasma membrane, and protein components of neuronal secretory organelle membranes are located on hormone-containing secretory granules and synaptic-like microvesicles (SLMV) (Wendland & Scheller 1994, Aguado *et al.* 1996, Jacobsson & Meister 1996, Majó

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Table 1 Primary antibodies used in this study

	Raised in	Code	Work dilution	Source and/or reference
Antibody to				
PRL	Rabbit	AFP425-10-91	1:3000	A F Parlow, NIDDK
GH	Rabbit	AFP4115	1:3000	A F Parlow, NIDDK
βLH	Rabbit	AFP22238790	1:3000	A F Parlow, NIDDK
α-tubulin	Mouse	T9026	1:2000	Sigma
SNAP-25	Mouse	SMI-81	1:1000	Sternberger Monocl. Inc. (Baltimore, MA, USA)
Synaptobrevin 2	Rabbit	18.2	1:2000	R Jahn (Max-Plank Institute, Gottingen, Germany), Edelmann *et al.* (1995)
Cellubrevin*	Rabbit	CB35	1:200	Majó *et al.* (1998)
Synaptotagmin I	Mouse	c1.41.1	1:3000	R Jahn, Brose *et al.* (1992)
Syntaxin 1	Mouse	HPC-1	1:2000	Barnstable *et al.* (1985)
Rab3A	Mouse	c1.42.2	1:1000	R Jahn, Matteoli *et al.* (1991)
Rab 5	Mouse	c1.621.2	1:500	R Jahn, Fischer-von-Mollard *et al.* (1994)
Na^{+}/K^{+}-ATPase	Rabbit	06-167	1:1000	Upstate Biotech. Inc. (Lake Placid, NY, USA)
Synaptophysin	Mouse	S5768	1:2000	Sigma

*The antibody raised against cellubrevin also recognizes its homolog synaptobrevin.

et al. 1998). It is thought that secretory granules are the counterpart of neuronal large dense-cored vesicles and SLMV of synaptic vesicles (Jahn & De Camilli 1991). Furthermore, the functional participation of some of these components in pituitary hormone exocytosis has been demonstrated. It has been shown that SNAP-25 is essential in the regulated release of adrenocorticotropin (ACTH) and prolactin (PRL) from AtT-20 and GH3 cell lines respectively (Aguado *et al.* 1997, Masumoto *et al.* 1997). In addition, different members of the Rab family are involved in secretory granule localization and calcium-induced exocytosis in anterior pituitary cells (Lledo *et al.* 1993, Nsee *et al.* 1993, Lledo *et al.* 1994, Perez *et al.* 1994).

It has been shown that SNAP-25 is overexpressed in human PRL cell adenomas (Majó *et al.* 1997). Since prolactinomas represent the most commonly occurring type of pituitary tumor in humans (Kovacs *et al.* 1977), it is important to study the possible regulation and functionality of the exocytotic molecular components in PRL-secreting tumor cells. The aim of this study was to analyze, by Western blotting and immunocytochemistry, the expression of several proteins involved in exocytosis during PRL-cell tumorigenesis induced by long-term 17β-estradiol (E_2) administration in Fischer 344 (F344) rats. In addition, we also compared the PRL secretion rates of control and E_2-treated pituitary cells.

Materials and Methods

Animals

Adult F344 female rats weighing 130–150 g (Criffa S.A., Barcelona, Spain) were housed in a controlled environment (22 °C; 12 h light/12 h darkness; 40–60% humidity) with food and water available *ad libitum*. Under deep anesthesia (ketamine hydrochloride 20 mg/kg, Parke-Davis, Morris Plains, NJ, USA) 30 animals were ovariectomized bilaterally and implanted subcutaneously with 1-cm silastic capsules (Dow-Corning (Midland, MI, USA); outside diameter, 3·125 mm; inside diameter, 1·875 mm) containing 17β-estradiol (Sigma, St Louis, MO, USA). Thirty-five ovariectomized control rats were implanted with blank silastic capsules. Animal surgery and care were carried out in accordance with the European Community Council Directive (86/609/EEC). Rats were killed at different times from one week up to seven weeks after treatment.

Western blotting analysis

The rats were decapitated and the pituitaries were excised. After removal of the neurointermediate lobe, the anterior pituitaries were homogenized with 10 mM HEPES pH 7·4, 1 mM EGTA, and 0·5 mM phenylmethylsulfonyl fluoride (Sigma). Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (12·5% SDS-PAGE) was carried out as described by Laemmli (1970) using a mini-protean system (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were electrotransferred to nitrocellulose membranes (Amersham Iberica, Madrid, Spain) according to Towbin *et al.* (1979). The membranes were blocked in a solution consisting of 5% non-fatty milk powder in TBS (140 mM NaCl, 50 mM Tris–HCl pH 7·4 with 0·1% Tween-20) for 1 h at room temperature and then incubated at 4 °C overnight with the appropriate primary antibody diluted in blocking buffer. The primary antibodies used are shown in Table 1. After several washes with blocking solution, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody, and peroxidase activity was detected using the enhanced chemiluminescent method (ECL, Amersham).

Immunocytochemistry

Under deep diethyl ether anesthesia, the rats were perfused through the heart with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0·1 M phosphate buffer, pH 7·4. The pituitaries were removed, post-fixed for 4 h in the same fixative solution and cryoprotected with 30% sucrose solution in 0·1 M phosphate buffer at 4 °C overnight. Sections (40 µm) were obtained with a cryostat and collected in PBS. Pituitary sections were soaked for 1 h in PBS containing 10% methanol and 3% H_2O_2, washed in PBS and preincubated for 1 h in 5% fetal calf serum in PBS containing 0·3% Triton X-100 and 0·2% gelatin. Overnight incubations with the primary antibodies indicated in Table 1 were carried out at 4 °C in PBS containing 1% fetal calf serum, 0·3% Triton X-100 and 0·2% gelatin. The immunocytochemical reaction was developed with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The peroxidase complex was visualized by incubating the sections with 0·05% diaminobenzidine and 0·01% H_2O_2 in PBS. Sections were mounted, dehydrated and coverslipped in DPX. The specificity of the immunoreaction was tested by omitting the primary antibody or by replacing it with an equivalent concentration of non-specific rabbit or mouse immunoglobulin G. No immunostaining was observed in these sections. Double-labeling immunocytochemistry was carried out in sections to detect different proteins and pituitary hormones simultaneously by a combined light microscopic procedure according to Levey *et al.* (1986). The immunostaining of SNAP-25 was performed using the monoclonal antibody SMI-81, as described above for single labeling studies. After further rinsing, sections were processed to reveal the presence of growth hormone (GH) or PRL using polyclonal antibodies to rat hormones (1:1000). The immunocytochemical reaction, after peroxidase–anti-peroxidase procedures was developed in a medium containing 0·01% benzidine dihydrochloride (Sigma) and 0·025% sodium nitroprusside (Merck, Darmstadt, Germany) in PBS pH 6 for 10 min, and then in the same solution containing 0·05% H_2O_2 until the signal developed. The double labeling was done both ways round with equivalent results.

Primary cell cultures and RIA for PRL

Short-term pituitary cell cultures were prepared according to a modification of the method of Ben-Jonathan *et al.* (1983) as described previously (Aguado *et al.* 1996). Seven weeks after surgery, control and treated ovariectomized animals were decapitated, the pituitary glands were removed and the neurointermediate lobes discarded. After rinsing in Krebs-Ringer's bicarbonate buffer without Ca^{2+} and Mg^{2+} (KRBGA), the tissues were cut into small pieces. The fragments were incubated in KRBGA containing 0·2% trypsin (Sigma) for 1 h in a metabolic incubator at 37 °C under CO_2 and dissociated mechanically by gentle passage through a Pasteur pipette. The tissue suspension was harvested by centrifugation and incubated in KRBGA with DNAase (1 mg/ml; Sigma) for 5 min. Dispersed individual cells were harvested by centrifugation and resuspended at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Life Technologies, Gaithersburg, MD, USA). Viability, as evaluated by Trypan Blue exclusion, was greater than 90%. Finally, 10^5 cells/well were seeded in 24-well tissue culture plates (Costar, Acton, MA, USA) coated with poly-D-lysine (Sigma) to minimize cell loss, and cultured in a metabolic incubator at 37 °C with 5% CO_2 and 95% air for two days. Before the experiments, the cells were rinsed twice with serum-free Dulbecco's modified Eagle's medium and then incubated at 37 °C in 5% CO_2 in 0·5 ml of the same medium containing the appropriate test substances. After two hours incubation, conditioned medium was collected from each well, centrifuged at 14 000 ***g*** for 5 min, and stored at −20 °C until assayed for PRL content. Attached cells were washed in PBS and fixed with 4% paraformaldehyde in 0·1 M phosphate buffer, pH 7·4 for 30 min. Following further rinsing, immunocytochemical procedures to identify PRL-positive cells were carried out as described above for tissue sections. Incubation of the cells with 1 µM thyrotropin-releasing hormone (TRH) and 5 mM 8-Br-cAMP (Sigma) for 1 h was used to stimulate secretion. All tests were done at least in triplicate in three independent experiments. PRL was determined by RIA using a National Pituitary Hormone Distribution Program (NIAMDD, Bethesda, MD, USA) rat hormone kit. The initial dilution of the antibody was 1:20 000. The double antibody method for separating the bound from the free fraction was used. Assay sensitivity was 3 ng/ml, while the intra-assay and interassay variations were 3% and 10% respectively. Data were analyzed by the Mann-Whitney U test to identify which groups had significant differences.

Results

Exocytotic machinery in estrogen-induced tumoral pituitary cells

One week of treatment with E_2 resulted in a significant increase in anterior pituitary weight (30 ± 2·82 mg vs 12·25 ± 2·87 mg; $P<0·02$) and pituitary PRL (Fig. 1). These differences were dependent on the time course of E_2 treatment (data not shown).

No differences were observed in the amount of t- and v-SNAREs in brain homogenates from E_2-treated rats 1, 3, and 6 weeks after surgery. Representative examples are shown in Fig. 2A. In striking contrast, the levels of all of these exocytotic proteins were notably decreased in total homogenates from pituitary glands of E_2-treated animals at

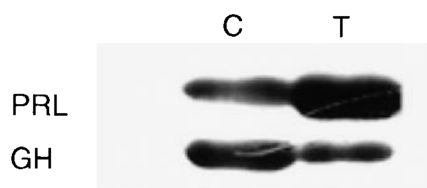

Figure 1 Western blotting analysis of PRL and GH in anterior pituitaries from control (C) and E_2-treated (T) animals one week after surgery. Each lane was loaded with 10 µg protein.

any time analyzed (Figs 2B and 3A). In addition, no changes were detected in the levels of the cytoskeletal component α-tubulin, the plasma membrane Na^+/K^+-ATPase, the SLMV marker synaptophysin, and the endosome marker linked to endocytotic pathway Rab5 (Gorvel *et al.* 1991) (Fig. 3B).

Immunocytochemical methods revealed that most of the pituitary cell population in the four-week E_2-treated rats consisted of PRL-positive cells (Fig. 4A,B), whereas immunoreactive cells for the rest of the hormones constituted a small percentage (data not shown). We found a lower immunostaining intensity for all the exocytotic protein components analyzed in most of the endocrine cells from pituitary glands of E_2-treated rats compared with controls. A representative immunostaining example is shown in Fig. 4C,D. Double-labeling immunocytochemistry showed a weak immunoreactivity for different exocytotic protein components in PRL-containing cells from E_2-treated animals compared with that found in

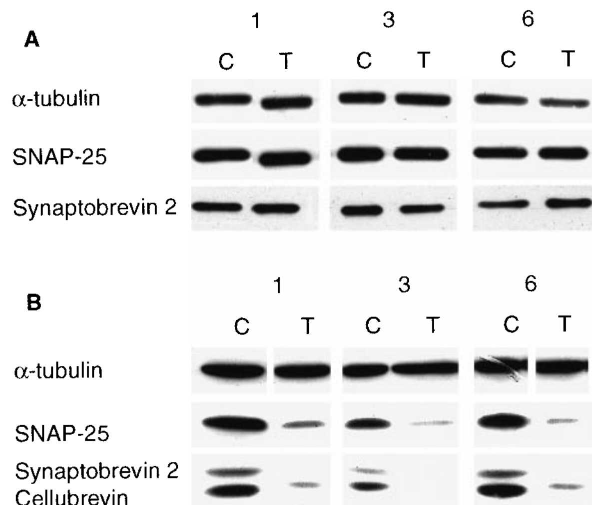

Figure 2 Western blotting analysis of α-tubulin, SNAP-25, synaptobrevin 2 and cellubrevin from (A) brain and (B) pituitary of control (C) and E_2-treated (T) rats 1, 3 and 6 weeks after surgery. In (A) a specific antibody against synaptobrevin 2 (18·2) was used; in (B) an antibody against cellubrevin (CB35), which also recognizes its homolog synaptobrevin was used. Note the specific decrease of SNAP-25 and synaptobrevin 2/cellubrevin in pituitary glands of E_2-treated rats.

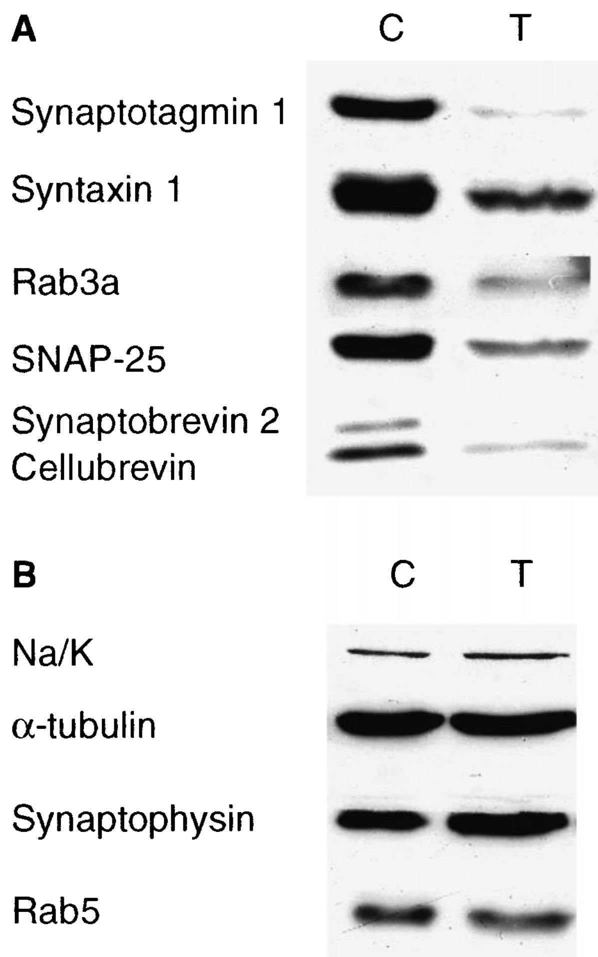

Figure 3 Western blotting analysis of different proteins from pituitary gland homogenates of control (C) and E_2-treated (T) rats 4 weeks after treatment. Several exocytotic proteins are decreased in pituitaries of E_2-treated rats (A), whereas no changes are observed in the amount of other proteins (B).

PRL-containing cells from control animals (Fig. 5A–C). It is noteworthy that in addition to the common immunostaining pattern for SNAP-25, associated with the plasma membrane, luteinizing hormone (LH)-containing cells from ovariectomized control animals consistently exhibited immunoreaction products for SNAP-25 in ring-shaped structures within the cells (Fig. 5D–F).

PRL secretion in E_2-treated pituitary in culture

In an attempt to compare the PRL release rates between normal and E_2-treated cells, we analyzed the amount of PRL released per number of PRL immunostained cells in pituitary primary cell cultures from control and E_2-treated animals seven weeks after surgery (Table 2). No differences were observed in the basal PRL release from

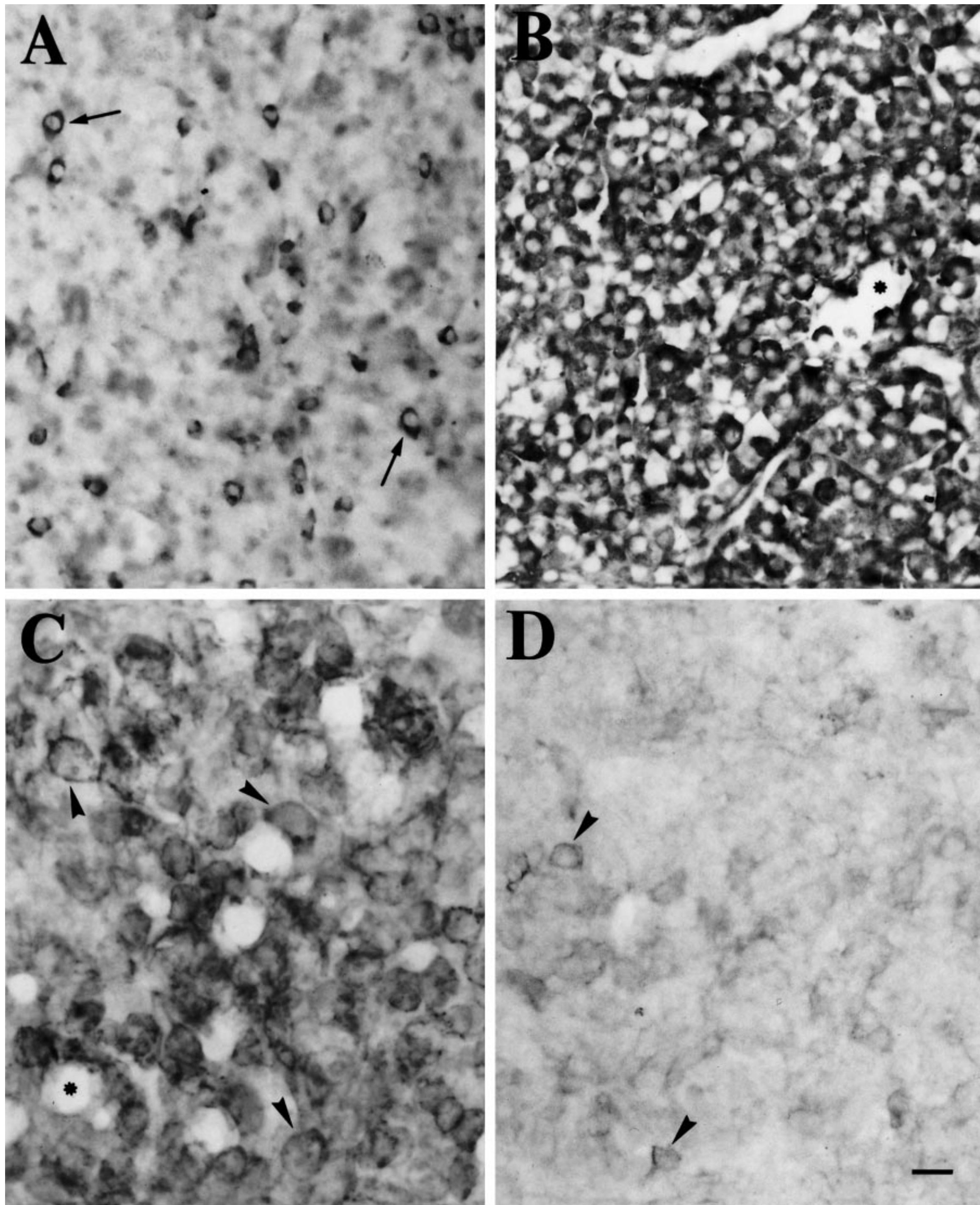

Figure 4 Photomicrographs of anterior pituitary sections of control (A,C) and E_2-treated (B,D) animals immunostained for PRL (A,B) and SNAP-25 (C,D). Note the increase in PRL-positive cells in pituitary glands of E_2-treated rats, whereas SNAP-25 is decreased. Arrows, PRL-positive cells; arrowheads, SNAP-25-positive immunostaining; asterisks, blood vessels. Scale bar: 20 µm.

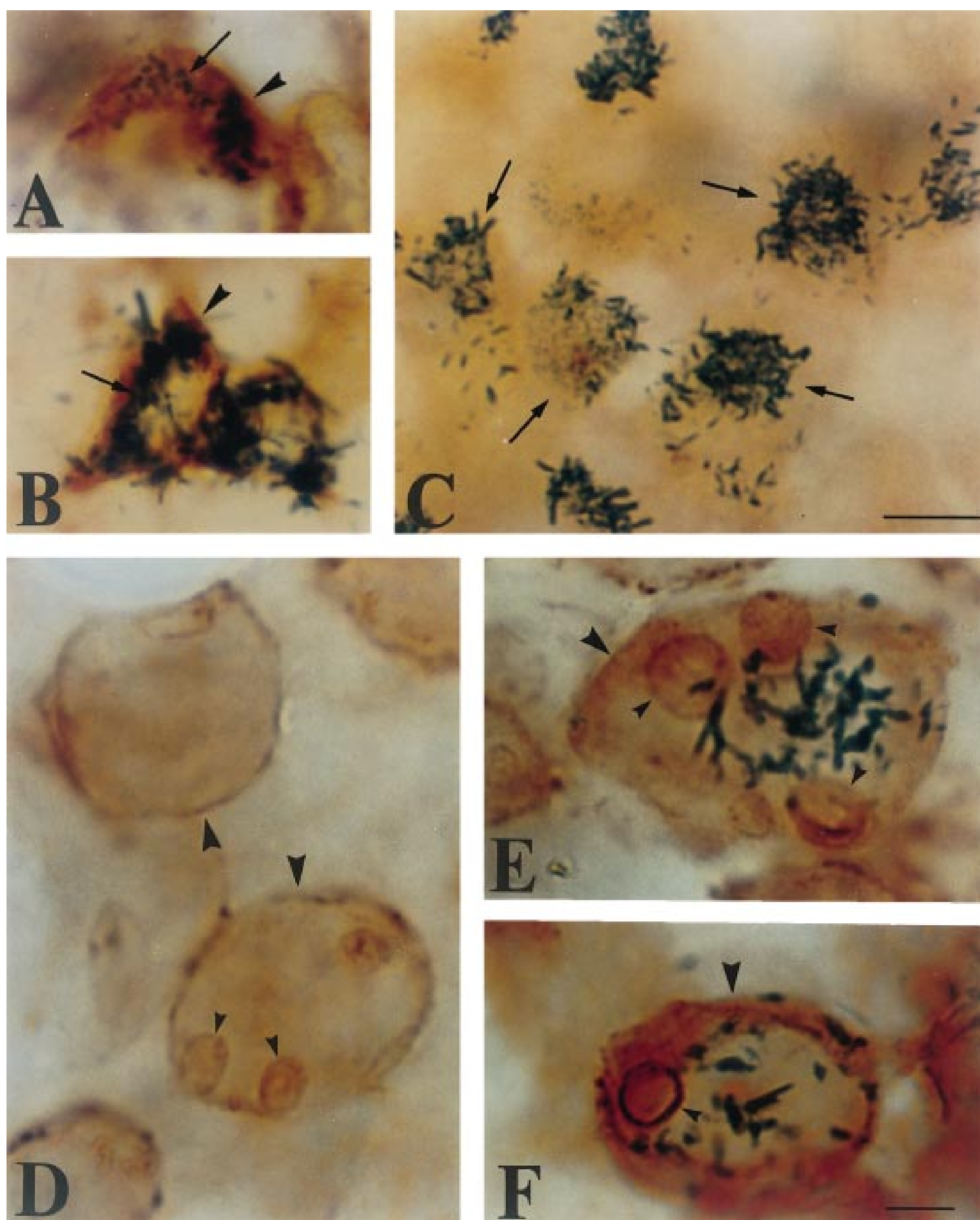

Figure 5 Double labeling immunocytochemistry of PRL (A–C) or LH (E,F) and SNAP-25 in control (A,B,D–F) and E_2-treated (C) pituitary sections 4 weeks after treatment. In all illustrations, SNAP-25 was visualized as a brown precipitate at the plasma membrane. PRL and LH immunoreactivity was detected as a granular blue precipitate located in cytoplasmic areas. A–C, PRL-positive cells (arrows) from control animals exhibit SNAP-25 immunoreactivity (arrowheads) (A,B). In E_2-treated pituitary, SNAP-25 immunostaining is near background in PRL-positive cells (arrows) (C). D–F, In addition to the plasma membrane (big arrowheads), SNAP-25 immunostaining is also located in ring-shaped structures within certain cells (small arrowheads) in ovariectomized control pituitary sections. Double labeling with LH (E,F) reveals the gonadotrope identity. Scale bars: A–C, 30 μm; D–F, 10 μm.

Table 2 PRL secretion from pituitary primary cell cultures of control and E_2-treated rats. Basal (non stimulated, $n=5$) and 8-Br-cAMP (5 mM, $n=4$) or TRH (1 μM, $n=4$)-induced PRL secretion was measured by RIA

	PRL release (pg/lactotrope cell)	
	Median	Range
Basal		
Control	0·41	0·272
E_2-treated	0·488	0·192
8-Br-cAMP		
Control	0·595	1·254
E_2-treated	1·084	0·319
TRH		
Control	0·497	0·518
E_2-treated	1·712	1·821

8-Br-cAMP significantly stimulated PRL secretion from pituitary cells from both control and E_2-treated rats ($P<0{\cdot}02$) whereas TRH significantly stimulated PRL secretion from pituitary cells from E_2-treated rats only ($P<0{\cdot}015$).

pituitary cells of control and E_2-treated rats. Incubation with the cAMP analog 8-Br-cAMP resulted in a similar increase in PRL secretion in cultured pituitary cells from control and E_2-treated animals. The rate of TRH-induced PRL secretion in anterior pituitary cells from control rats was not increased. However, TRH had a notable effect on the rate of PRL secretion in pituitary cell cultures obtained from E_2-treated animals (more than 3 times the basal secretion).

Discussion

In recent years, a group of protein components of the exocytotic machinery has been shown to be present in the rat anterior pituitary gland (Wendland & Scheller 1994, Marquéze *et al.* 1995, Oho *et al.* 1995, Redecker *et al.* 1995, Aguado *et al.* 1996, Jacobsson & Meister 1996, Majó *et al.* 1997, 1998). The amount and immunocytochemical location of all of these proteins shown in the present study agrees with previous reports. Interestingly, SNAP-25 immunoreactivity is also located in ring-shaped structures within gonadotrope cells from ovariectomized rats. Although the identity of these structures is still unknown, these may be related to the ultrastructural changes described in rat gonadotrope cells after ovariectomy (Garner & Blake 1981). Future electron microscopic studies may shed light on this issue.

In this study we have shown that long-term E_2 administration in F344 rats causes a decrease in SNAP-25, synaptobrevin 2, cellubrevin, syntaxin 1, synaptotagmin I, and Rab3a levels in the anterior pituitary gland. Since total brain homogenates show no differences in the levels of the exocytotic machinery component proteins after E_2 treatment, the decrease observed in the pituitary gland of E_2-treated animals seems to be specific. It has been shown that neonatal sex hormone manipulations can influence the expression of several synaptic proteins in the nervous system (Lustig *et al.* 1993). Administration of estrogen and estrogen inhibitor during the 'critical period' induces up- and down-regulation respectively of SNAP-25 mRNA expression (Lustig *et al.* 1993). The present results showing a lack of change in SNAP-25 protein levels after E_2 treatment in the adult brain may be related to the administration period. In addition, it is very likely that Western blotting analysis from whole brain homogenates could mask possible area-specific differences.

It is well established that long-term E_2 administration in rats produces PRL-cell adenomas and chronic hyperprolactinemia (McEuen *et al.* 1936, Meyer & Clifton 1956), with the F344 strain being particularly sensitive to developing E_2-induced pituitary tumors (Dunning *et al.* 1947, Lloyd 1983, Phelps & Hymer 1983). Ultrastructural examination of the E_2-induced pituitary tumor cells reveals that most of them are sparsely granulated, suggesting a low storage capacity for these cells (Lloyd 1983, Hymer & Motter 1988, G Majó, I Ferrer & F Aguado (personal observations)). Recent reports have shown that most exocytotic proteins located on synaptic vesicles are also present in secretory granules and SLMV in anterior pituitary endocrine cells (Wendland & Scheller 1994, Jacobsson & Meister 1996, Majó *et al.* 1998). Interestingly, the levels of most of the proteins located on secretory organelles are decreased in pituitary cells of E_2-treated rats, whereas no changes are observed for synaptophysin. Since synaptophysin is located exclusively on SLMV (Jahn & De Camilli 1991, Majó *et al.* 1998), the decrease in the levels of synaptobrevin 2, cellubrevin, synaptotagmin I, and Rab3a in E_2-treated pituitaries could reflect the low number of secretory granules in PRL cells of E_2-treated animals. In parallel with the levels of the exocytotic proteins present on secretory granules, the t-SNAREs, SNAP-25 and syntaxin 1 levels are also decreased in pituitary cells of E_2-treated rats. In agreement with this, a down-regulation of SNAP-25 expression in the rat pituitary has recently been shown after E_2 administration (Jacobsson *et al.* 1998). On the other hand, it has been reported that SNAP-25 immunoreactivity is increased in human prolactinomas (Majó *et al.* 1997).

Since several exocytotic proteins have been shown to be essential for adenohypophyseal hormone release (Lledo *et al.* 1994, Aguado *et al.* 1997, Masumoto *et al.* 1997), the observed low levels of these proteins in the pituitaries of E_2-treated rats may be correlated with the secretion rates. It has been established that the hyperprolactinemia observed in E_2-treated animals is due to the increased number of PRL cells produced by lactotrope proliferation (Lloyd 1983) and recruitment of nonsecreting cells into an active PRL secreting pool (Boockfor *et al.* 1986, Scarbrough *et al.* 1991), as well as the E_2-stimulated PRL synthesis (Scarbrough *et al.* 1991). Whether PRL release is increased or decreased in lactotropes from E_2-treated

animals is not well established yet. Our results show that neither the basal nor 8-Br-cAMP-induced PRL release in cultured cells from E_2-treated animals differs from that in control donors. These results agree with a previous report showing no differences in basal PRL secretion between E_2-induced adenoma and normal pituitaries, estimated by RIA (Yoshikawa *et al.* 1995). In addition, these authors found a smaller mean plaque area for E_2-treated cells from F344 rats using the reverse hemolytic plaque assay (RHPA), suggesting a lower PRL release rate in E_2-induced tumor cells (Yoshikawa *et al.* 1995). This observation could be in line with the low levels of exocytotic protein components detected in pituitary glands of E_2-treated rats. However, by using the same RHPA technique, other authors found that PRL release is increased in cultured lactotropes from estrogen-primed rats (Kendall & Hymer 1987, Lloyd *et al.* 1987, Zhang *et al.* 1990, Scarbrough *et al.* 1991). Although the meaning of these differences is unknown, they may be related to the rat strains and experimental conditions used.

We observed that TRH induced higher PRL secretion in cultured lactotropes from E_2-induced tumors than in any other condition tested. In like measure, it has been shown that pretreatment with E_2 enhances the TRH-stimulated PRL release in cultured rat lactotropes (Zhang *et al.* 1990, Scarbrough *et al.* 1991). The increased response to TRH observed in E_2-treated animals could be related to an increase in the number of TRH receptors (Gershengorn *et al.* 1979) or alterations in the intracellular transduction signaling (Tang *et al.* 1982).

In conclusion, this study shows that protein components of the exocytotic machinery are down-regulated in the pituitary of F344 rats by E_2 administration. The functional significance of the low exocytotic protein levels found in pituitary glands of E_2-treated animals within the exocytotic process remains to be elucidated.

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