Estradiol differentially modulates the exocytotic proteins SNAP-25 and munc-18 in pituitary gonadotrophs

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Abstract

Long-term treatment with estradiol increases LH secretion from female gonadotrophs. The mechanisms are not fully clarified yet. Our previous data indicated that sexual steroids might affect late steps in GnRH signal transduction such as exocytosis. The secretion of hormones from neuroendocrine cells requires the merger of secretory vesicles with the plasma membrane. This regulated exocytosis is mediated by specific proteins, which are present in the pituitary gland. Here, we examined whether two of these crucial exocytotic proteins, SNAP-25 and munc-18, are affected by estradiol in female gonadotrophs. Female rat anterior pituitary cells and α T3-1 cells, derived from a murine immortalized gonadotroph cell line, were treated with 100 pM estradiol for 48 h. LH secretion of anterior pituitary cells, additionally stimulated with eight consecutive pulses of 1 nM GnRH for 15 min at an interval of 1 h, was determined by RIA. Gene expression was measured by quantitative RT-PCR and protein expression by immunoblotting. Additionally, quantitative RT-PCR was performed in single rat gonadotrophs to ascribe effects exclusively to intact gonadotrophs. Pulsatile GnRH enhanced the mRNA expression of SNAP-25 and munc-18 in accordance with the LH secretory response with the greatest increase at the third pulse of GnRH. Estradiol treatment further increased GnRH-induced LH secretion at all GnRH pulses. *SNAP-25* gene expression was significantly decreased at the fifth GnRH pulse and unaffected at basal after 48 h of estradiol treatment. In contrast, *munc-18* mRNA levels were not significantly affected by estradiol at different GnRH-pulses in mixed anterior pituitary cells, whereas *munc-18* gene expression was significantly increased at basal. In α T3-1 cells and single gonadotrophs, long-term estradiol treatment significantly reduced SNAP-25 protein and gene expression. In contrast, the protein and gene expression of munc-18 was significantly enhanced in both α T3-1 cells and single gonadotrophs. In conclusion, munc-18 and SNAP-25 were oppositionally influenced by estradiol. The results suggest that estradiol modulates the expression of exocytotic proteins in gonadotrophs and thus affects LH secretion.

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Introduction

Previous studies demonstrated that long-term estradiol treatment of rat pituitary gonadotrophs increased basal and agonist-induced LH secretion. The mechanisms are not fully elucidated yet. Modulations of GnRH signal transduction such as calcium signaling and inositol phosphates are in part responsible for the effects of estradiol (Ortmann *et al.* 1992, 1995). It might well be that steroids affects late steps in GnRH signal transduction like exocytosis (Ortmann *et al.* 1998).

Exocytosis, the fusion of secretory vesicles with the plasma membrane, is the final step of signal transduction in neuroendocrine cells such as gonadotrophs to release hormones. Before the secretory vesicles, processed at the trans-Golgi region of the cells, fuse with the plasma membrane, they undergo several modifications such as translocation (transport close to the plasma membrane), docking (attachment to the

plasma membrane), and priming (maturation that makes them ready for the final Ca^{2+} -triggered step; Stojilkovic 2005). Functional studies have provided intriguing details into how SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) proteins interact with each other to generate the fusion of lipid bilayers. Regulated exocytosis is controlled and mediated by SNARE proteins. They play a crucial role in the intracellular membrane fusion (Rettig & Neher 2002). SNARE proteins are either residing in the vesicle membrane (v-SNAREs like synaptobrevin=vesicle-associated membrane protein (VAMP) and synaptotagmin; Borisovska *et al.* 2005) or in the plasma membrane (syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25); Sorensen *et al.* 2002). SNAREs bind to each other by forming a very stable four-stranded coiled-coil core complex, constituted by one coil each from syntaxin and VAMP, and two coils from SNAP-25 (Chen & Scheller 2001).

Recently, many proteins have been identified that participate in the exocytosis of the pituitary gland (Thomas *et al.* 1998). Their exact function, however, remains unclear. SNAP-25, originally identified at neuronal synapses, was found in pituitaries (Aguado 1996, Jacobsson & Meister 1996, Kolk *et al.* 2004). Since the antisense oligonucleotide of SNAP-25 inhibited TRH-induced prolactin release, SNAP-25 is essential for the release of prolactin in pituitary cell lines (Masumoto *et al.* 1997). Immunoreactivity of SNAP-25 is enhanced in human prolactinomas as well (Majo *et al.* 1997). Ca^{2+} -induced LH secretion was significantly decreased after treatment with SNAP-25 antibodies. Furthermore, incubation with GnRH decreased SNAP-25 expression in western blots (Quintanar *et al.* 2004). Taken together, these results show that SNAP-25 is essential for the secretion of pituitary cells.

Munc-18 seems to be involved in all eukaryotic vesicle trafficking and fusion (Jahn 2000). It lacks membrane targeting domains and is located in the cytoplasm. Munc-18 binds tightly to syntaxin which blocks its interaction with SNAP-25. The dissociation of munc-18 allows the formation of a complex between syntaxin and SNAP-25. VAMP binds to syntaxin (Sudhof 1995). Plasma membrane and vesicle membrane are now capable of fusing and hormones are released. Munc-18 null mutant mice have no synaptic activity in neurons (Verhage *et al.* 2000). In the absence of munc-18, Ca^{2+} -triggered exocytosis of neuroendocrine cells is minimized, whereas the overexpression of munc-18 increased exocytosis (Voets *et al.* 2001). These facts demonstrate the importance of munc-18 in exocytosis. Although many details on munc-18 function in different cell types are known, the function of munc-18 in gonadotrophs and its interaction with estradiol remain unclear.

To shed light on the actions of estradiol, we examined for the first time the effects of estradiol on the exocytotic proteins SNAP-25 and munc-18 in female gonadotrophs. We concentrated on these proteins, because both are critical components for the exocytosis in pituitaries.

Materials and methods

Pituitary cell preparation and culture conditions

Pituitary cells were obtained from ovariectomized adult (200–250 g) female Sprague Dawley rats (Charles River, Kirchborchen, Germany). Single-cell suspensions were prepared by controlled trypsinization as described previously (Ortmann *et al.* 1990). Briefly, pituitary glands were collected after decapitation and neural lobe was separated. Anterior pituitaries were cut into small pieces. After digestion with 0.5% trypsin, they

were treated with DNase and 0.1% trypsin inhibitor. After gentle pipetting, dispersed cells were separated by centrifugation. Cell viability was determined using the Trypan Blue exclusion method (usually >95%). Cells were incubated in medium 199 (Biochrom KG, Berlin, Germany) with Hank's salts and L-glutamine, supplemented with 1.4 g/l sodium bicarbonate, 10 µg/ml streptomycin, 100 U/ml penicillin, and 10% horse serum, pretreated with 2% charcoal (Norit A) and 0.2% Dextran T 70 (Pharmacia, Uppsala, Sweden) to eliminate steroids. To allow cell attachment, cultures were incubated for 48 h in humidified incubators at 37 °C in an atmosphere of 5% CO_2 and 95% air.

Estradiol and GnRH treatment

After 48 h, cells were treated for another 48 h period with 100 pM estradiol in serum-free media. Estradiol was dissolved in ethanol and the final concentration of ethanol in the culture media was 0.2%. Controls received media containing 0.2% ethanol alone (vehicle). After 48 h treatment with estradiol, mixed anterior pituitary cells were washed and stimulated with eight consecutive GnRH pulses of 1 nM GnRH for 15 min at an interval of 1 h in a static culture system. GnRH pulses were administered by a pipette. After each GnRH pulse, media were removed and replaced by fresh media and kept for 45 min. Media for LH measurements were taken before and after each pulse. Only gonadotrophs react to their physiological agonistic stimulus GnRH. Therefore, we assume that the described effects could be ascribed to gonadotrophs.

LH measurement

Media were collected and analyzed for their hormone content by RIA, using the reference preparation RP-3 from the National Pituitary Agency and Dr Parlow (Harbor-University of California-Los Angeles Medical Center, Torrance, CA, USA). The intra- and inter-assay variations were below 8%.

α T3-1 cell culture

Since mixed populations of pituitary cells contain only between 5 and 10% of gonadotrophs we employed α T3-1 cells, an immortalized gonadotroph-derived cell line was kindly provided by Dr P L Mellon (San Diego, CA, USA), to save rats and to attribute effects to gonadotrophs. Cells were cultured on six-well dishes (500 000 cells/well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies, Karlsruhe, Germany) and 100 mg/l gentamycin (Biochrom KG). To allow cell attachment, cells were kept for 36 h in humidified

incubators at 37 °C in an atmosphere of 5% CO₂ and 95% air. At 70–80% confluency, cells were harvested after detachment with accutase (GibCO, Wiesbaden, Germany) and treated with 100 pM estradiol in serum-free medium for 48 h.

Immunoblotting

α T3-1 cells were lysed in RIPA buffer (containing 1 mM PMSF) for 1 h at 4 °C. Nuclei were removed by centrifugation. Protein concentration was determined according to the method of Bradford (Bio-Rad). Proteins were separated by SDS-polyacrylamide gels (SDS-PAGE), transferred on PVDF membranes and analyzed by immunoblotting as follows: the membranes were blocked in a solution of 5% milkpowder and 3% BSA for 1 h. Primary antibody was diluted in blocking buffer (SNAP-25, 1:3000; munc-18, 1:500) and incubated with the membrane overnight. After washing, peroxidase-conjugated secondary antibody (IgG 1:2000) was added for 1 h. Detection of peroxidase activity was performed with enhanced chemiluminescence hyperfilm and kits (Amersham, Freiburg, Germany).

Antibodies used

SNAP-25a (1:3000), BabCO, Berkley, CA, USA; munc-18-2 (1:500), Becton Dickinson, Heidelberg, Germany; β -actin (1:7500), Sigma; IgG (1:2000), Amersham, Braunschweig, Germany.

Quantitative RT-PCR

RNA was isolated from rat pituitary cells and α T3-1 cells using TRI reagent (Sigma) according to the manufacturer's instructions. Total RNA (0.5–2 μ g) was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technologies, Inc.) and 10 pM poly-(deoxythymidine) primer in a 10 μ l total volume reaction. The first cDNA synthesis reaction was heated to 55 °C for 5 min to inactivate the reverse transcriptase and then diluted with 10 mM Tris-HCl (pH 7.5) to a final volume of 100–300 μ l. An aliquot (4 μ l) of cDNA was used for quantification of gene expression. We used DNase digestion after RNeasy silica membrane-based RNA purification to remove any trace of DNA. Specific primers for LH, SNAP-25, and munc-18 were used (Tables 1 and 2). All primers were designed over introns based on the exon/intron gene structures. MgCl₂ concentrations were optimized. A typical reaction involved an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 50 °C for 1 min, and elongation at 60 °C for 30 s. A standard melting curve cycle was used to check the quality of amplification and to

Table 1 Primers for quantitative RT-PCR in rat pituitary cells

<i>β-Actin</i>	
Forward	5' CTCTTCCAGCCTTCCTTCCT 3'
Reverse	5' GCACTGTGTTGGCATAGAGG 3'
<i>SNAP-25</i>	
Forward	5' CCTCCACTCTTGCTACCTGC 3'
Reverse	5' TCCTCTGCATCTCCTCCAGT 3'
<i>Munc-18</i>	
Forward	5' CTGTGTGCCACTCTTCAGGA 3'
Reverse	5' TGTGAGCGTGTTTCTCTGG 3'

confirm the absence of primer dimer formation. To ensure accuracy, each reaction was repeated 2–5 times per sample. The mean value of the repeat was used for each gene per sample to calculate the ratio between the mean value of the target gene and the internal control gene. The relative level of each gene was expressed by the ratio of the number of transcripts of the gene to an internal control gene which was β -actin. The data were analyzed using the comparative threshold cycle method, because the efficiency of the target amplification was approximately equal to that of the β -actin amplification.

Single-cell quantitative RT-PCR

We have established a quantitative reverse transcriptase-PCR (RT-PCR) approach for the analysis of RNA transcript levels in single rat pituitary cells. This approach allowed us to trace back the effects exclusively to intact rat gonadotrophs. Primary pituitary cell cultures were dispersed in 60 mm tissue culture plates at a density of 1×10^4 cells and incubated as described before. Cells were detached with accutase (PAA-Laboratories GmbH Catalogue no. L11-007, Vienna, Austria). The pellet was washed twice in cold (4 °C) Dulbecco's PBS (without Mg²⁺ and Ca²⁺) and diluted in 1 ml 10% polyvinylpyrrolidone (PVP; Sigma P-5288) to ease cell collection in viscous medium.

A drop of the cell mixture was observed under an inverted microscope. Single cells were isolated with a micromanipulator fitted with a pulled microcapillary. A single cell was expelled into a PCR tube filled with

Table 2 Primers for quantitative RT-PCR in α T3-1 cells

<i>β-Actin</i>	
Forward	5' GCTCGTCGTCGACAACGGCTC 3'
Reverse	5' CAAACATGATCTGGGTCATCTTCTC 3'
<i>Mouse SNAP-25</i>	
Forward	5' CCTCCACTCTTGCTACCTGC 3'
Reverse	5' CTCCTCTGCATCTCCTCCAG 3'
<i>Mouse munc-18-2</i>	
Forward	5' ACTCCGCTGACTCTTTCCAA 3'
Reverse	5' TCTGGTACTGGGGCATTTTC 3'

10 µl ice-cold 1× PCR buffer and frozen immediately in –80 °C overnight. First-strand cDNA synthesis was performed using standard protocol with SuperScript II Reverse Transcriptase (Invitrogen). The PCR program consisted of the following cycles: one cycle (2 min at 50 °C and 2 min at 95 °C) followed by 50 cycles (95 °C for 15 s, 59 °C for 15 s, and 72 °C for 20 s.).

All primer pairs span at least one intron so that the PCR product could be distinguished by size from contaminating genomic DNA. All primer pairs were designed to anneal at 59 °C optimally. Primers are listed in Table 3. Real-time PCR was performed on a DNA Engine Opticon 2 System (MJ Research, Waltham, USA) in a volume of 25 µl including 12.5 µl 2× Platinum SYBR Green qPCR SuperMix (Invitrogen), 0.4 µM primer (final concentration) and 2 µl template cDNA. All components without cDNA, but DEPC water instead served as negative control. To ensure ‘hot start conditions’, the Platinum *Taq* DNA polymerase was complexed with a proprietary antibody that blocks polymerase activity at ambient temperatures. Activity is restored after a denaturation step in PCR cycling, providing an automatic hot start in PCR for increased sensitivity, specificity, and yield. PCRs were optimized for magnesium concentration, primer concentration, and annealing temperature. PCR products were verified with both melting curve analysis and DNA gel electrophoresis. Gonadotrophs were identified by simultaneous detection of LH-β, which is unique for gonadotrophs.

Quantification with gene-specific standard curves

Unknown amounts of molecules were determined from gene-specific standard curves using the Opticon Monitor Analysis Software (MJ Research). The cDNA standards were prepared from purified PCR products

Table 3 Primers for single-cell quantitative RT-PCR in rat gonadotrophs

Rat β-actin	
Product size	92 bp
Forward	5'-GCTCTCTTCCAGCCTT -3'
Reverse	5'-CGGATGTCAACGTCACACTT-3'
Rat LH-β	
Product size	99 bp
Forward	5'-ATCACCTTCACCACCAGCAT -3'
Reverse	5'- GTAGGTGCACACTGGCTGAG -3'
Rat SNAP-25	
Product size	102 bp
Forward	5'-AGCAGAGCTCACGTTGCATT-3'
Reverse	5'-CCTGCAGGTTAGCAAGAGTGG-3'
Rat Munc-18-2	
Product size	137 bp
Forward	5'-TATGAGGCCAGGTGTTCTC-3'
Reverse	5'-TCCTGAAGAGTGGCACACAG-3'

(NucleoSpin Extract II, Machery-Nagel, Düren, Germany). The purified PCR products were analyzed and quantified photometrically at 260 nm. Quantified DNA was diluted from 10⁷ to 10² molecules (standard curve).

Statistical analysis

Data obtained from 3–4 experiments run in triplicate were pooled, and all the results are expressed as percentage (%) of control (vehicle-treated), set to 100%. *P* values <0.05 were considered as statistically significant. Statistical differences between each experimental and the control group were analyzed using two-way ANOVA repeated measures and Bonferroni post-test, one-way ANOVA with Dunnett’s post-test or unpaired *t*-test respectively.

Results

Effects of GnRH pulses and estradiol on LH secretion and SNAP-25 and munc-18 gene expression in mixed anterior pituitary cells

LH secretion was highest and significantly higher than control (from 4 up to 18 ng/ml, *P*<0.01) at the fourth GnRH pulse. Estradiol further increased the LH response of mixed anterior pituitary cells to GnRH pulses with the highest LH secretion of 22 ng/ml (*P*<0.01) at the third GnRH pulse (Fig. 1A).

Pulsatile GnRH significantly increased the mRNA expression of SNAP-25 and munc-18 in untreated cells in accordance with the LH secretory response compared with basal (*P*<0.01 for SNAP-25 and munc-18 mRNA). The greatest increase at the third pulse of GnRH is significantly higher than that at all other GnRH pulses (*P*<0.001 for both proteins; Fig. 1B).

After 48 h of estradiol treatment, the gene expression of SNAP-25 was diminished, whereas munc-18 mRNA levels were significantly enhanced (*P*=0.012; basal in Fig. 2B).

SNAP-25 levels were significantly decreased by estradiol at the fifth GnRH pulse (*P*=0.028). In contrast, munc-18 mRNA was increased in estradiol-treated cells at the first and third pulse, but this increase did not reach statistical significance (Fig. 2B).

Presence of SNAP-25 and munc-18 in αT3-1 cells

Since it was not known whether SNAP-25 and munc-18 are present in αT3-1 cells, we performed immunoblots to identify these proteins in our cell line. We could demonstrate the presence of SNAP-25 and munc-18 in our αT3-1 cells (Fig. 3).

Figure 1 Effects of GnRH pulses and estradiol on LH secretion and the gene expression of *SNAP-25* and *munc-18*. (A) Mixed anterior pituitary cells were treated for 48 h with 100 pM estradiol. Cells were stimulated with eight pulses of 1 nM GnRH for 15 min at 1 h intervals. LH was measured by radioimmunoassay before and after each GnRH pulse. Data from six independent experiments performed in triplicate were pooled. Asterisk indicates significance against control. Two-way ANOVA (repeated measures) with consecutive Bonferroni post-tests were applied. (B) Gene expression of *SNAP-25* and *munc-18* from control cells were determined immediately after GnRH pulses 1, 3, 5, and 8. Levels of mRNA after 48 h (basal) were set to 100% as controls. Data from three independent experiments performed in triplets were pooled. Asterisk indicates significance against basal. ** indicates significance of the third pulse versus all other pulses for both *SNAP-25* and *munc-18* ($P < 0.001$). ANOVA with consecutive Dunnett's multiple comparison post-test were applied. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Effects of estradiol on *SNAP-25* and *munc-18* gene expression in α T3-1 cells

Figure 4A shows a significant decrease (-22%) in *SNAP-25* gene expression by treatment with 100 pM estradiol for 48 h ($P < 0.01$). On the contrary, the gene expression of *munc-18* was significantly increased ($+33\%$; $P < 0.05$) by 100 pM estradiol for 48 h (Fig. 4B). Incubation with 100 pM estradiol for only 24 h had no

Figure 2 Effects of GnRH pulses and estradiol on the gene expression of *SNAP-25* and *munc-18*. Mixed anterior pituitary cells were treated for 48 h with 100 pM estradiol and stimulated with eight pulses of 1 nM GnRH for 15 min at 1 h intervals. *SNAP-25* (A) and *munc-18* (B) mRNA levels were determined at basal (after 48 h of estradiol treatment) and directly after GnRH pulses 1, 3, 5, and 8. Gene expression of untreated cells at each GnRH pulse was set to 100%. Data from three independent experiments performed in triplicate were pooled. Asterisk indicates significance against control (* $P = 0.028$ for *SNAP-25* and ** $P = 0.012$ for *munc-18*). Unpaired *t*-test was performed for each pulse.

effect on the gene expression of neither *SNAP-25* nor *munc-18* (data not shown).

Effects of estradiol on *SNAP-25* and *munc-18* protein expression in α T3-1 cells

The data on the protein expression of the exocytotic proteins are in keeping with the results on the gene expression. Again, long-term treatment with 100 pM estradiol significantly decreased *SNAP-25* protein expression by 34% ($P < 0.0001$, Fig. 5A), whereas the expression of *munc-18* was significantly enhanced by

Figure 3 Representative immunoblots in α T3-1 cells. Immunoblots of SNAP-25 and munc-18 in α T3-1 cells. β -Actin was used as internal standard. Panel A shows SNAP-25 and Panel B shows munc-18. Lane 1 shows untreated control cells and lane 2 cells treated with estradiol.

46% ($P < 0.01$, Fig. 5B). The 24 h incubation of the cells with 100 pM estradiol had no effect on SNAP-25 and munc-18 protein expression (data not shown).

Effects of estradiol on SNAP-25 and munc-18 gene expression in single gonadotrophs

To confirm our previous results, we performed experiments in single cells of rat pituitary gonadotrophs. Mixed populations of anterior pituitary cells contain around 10% gonadotrophs in intact rats and up to 30% in ovariectomized rats. Therefore, it is not possible to trace back effects exclusively to gonadotrophs in mixed anterior pituitary cell cultures. This was the reason why we here used immortalized murine-derived α T3-1 tumor cells consisting only of gonadotrophs. The normal secretion of the complete LH in this cell line is disturbed, however. We cannot exclude the possibility that this disturbed secretion is due to a defect in the exocytotic machinery. To solve that dilemma, we used single rat pituitary gonadotrophs. To identify gonadotrophs, we performed single-cell quantitative RT-PCR simultaneously always for LH- β and either SNAP-25 or munc-18.

Our results in single rat gonadotrophs were congruent to the results gained in α T3-1 cells. SNAP-25 gene expression (Fig. 6A) was significantly decreased by 87% ($P = 0.014$) and munc-18 gene expression (Fig. 6B) was significantly increased (+181%) in single gonadotrophs after long-term (48 h) estradiol treatment with 100 pM ($P < 0.01$). Figure 6C shows a representative gel with the PCR products for LH- β , munc-18, SNAP-25, and β -actin.

Discussion

Here, we demonstrated that long-term treatment of female gonadotrophs with estradiol oppositionally affected two important components of the exocytotic machinery of the pituitary gland. Estradiol suppressed SNAP-25 levels, whereas the expression of munc-18 was increased.

Only a few other investigators have tested the effects of estradiol on exocytotic proteins and the results have been

Figure 4 RT-PCR in α T3-1 cells. Cells were incubated with 100 pM estradiol for 48 h. mRNA of SNAP-25 (A) and munc-18 (B) were quantified by real-time RT-PCR. Data from 10 independent experiments performed in triplicates were pooled. Untreated controls were set to 100%. * $P < 0.01$; † $P < 0.05$ compared with control (unpaired t -test).

conflicting. Jacobsson *et al.* (1998) demonstrated that estradiol *in vivo* suppressed SNAP-25 gene expression in anterior lobes of pituitaries. Rats were treated for 21 days with estradiol capsules. They, however, found no influence of estradiol on other exocytotic proteins like VAMP-2, Hrs-2 and munc-18. In keeping with that line, a lower expression of SNAP-25 protein in estrogen-induced tumors of the pituitary gland in Fisher 344 rats was detected (Majo *et al.* 1999). In contrast, others concluded that the expression of synaptic proteins including SNAP-25 and munc-18 in ewes is not changed in the anterior pituitary at the time when LH secretion is maximal, i.e. after estradiol treatment (Thomas *et al.* 1998). Estradiol treatment of GH₄C₁ rat pituitary cells even increased SNAP-25 levels about 1.5-fold (Lee *et al.* 2000).

These results are in part contradictory to our findings. The differences might be due to different species and different cell lines used. These data suggest

Figure 5 Immunoblots in α T3-1 cells. Cells were incubated with 100 pM estradiol for 8 h. SNAP-25 (A) and munc-18 (B) protein concentrations were quantified by western blot in relation to β -actin. Data from 12–14 independent experiments performed in triplicates were pooled. Untreated controls were set to 100%. * $P < 0.01$; [†] $P < 0.0001$ compared with control (unpaired *t*-test).

that SNAP-25 regulation is not the same in GH₄C₁ cells, a prolactin-secreting cell line, and in ewes as it is in the normal rodent pituitary gland. Since no investigator examined the effects of estradiol particularly on gonadotrophs but on the entire anterior lobe of the pituitary, the differences in estradiol regulation might be due to the dominance of other compartments as well. The pituitary gland consists of several hormone-secreting cell types with only a small fraction of gonadotrophs (5–10%). The literature to date deals either with the anterior pituitary on the whole, prolactin secreting cells, or tumors. Reviewing these articles concerning the effects of estradiol on SNAP-25, one may speculate that estradiol treatment decreased SNAP-25 in gonadotrophs, whereas in prolactin-secreting cells, SNAP-25 is increased after estradiol treatment. It fits well that human prolactinomas express more SNAP-25 than normal glands (Majo *et al.* 1997). That

points at a different regulation of exocytosis in gonadotrophs and lactotrophs. Further studies on the different regulation of SNAP-25 mediated exocytosis in lactotrophs and gonadotrophs are warranted.

We aimed to delineate, how our findings fit into the current concepts of exocytosis and its regulation. Treatment of permeabilized male rat gonadotrophs with SNAP-25 antibodies diminished calcium-induced LH secretion, but did not influence basal LH secretion. Treatment with 100 nM GnRH, either *in vitro* for 4 or 72 h or *in vivo* for 5 days twice a day, decreased SNAP-25 protein expression (Quintanar *et al.* 2004). This is in striking contrast to our results. We found a profound increase in both SNAP-25 and munc-18 gene expression after treatment with GnRH pulses. These differences might well be due to a very different experimental setting: we used female, not male rats and we measured gene, not protein expression *in vitro*. Furthermore, we applied 1 nM GnRH for 15 min at 1 h intervals. This procedure is well established to mimic the physiological situation and has been used by other groups (Turgeon & Waring 1999). Furthermore, we could demonstrate a significant decrease of SNAP-25 in GnRH-stimulated gonadotrophs by estradiol. On the other hand, the expression of munc-18 was significantly increased in non-agonist stimulated gonadotrophs treated with estradiol. The effects of estradiol on GnRH-stimulated cells were not that distinctive, since GnRH pulses alone led to a higher increase in munc-18 than in SNAP-25 expression. This vigorous rise might mask the additional effects of estradiol.

In our experiments, the LH secretory pattern in response to the agonistic stimulus GnRH is congruent to the response of SNAP-25 and munc-18 to GnRH with the maximal increase at the third pulse of GnRH. It is therefore conceivable that GnRH-induced LH secretion is associated with corresponding changes in the exocytotic proteins SNAP-25 and munc-18. As yet another contrast, here we gained evidence that the estradiol-induced increase in basal LH release leads to a reduced SNAP-25 expression. This indicates that basal LH release is not independent of SNAP-25. This may be explained by the fact that the enhanced LH secretion precipitates a depletion of SNAP-25. The function of SNAP-25 in the modulation of LH secretion by estradiol remains not fully elucidated. It is not clear what happens to SNAP-25 after membrane merger. Walch-Solimena *et al.* (1995) found evidence that SNAP-25 is at least partially recycled at the synaptic cleft of neurons. Pituitary cells are able to recycle components of the exocytotic machinery (Ferraro *et al.* 2005). Massive stimulation of prolactin release *in vitro* leads to a depletion of the cellular levels of prolactin up to 80%. Prolactin, for example, remains connected with the cell membrane after exocytosis and is ready for recycling and reuse. Others components like PAM-1, a

membrane enzyme participating in the final steps of the biosynthesis of hormones, can be functionally recycled. There are no reports that clarify the fate of SNAP-25 after exocytosis in the pituitary gland occurred.

Korteweg *et al.* (2005) tested hormone levels in the anterior pituitaries of munc-18 null mutant mice. They found very low levels of circulating hormones and enhanced intracellular hormones. This underlines the importance of munc-18 for the secretion of hormones from anterior pituitary cells. They suggested that munc-18 is important for the docking of vesicles, because large dense-core vesicles assembled close to, but not directly at the terminal membrane (Korteweg *et al.* 2005). This is in contrast to the findings of Verhage *et al.* (2000) that synaptic vesicles are distributed normally in synapses of munc-18 null mutant mice. This shows that the transfer of results derived from experiments in neuronal synapses to gonadotrophs is restricted.

Since it has been suggested that munc-18 hampers the ability of syntaxin to form SNARE complexes, munc-18 might be a negative regulator of exocytosis and an overexpression of munc-18 should reduce hormone release. This could be true if the concentration of munc-18 and syntaxin is equal, because they interact with a 1:1 stoichiometry (Misura *et al.* 2000). Schütz *et al.* (2005) found that syntaxin 1 expression is 20 times higher than munc-18 expression. In accordance with that, Toonen *et al.* (2005) argued against the possibility that munc-18 acts as a simple chaperone for syntaxin. Munc-18 has recently been identified as a positive regulator of exocytosis in PC12 cells (Schütz *et al.* 2005). They proposed a dual role for munc-18 switching between syntaxin binding and mint1 binding. Overexpression of mint1 inhibits exocytosis in PC12 cells. Binding of munc-18 to mint1 prevents mint1's negative effect on exocytosis. The first target of munc-18 is syntaxin, because of the high-affinity of their binding. Higher cytosolic munc-18 however leads to a higher binding to mint1 and consecutively to an increase in hormone release. An overexpression of the cytoplasmic region of syntaxin thus leads to an inhibition of secretion from PC12 cells (Dulubova *et al.* 1999). Furthermore, munc-18 is required for the stability of syntaxin. Munc-18 null mutant mice expressed lower levels of syntaxin 1, but other proteins of the exocytotic machinery remained unaffected. Even in the absence of munc-18, syntaxin 1 can be targeted properly (Toonen *et al.* 2005). The lack of munc-18 may lead to an uncontrolled binding of syntaxin 1 to other, non-cognate, exocytotic proteins, which in turn could hamper cell viability. Toonen *et al.* (2005) did not find evidence for

Figure 6 RT-PCR in single gonadotrophs. Rat anterior pituitary cells were incubated with 100 pM estradiol for 48 h. Single gonadotrophs were extracted and identified. mRNA of SNAP-25 (A) and munc-18 (B) from a single cell was quantified by real-time RT-PCR in relation to β-actin. Data from 13–19 experiments performed in duplicates were pooled. Untreated controls were set to 100%. A: * $P < 0.0034$; B: † $P < 0.0141$ (Unpaired *t*-test was applied). C shows a representative gel with the PCR products for LH-β, munc-18, SNAP-25, and β-actin.

detrimental effects of the promiscuous syntaxin 1 binding on cell viability. The SNARE complexes formed might not be accessible for normal exocytosis, since munc-18 null-mutant mice have no transmitter release (Verhage *et al.* 2000). Munc-18 may have a crucial and autonomous function in conjunction with syntaxin 1 in exocytosis (Toonen *et al.* 2005).

The concepts of Schütz *et al.* (2005) and Toonen *et al.* (2005) are in keeping with our results. Estradiol treatment leads to an up-regulation of munc-18 and consecutively to increased LH secretion. Therefore, munc-18 is a crucial component of the modulation of LH secretion by estradiol.

Other concepts on how estradiol could positively regulate LH secretion are well established. Estradiol modulates GnRH receptor number (Gregg *et al.* 1990) and its gene expression (Bauer-Dantoin *et al.* 1993). Moreover, estradiol could affect components of the GnRH signal transduction such as calcium signaling and inositol phosphates (Ortmann *et al.* 1992, 1995).

However, those concepts could not fully explain the positive modulatory effect of estradiol on basal and agonist induced LH secretion. Therefore, our findings add novel evidence to the hypothesis that estradiol affects even distant mechanisms of signal transduction like exocytosis.

In conclusion, long-term estradiol treatment negatively regulates the exocytotic protein SNAP-25 and positively affects the regulation of munc-18 in female gonadotrophs. The up-regulation of munc-18 is another mechanism by which estradiol might enhance LH secretion from female gonadotrophs.

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References

- Aguado F, Majo G, Ruiz-Montasell B, Canals JM, Casanova A, Marsal J & Blasi J 1996 Expression of synaptosomal-associated protein SNAP-25 in endocrine anterior pituitary cells. *European Journal of Cell Biology* **69** 351–359.
- Bauer-Dantoin AC, Hollenberg AN & Jameson JL 1993 Dynamic regulation of gonadotropin-releasing hormone receptor mRNA levels in the anterior pituitary gland during the rat estrous cycle. *Endocrinology* **133** 1911–1914.
- Borisovska M, Zhao Y, Tsytsyura Y, Glyvuk N, Takamori S, Matti U, Rettig J, Sudhof T & Bruns D 2005 v-SNAREs control exocytosis of vesicles from priming to fusion. *EMBO Journal* **24** 2114–2126.
- Chen YA & Scheller RH 2001 SNARE-mediated membrane fusion. *Nature Reviews Molecular Cell Biology* **2** 98–106.
- Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC & Rizo J 1999 A conformational switch in syntaxin during exocytosis: role of munc18. *EMBO Journal* **18** 4372–4382.
- Ferraro F, Eipper BA & Mains RE 2005 Retrieval and reuse of pituitary secretory granule proteins. *Journal of Biological Chemistry* **280** 25424–25435.
- Gregg DW, Allen MC & Nett TM 1990 Estradiol-induced increase in number of gonadotropin releasing hormone receptors in cultured ovine pituitary cells. *Biology of Reproduction* **43** 1032–1036.
- Jacobsson G & Meister B 1996 Molecular components of the exocytotic machinery in the rat pituitary gland. *Endocrinology* **137** 5344–5356.
- Jacobsson G, Razani H, Ogren SO & Meister B 1998 Estrogen down-regulates mRNA encoding the exocytotic protein SNAP-25 in the rat pituitary gland. *Journal of Neuroendocrinology* **10** 157–163.
- Jahn R 2000 Sec1/Munc18 proteins: mediators of membrane fusion moving to center stage. *Neuron* **27** 201–204.
- Kolk SM, Groffen AJ, Tuinhof R, Ouwens DT, Cools AR, Jenks BG, Verhage M & Roubos EW 2004 Differential distribution and regulation of expression of synaptosomal-associated protein of 25 kDa isoforms in the *Xenopus* pituitary gland and brain. *Neuroscience* **128** 531–543.
- Korteweg N, Maia AS, Thompson B, Roubos EW, Burbach JP & Verhage M 2005 The role of Munc18-1 in docking and exocytosis of peptide hormone vesicles in the anterior pituitary. *Biology of the Cell* **97** 445–455.
- Lee MS, Zhu YL, Sun Z, Rhee H, Jeromin A, Roder J & Dannies PS 2000 Accumulation of synaptosomal-associated protein of 25 kDa (SNAP-25) and other proteins associated with the secretory pathway in GH4C1 cells upon treatment with estradiol, insulin, and epidermal growth factor. *Endocrinology* **141** 3485–3492.
- Majo G, Ferrer I, Marsal J, Blasi J & Aguado F 1997 Immunocytochemical analysis of the synaptic proteins SNAP-25 and Rab3A in human pituitary adenomas. Overexpression of SNAP-25 in the mammosomatotroph lineages. *Journal of Pathology* **183** 440–446.
- Majo G, Lorenzo MJ, Blasi J & Aguado F 1999 Exocytotic protein components in rat pituitary gland after long-term estrogen administration. *Journal of Endocrinology* **161** 323–331.
- Masumoto N, Ikebuchi Y, Matsuoka T, Tasaka K, Miyake A & Murata Y 1997 Involvement of SNAP-25 in TRH-induced exocytosis in pituitary GH4C1 cells. *Journal of Endocrinology* **153** R5–R10.
- Misura KM, Scheller RH & Weiss WI 2000 Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* **404** 355–362.
- Ortmann O, Sturm R, Knuppen R & Emons G 1990 Weak estrogenic activity of phenol red in the pituitary gonadotroph: re-evaluation of estrogen and antiestrogen effects. *Journal of Steroid Biochemistry* **35** 17–22.
- Ortmann O, Stojilkovic SS, Cesnjaj M, Emons G & Catt KJ 1992 Modulation of cytoplasmic calcium signaling in rat pituitary gonadotrophs by estradiol and progesterone. *Endocrinology* **131** 1565–1567.
- Ortmann O, Bakhit M, Bloh P, Schulz KD & Emons G 1995 Ovarian steroids modulate gonadotropin-releasing hormone-induced biphasic luteinizing hormone secretory responses and inositol phosphate accumulation in rat anterior pituitary cells and alpha T3-1 gonadotrophs. *Journal of Steroid Biochemistry and Molecular Biology* **54** 101–109.
- Ortmann O, Tomic M, Weiss JM, Diedrich K & Stojilkovic SS 1998 Dual action of androgen on calcium signaling and luteinizing hormone secretion in pituitary gonadotrophs. *Cell Calcium* **24** 223–231.

- Quintanar JL, Salinas E, Chavez-Morales RM & Quintanar-Stephano A 2004 Pituitary synaptic protein SNAP-25 sensitive to GnRH is necessary for LH release. *Endocrine Regulations* **38** 1–6.
- Rettig J & Neher E 2002 Emerging roles of presynaptic proteins in Ca^{2+} -triggered exocytosis. *Science* **298** 781–785. Erratum in: *Science* **298** 1336.
- Schütz D, Zilly F, Lang T, Jahn R & Bruns DA 2005 A dual function for Munc-18 in exocytosis of PC12 cells. *European Journal of Neuroscience* **21** 2419–2432.
- Sorensen JB, Matti U, Wei SH, Nehring RB, Voets T, Ashery U, Binz T, Neher E & Rettig J 2002 The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. *PNAS* **99** 1627–1632. Erratum in: *PNAS* **99** 6449.
- Stojilkovic SS 2005 Ca^{2+} -regulated exocytosis and SNARE function. *Trends in Endocrinology and Metabolism* **16** 81–83.
- Sudhof TC 1995 The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375** 645–653.
- Thomas SG, Takahashi M, Neill JD & Clarke IJ 1998 Components of the neuronal exocytotic machinery in the anterior pituitary of the ovariectomised ewe and the effects of oestrogen in gonadotrophs as studied with confocal microscopy. *Neuroendocrinology* **67** 244–259.
- Toonen RF, de Vries KJ, Zalm R, Sudhof TC & Verhage M 2005 Munc18-1 stabilizes syntaxin 1, but is not essential for syntaxin 1 targeting and SNARE complex formation. *Journal of Neurochemistry* **93** 1393–1400.
- Turgeon JL & Waring DW 1999 Androgen modulation of luteinizing hormone secretion by female rat gonadotrophs. *Endocrinology* **140** 1767–1774.
- Verhage M, Maia AS, Plomp JJ, Brussaard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, van den Berg TK, Missler M *et al.* 2000 Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* **287** 864–869.
- Voets T, Toonen RF, Brian EC, de Wit H, Moser T, Rettig J, Sudhof TC, Neher E & Verhage M 2001 Munc18-1 promotes large dense-core vesicle docking. *Neuron* **31** 581–591.
- Walch-Solimena C, Blasi J, Edelmann L, Chapman ER, von Mollard GF & Jahn R 1995 The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *Journal of Cellular Biology* **128** 637–645.

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