PITUITARY SYNAPTIC PROTEIN SNAP-25 SENSITIVE TO GnRH IS NECESSARY FOR LH RELEASE

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Objective. The protein SNAP-25 is located in the plasma membrane and is known to participate in hormone exocytosis process. In the present work we studied the role of SNAP-25 on LH secretion in permeabilized adenohypophyseal cultured cells. The question of whether GnRH regulates SNAP-25 expression in adenohypophyseal cultured cells and in the adenohypophyses *in vivo* was also investigated.

Methods. In digitonin-permeabilized cells incubated with anti-SNAP-25, stimulated LH secretion with Ca^{2+} was analysed. The presence and expression of SNAP-25 in adenohypophyseal cultured cells incubated with GnRH and in adenohypophyses of orchidectomized rats with GnRH administration was studied by immunochemistry and immunoblotting.

Results. Immunochemical study revealed that SNAP-25 was present in cultured adenohypophyseal cells and in adenohypophysis of orchidectomized rats both with GnRH treatment. We found that LH secretion can be blocked by antobodies raised against SNAP-25 in permeabilized cells. Likewise, GnRH administration induced a significant decrease of SNAP-25 expression in cultured adenohypophyseal cells and in adenohypophysis of orchidectomized rats.

Conclusion. Our study showed that SNAP-25 is present in adenohypophyses in vitro as well as in vivo and that is involved in LH release and that GnRH can modify its expression.

Key words: Gonadotrophs – Immunohistochemistry – Immunoblot – Permeabilized cells – Digitonin

Recently, exocytotic proteins of plasma membrane have been found in various endocrine tissues. Synaptosomal-associated protein of 25 kDa (SNAP-25) was originally identified in the nervous system, but is also expressed in the pituitary (Aguado et al. 1996; Jacobsson and Meister 1996; Salinas and Quintanar 1999). This protein functions in association with syntaxin, an other plasma membrane protein; both interact with synaptobrevin, a membrane vesicular protein to cause docking and fusion of vesicles with the plasma membrane resulting in hormone release (Gerst 1999). It has been shown that SNAP-25 is crucial for the release of PRL and ACTH in the GH_4C_1 (Masumoto et al. 1997) and AtT-20 (Aguado et al. 1997) pituitary cell lines. It was also established that expression of SNAP-25 can be correlated with hormone secretion rates. In the pituitaries of 17β-estradiol treated rats, low levels of SNAP-25 and elevated concentrations of PRL were observed (Majó et al. 1999). It was also reported that SNAP-25 immunoreactivity is increased in human prolactinomas (Majó et al. 1997). In our studies, thyroidectomy resulted in increased pituitary SNAP-25 expression as well as TSH secretion in rats (Quintanar and Salinas 2002).

The hypothalamic peptide gonadotropin-releasing hormone (GnRH) is the principal stimulator of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from pituitary gonadotrophs (Millar 2003). GnRH causes dose-dependent increase in intracellular calcium that induces LH release (Stojilkovic et al. 1994).

In the present work we studied the role of SNAP-25 on LH secretion in adenohypophyseal cultured cells.

The question of whether GnRH regulates SNAP-25 expression *in vitro* and *in vivo* was also investigated.

Material and Methods

In vitro experiments

Cell culture. Forty adult Wistar male rats weighting 200-250 g were maintained in a temperature- and light-controlled room and given food and water *ad libitum*. Animals, sacrificed by intraperitoneal injection of sodium pentobarbital (50 mg/kg i.p.), were used as donors of pituitaries. Anterior lobes were dispersed by 0.5 % collagenase (type II Gibco G.I., NY, USA) and 0.17 % hyaluronidase (type VIII Gibco G.I., NY, USA) as described by Wynick and Bloom (1990). Dispersed cells were plated at different densities in plastic culture dish (Costar, Cambridge, MA,USA) and maintained in Dulbecco's Modified Eagle's Medium containing 10 % fetal calf serum (Gibco G.I., NY, USA). The cultures were maintained at 37 ºC under an atmosphere of 95 % O_2 and 5 % CO_2 for 24 h before to be used.

Immunocytochemistry for SNAP-25 in cultured cells incubated with GnRH The presence of SNAP-25 was studied by immunocytochemistry using cells plated at different times (4 or 72 h, 100,000 cells/dish) and incubated with GnRH (100 nM/twice/day) (Sigma Chem. Co., St. Louis, MO, USA). Cells were fixed in 10 % buffered formalin for 1 h. After fixation, cells were washed with 1 % bovine serum albumin in phosphate buffer saline and incubated at 4 ºC overnight with 1:100 dilution of the SNAP-25 antibody (Monoclonal anti-SNAP-25; Sternberg Monoclonals Inc., Baltimore, MD. USA) The avidin-biotin peroxidase complex method was applied as described elsewhere (Stefaneanu et al. 1990) using a kit (Vectastin ABC kit; Dimension Laboratories Inc., Carpinteria, CA, USA). The reaction product was developed with diaminobenzidine. The nuclei were slightly counterstained with hematoxylin. For controls, the cells were incubated either without the primary antibody or non-specific rabbit immunoglobulin G (IgG).

Effect of SNAP-25 antibody on LH secretion from permeabilized cells. As described by Gutierrez et al. (1995), digitonin-permeabilized cells were used with minor modifications. During the permeabilization period with digitonin 20 μM (10 min), adenohypophysial cells ($5x10^5$ cells/dish) were incubated in the presence of the anti-SNAP-25 or denatured anti-SNAP-25 (100 °C/10 min) as control. After this treatment, the media were discarded and cells treated for 10 additional min with the same media lacking digitonin, in the presence of 10 μM buffered Ca^{2+} concentration or ethylene-glycol-tetraacetic acid (EGTA) (5 mM) containing the antibodies assayed.

Enzyme-linked immunosorbent assay (ELISA) described by Signorella and Hymer (1984) was used for measured LH in the supernatant.

Expression of SNAP-25 in adenohypophyseal cells incubated with GnRH.

Adenohypophyseal cells ($5x10^6$ cells/dish) were incubated with GnRH (100 nM) for 4 or 72 h (twice/day) and the LH was measuring by ELISA in the supernatant. The cells were homogenized in a manual tissue grinder in ice-cold buffer Tris-HCl, pH 7.4. Electrophoresis was carried out as described by Laemmli (1970) using the mini-protean system (Bio-Rad, Hercules, CA, USA) and running on 100 μg of protein of each sample. After electrophoresis, gels were electrotransferred to polyvinylidene difluoride membranes (Sigma, St. Louis, MO) according to the procedure of Towbin et al. (1979). Blots were treated with 3 % bovine serum albumin for 1 h at room temperature and then incubated at 4 °C overnight with the monoclonal anti-SNAP-25. The membranes were incubated for 2 h with alkaline phosphatase-conjugated secondary antibody (1:20,000 dilution), and color was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma, St. Louis, MO). Quantification of protein bands was carried out by densitometry, using a Kodak Digital Science imaging system (Eastman Kodak Company, Rochester, NY) and the values were expressed as Media of Intensity (M.I.) per 100 μg of protein.

In vivo experiments

Effect of GnRH on adenohypophyseal SNAP-25 expression in orchidectomized rats

Since various steroid hormones induce changes in SNAP-25 expression (Majó et al. 1999), to avoid possible interference, 20 male Wistar rats weighing 250 g were orchidectomized under ether anesthesia. They were maintained on standard Purina chow and drinking water *ad libitum* under a 12 h light: 12 h darkness cycle and were treated according to the Institutional Normative on animal welfare (Universidad Autónoma de Aguascalientes). Fifteen days after surgery the rats were divided into two

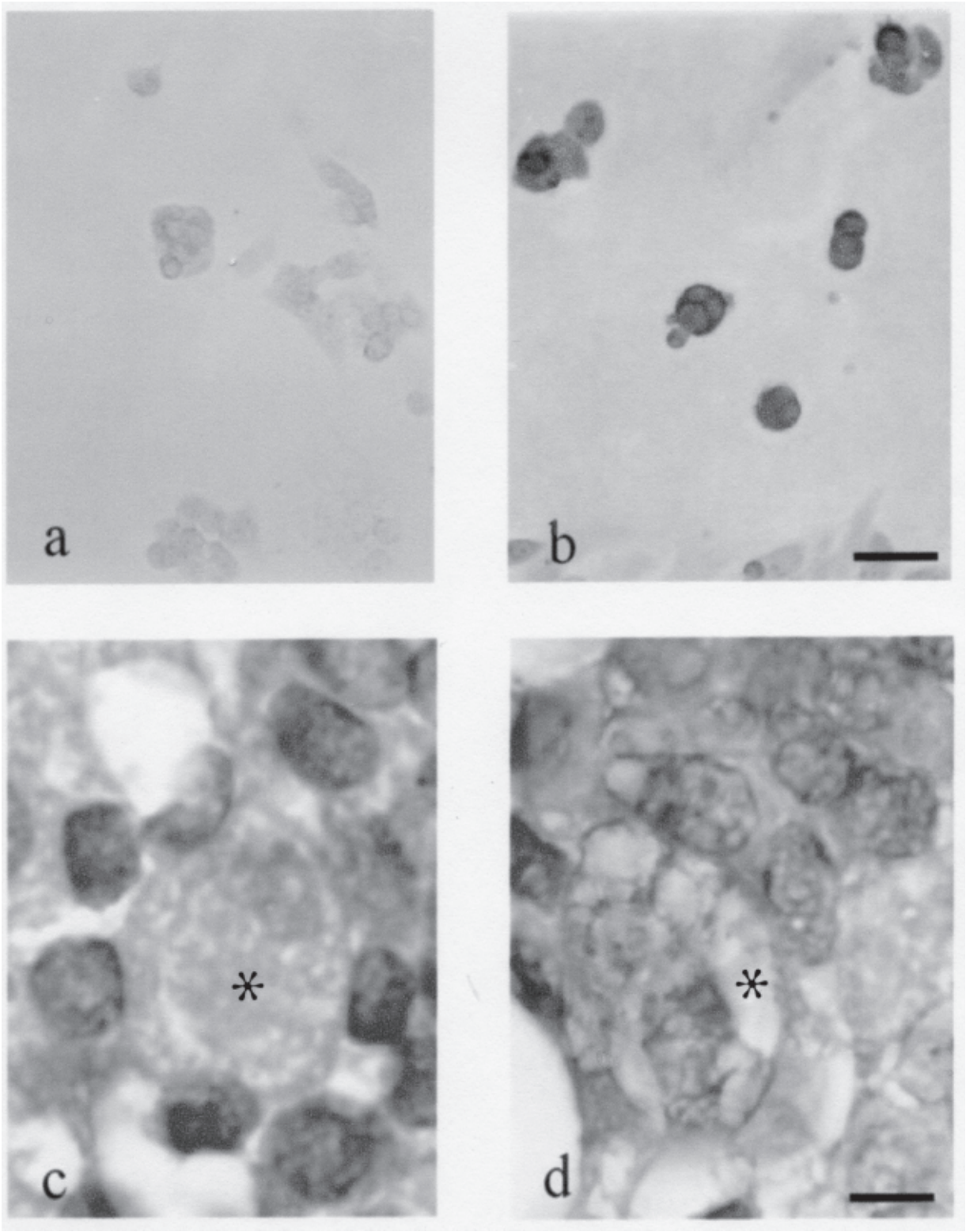

Fig 1 Photomicrographs of cultured adenohypophyseal cells immunostained for SNAP-25 exposed to GnRH (100 nM, 72 h). Control without antibody (a) **and anti-SNAP-25** (b)**. Adenohypophyseal cells of orchidectomized rats given GnRH (100 nM). Control without antibody** (c) **and anti-SNAP-25** (d)**. Asterix = castration cell. Optimal magnification in (a) and (b): x200, bar = 50 mm; in (c) and (d): x1000, bar = 10 mm.**

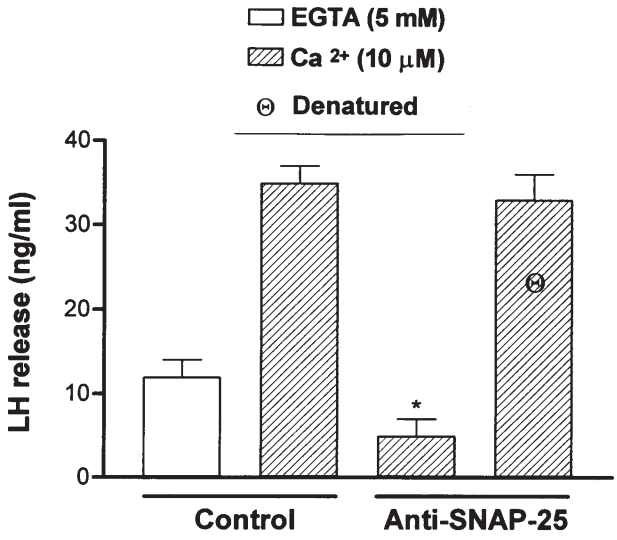

Fig 2 LH secretion in cultured adenohypophyseal cells. Digitonin (10 μM for 3 min) permeabilized cells (1x10^5 cells/dish) were incubated for 10 min with SNAP-25 antibody or the denatured antibody (100 °C, 10 min) and subsequently stimulated by calcium for 10 min at 37 °C. Experiments were performed three times and were triplicated. Data are presented as mean ± SEM. * p<0.001 compared with calcium stimulated control.

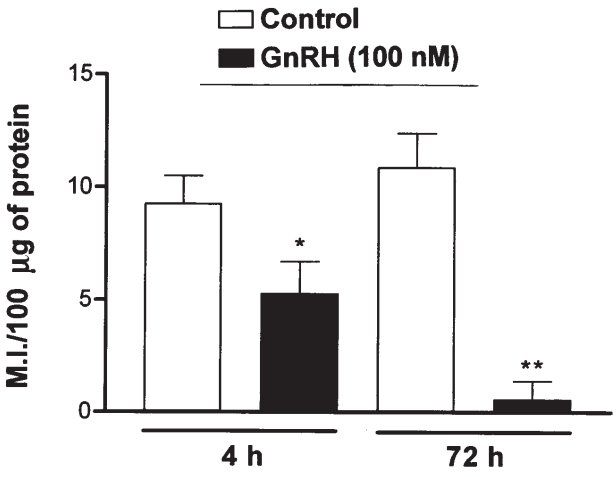

Fig 3 Western blot densitometric analysis of SNAP-25 from cultured adenohypophyseal cells (5x10^6 cells/dish) incubated with GnRH (100 nM) at 4 and 72 h. Values were expressed as media of intensity (M.I) per μg of protein. 100 μg of protein/lane were processed for SDS-PAGE. Experiments were performed five times and were triplicated. Data are presented as mean ± SEM. * p<0.01 and ** *p*<0.001.

groups: 1. Treated with GnRH (100 ng/twice/day for 5 days s.c.) and 2. Treated with NaCl (0.85 per cent) as Controls. Rats were sacrificed by decapitation under deep sodium pentobarbital anesthesia (50 mg/kg i.p). After removal of the neurointermediate lobe, the anterior pituitaries were weighted and used for immunohistochemical and Western blot analysis.

Immunohistochemical analysis. Five anterior pituitaries per group (GnRH treated and Control) were fixed in 10 % buffered formalin, dehydrated in graded ethanol and embedded in paraffin. Consecutive horizontal 5 μm-thick sections were prepared. For immunohistochemical analysis of SNAP-25, the streptavidin-biotin-peroxidase complex method was used as described previously (Stefaneanu et al. 1990).

Western blot analysis. Five anterior pituitaries per group were homogenized in a manual tissue grinder in ice-cold buffer Tris-HCl, pH 7.4. The samples were analyzed by western blot analysis and SNAP-25 quantification was by densitometry as described above. The values were expressed as Media of Intensity (M.I.) per μg of protein.

Statistical evaluation. Data are expressed as means ± SEM. Statistical significance was analyzed by the Tukey-Kramer multiple comparisons test.

Results

Immunocytochemical and immunohistochemical findings.

Immunoreactivity for SNAP-25 was found in adenohypophyseal cells cultured for 4 and 72 h and incubated with GnRH 100 nM. Immunopositivity was evident in the plasma membrane and it was specially intense (Figure 1b). Similar results were obtained in adenohypophyseal cells without GnRH. In the adenohypophyses of orchidectomized rats, SNAP-25 was present in almost every cell. It was evident that immunoreactivity was very strong in castration cells (Figure 1d).

Effect of SNAP-25 antibodies on LH secretion in permeabilized cells

Permeabilized cells were incubated with native or denatured SNAP-25 antibodies in order to block SNAP-25 protein and decrease LH secretion under calcium stimulation. The results showed that LH secretion induced by calcium stimulation decreased significantly (85%) after SNAP-25 antibody incubation. The denatured antibody had no effect on LH secretion (Figure 2). No changes in basal secretion of LH were observed when the cells were incubated with anti-SNAP-25.

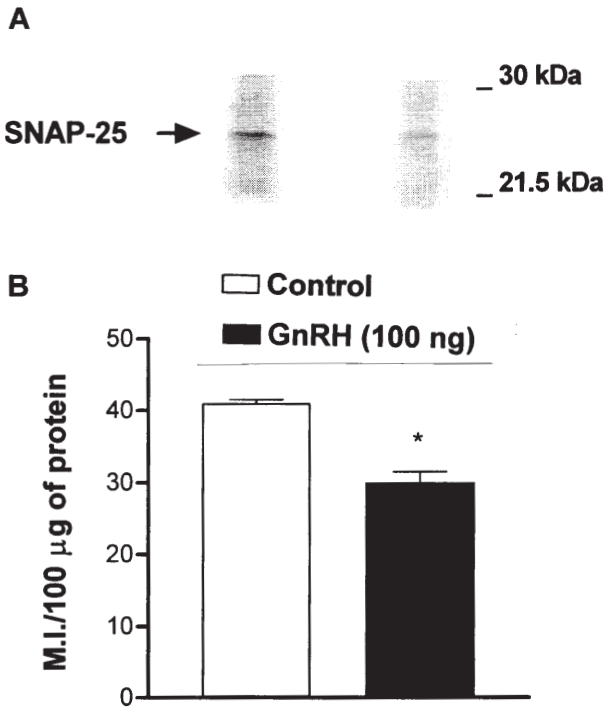

Fig 4 Western blot analysis of SNAP-25 from 5 anterior pituitaries of orchidectomized rats treated given GnRH (100 ng/twice/day for 5 days). (A) **immunoblot of SNAP-25 and** (B) **data analysed by densitometry. Values are expressed as media of intensity (M.I) per μg of protein. 100 μg of protein/ lane were processed for SDS-PAGE. *p<0.001**

Expression of SNAP-25 in adenohypophyseal cells in vitro and in vivo treated with GnRH

Western blot analysis was performed in order to explore the level of SNAP-25 expression in adenohypophyseal cells *in vitro* incubated with GnRH. Densitometric quantification showed that SNAP-25 decreased by 50 and 90 % at 4 and 72 h of incubation with GnRH respectively (Figure 3). LH secretion measured by ELISA, increased after 4 and 72 h of stimulation with GnRH compared to basal secretion (2.0 ± 0.1 vs 4.2 ± 0.3 and 5.1 ± 0.2 ng/μl respectively). Likewise, in the adenohypophyses of orchidectomized rats, GnRH administration for 5 days reduced SNAP-25 expression (from 41.2 ± 0.3 to 30.2 ± 0.5; $p<0.001$) (Figure 4).

Discussion

In recent years, several proteins related to the membrane secretory machinery have been identified in the pituitaries of different species (Aguado et al. 1996; Jacobsson and Meister 1996; Salinas and Quintanar 1999). The presence of SNAP-25 in gonadotrophs cell has been described by Jacobsson and Meister (1996). We found similar results in gonadotrophs (castration cells) of orchidectomzed rats. The role of SNAP-25 during exocytosis, as well as its regulation has yet to be elucidated. Our results shown that SNAP-25 was present in cultured adenohypophyseal cells and that LH secretion can be blocked by antobodies raised against SNAP-25 in permeabilized cells. It was reported that the introduction of antisense oligonucleotide of SNAP-25 into the clonal pituitary cell line GH4C1, also inhibited TRH-induced prolactin release (Masumoto et al. 1997). These facts support the idea that this protein play a crucial role in hormone secretion. In our studies, the inhibition of LH release was 85 %, thus it is possible that other proteins are involved in hormone exocytosis as suggested by Coorssen et al. (2003). Likewise, it is possible that in our model of permeabilized cell incubated with anti-SNAP-25 and stimulated with Ca^{2+} any pituitary hormone (FSH, TSH, GH, etc.) could be inhibit.

Only few factors capable of regulating SNAP-25 levels have been identified in endocrine tissues so far; these include certain hormones, intracellular messengers and depolarization (Hepp and Langley 2001). In our study, GnRH administration induced a significant decrease of SNAP-25 expression in cultured adenohypophyseal cells and in adenohypophysis of orchidectomized rats. It is conceivable that GnRH administration reduced SNAP-25 mRNA levels as in pituitary glands of estrogen-treated ovariectomized rats (Jacobsson et al. 1998). It may also be that, GnRH down-regulation by increasing the degradation of SNAP-25.

It was reported that SNAP-25 expression may be correlated with PRL and ACTH release (Majo et al. 1997; Majo et al. 1999). In contrast to these findings, we observed low levels of SNAP-25 and high levels of LH after GnRH incubation. It is possible that low concentrations in SNAP-25 can evoke a physiological response.

In conclusion, our study showed that SNAP-25 is involved in LH release and that GnRH can modify its expression. The parallel changes in SNAP-25 mRNA expression and functional significance of the low expression of SNAP-25 found in culture adenohypophyseal cells or in the adenohypophyses during the secretion process, remain by to be elucidated.

Acknowledgements

We would like to express our sincere gratitude to Dr. Kalman Kovacs for reviewing the manuscript and AQBs Rosario Montoya and Alejandro Organista, Mr. Manuel Tinajero and TLQ Gabriela Orozco for the methodologic support.

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