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Thyroidectomy Induces Neurofilament Expression in Adenohypophyses of Rats

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We studied the effect of thyroidectomy on neurofilament expression in adenohypophyses of rats. The question of whether thyroxine (T_4) administration can reduce this effect was also investigated. Rats were divided into:

1. **Euthyroid controls,**
2. **Thyroidectomized 20 d (Tx 20 d),**
3. **Thyroidectomized 20 d with replacement of T_4 (Tx 20 d + T_4 20 d),**
4. **Thyroidectomized 40 d (Tx 40 d),**
5. **Thyroidectomized 40 d with replacement of T_4 20 d after surgery (Tx 40 d + T_4 20 d).**

Adenohypophyses were studied by immunohistochemistry and Western blot analysis using antibodies against neurofilament 200 kDa (NF-H) and thyroid-stimulating hormone (TSH). The number of thyrotrophs with immunoreactivity for NF-H was increased in Tx 20 d and Tx 40 d rats, whereas T_4 administration protected the effect of thyroidectomy. In the thyroidectomized animals, thyrotrophs showed eccentric nuclei and the cytoplasm was full of NF-H immunoreactivity, whereas in T_4 treated rats, the thyrotrophs were similar to control. Western blot analysis showed that NF-H expression increased in rats thyroidectomized for 20 and 40 d. T_4 given immediately or 20 d after thyroidectomy caused no changes in NF-H expression. We conclude that thyroidectomy induces NF-H expression in adenohypophyses of rats and administration of T_4 decreases this effect.

Key Words: Neurofilaments; adenohypophysis; thyroxine; thyroidectomy.

Introduction

Both thyroidectomy and prolonged propylthiouracil (PTU) administration increase pituitary weight, decrease blood level of thyroid hormones thyroxine (T_4), and Triiodothyronine (T_3), elevate serum thyroid-stimulating hormone (TSH) concentrations, induce hypertrophy and hyperplasia of thyrotrophs and result in the developement of thyroidectomy cells *(1,2)*.

The hyperplasia of thyrotrophs can be explained by the negative feedback effect in which reduced circulating levels of thyroid hormones result in overstimulation of thyrotrophs by thyrotropin-releasing hormone (TRH). Other factors such as epidermal growth factor and insulin may also be involved at pituitary level similar to direct thyroid hormone feedback *(1,3)*. In fact, replacement of thyroid hormones reverses thyrotroph hyperplasia *(4,5)*.

Neurofilaments (NFs), the major type of intermediate filaments (IFs) in neurons comprise three subunits: light 68 kDa (NF-L), medium 160 kDa (NF-M), and heavy 200 kDa (NF-H) *(6)*. It was revealed that NFs are present in endocrine cells of the anterior pituitary *(7)*, as well as in pituitary adenomas *(8–11)*. Recently, we demonstrated NF-H expression specifically in pituitary thyrotrophs of normal rats and in thyroidectomy cells induced by PTU administration *(12)*. The significance of NFs expression in non-neural cells is not clear; however, we found that antibodies against NF-H in digitonin-permeabilized cultured adenohypophysial cells suppresed calcium induced TSH release *(13)*. Likewise, is possible that the subunit proteins of IFs can act as signal transmitters from the plasma membrane to the nucleus *(14)* and NFs may be related to pituitary adenoma induction depending on the presence of thyroid hormones.

The aim of this study was to determine the effect of thyroidectomy on neurofilament expression in adenohypophyses of rats and to clarify whether thyroxine administration reduces this effect.

Results

Effect of Thyroidectomy and T_4 on Anterior Pituitary Weight, as well as TSH and T_4 Plasma Levels

The anterior pituitary weights of Tx 40 d rats were increased significantly, whereas they were similar to controls in Tx 20 d, Tx 20 d + T_4 20 d, and Tx 40 d + T_4 20 d rats. In animals Tx 40 d treated with T_4 for 20 d, the pituitary weights were significantly reduced compared to control euthyroid values (Table 1). Plasma TSH levels were increased significantly in Tx 20 d, Tx 20 d + T_4 20 d, Tx 40 d, and Tx 40 d + T_4

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Table 1
Adenohypophyses Weights, Plasma TSH and T_4 Levels of the Following Group of Rats:
(**A**) control; (**B**) thyroidectomized 20 d (Tx 20 d); (**C**) thyroidectomized 20 d with replacement of T_4 (Tx 20 d + T_4 20 d);
(**D**) thyroidectomized 40 d (Tx 40 d) and (**E**) thyroidectomized 40 d with replacement of T_4 20 d after surgery (Tx 40 d + T_4 20 d)

	Control	Tx 20 d	Tx 20 d + T_4 20 d	Tx 40 d	Tx 40 d + T_4 20 d
Adenohypophysis weights (mg/100 g BW)	3.6 ± 0.2	4.3 ± 0.3	4.2 ± 0.2	4.7 ± 0.1*	4.1 ± 0.2**
Plasma TSH (ng/mL)	7.8 ± 0.1	59.4 ± 0.1*	17.3 ± 0.1*	77.2 ± 0.1*	24.5 ± 0.1*
Plasma T_4 (ng/mL)	137.5 ± 3	12.2 ± 2*	202.0 ± 4*	13.1 ± 3*	304.1 ± 6*

*$p < 0.001$ as compared to control, **$p < 0.02$ as compared to Tx 40 d.

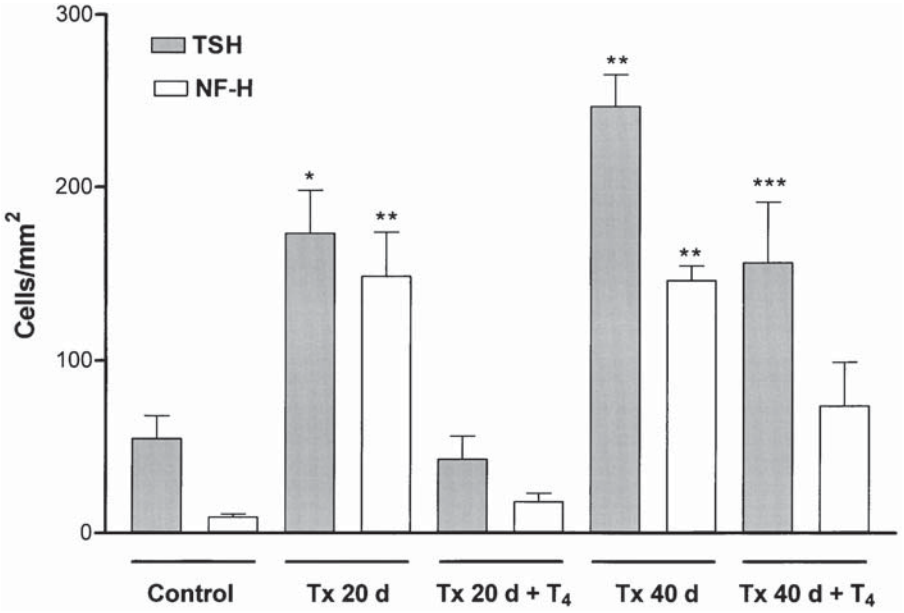

Fig. 1. Effect of thyroidectomy and T_4 on the number of immunoreactive thyrotrophs to NF-H and TSH in adenohypophyses of the following group of rats: (**A**) control; (**B**) thyroidectomized 20 d (Tx 20 d); (**C**) thyroidectomized 20 d with administration of T_4 (Tx 20 d + T_4 20 d); (**D**) thyroidectomized 40 d (Tx 40 d) and (**E**) thyroidectomized 40 d with treatment of T_4 20 days after surgery (Tx 40 d + T_4 20 d). *$p < 0.01$, **$p < 0.001$ and ***$p < 0.05$ as compared to control respective.

20 d compared to controls. The levels were highest in groups Tx 20 d and Tx 40 d (Table 1). T_4 plasma levels were lower in Tx 20 d and Tx 40 d rats compared to the con-trols. Administration of T_4 increased T_4 plasma concentrations (Table 1).

Thyrotrophs Expressing NF-H

To investigate whether immunoreactivity for NF-H was present in thyrotrophs and not in other pituitary cell types, consecutive sections were immunostained with NF-H, TSH, LH, FSH, ACTH, GH, and PRL antibodies. Only NF-H was demonstrated in TSH immunoreactive cells. In the adenohyphyses of control rats, the percentage of thyrotrophs immunoreactive for NF-H was 17.5 % (Fig. 1). The number of thyrotrophs that showed immunoreactivity for NF-H was increased significantly in Tx 20 d and Tx 40 d rats compared to controls. In Tx 20 d + T_4 20 d and Tx 40 d + T_4 20 d groups, T_4 administration caused no changes in the number of thyrotrophs immunoreactive for NF-H compared to controls. At 20 and 40 d after thyroidectomy, the number of thyrotrophs that expressed NF-H was similar. The number of thyroidectomy cells immunoreactive for TSH, was increased at 20 and 40 d after thyroidectomy and administration of T_4 in Tx 40 d + T_4 20 d rats did not prevent this effect.

Cytology of Thyrotrophs Immunoreactive for NF-H

Thyrotrophs immunoreactive for NF-H were evenly distributed in all adenohypophyses and exhibited a marked pleomorphism with a voluminous cytoplasm (Fig. 2). Cytologic changes were observed in different groups. In control rats, the adenohypophysial cells immunoreactive for NF-H,

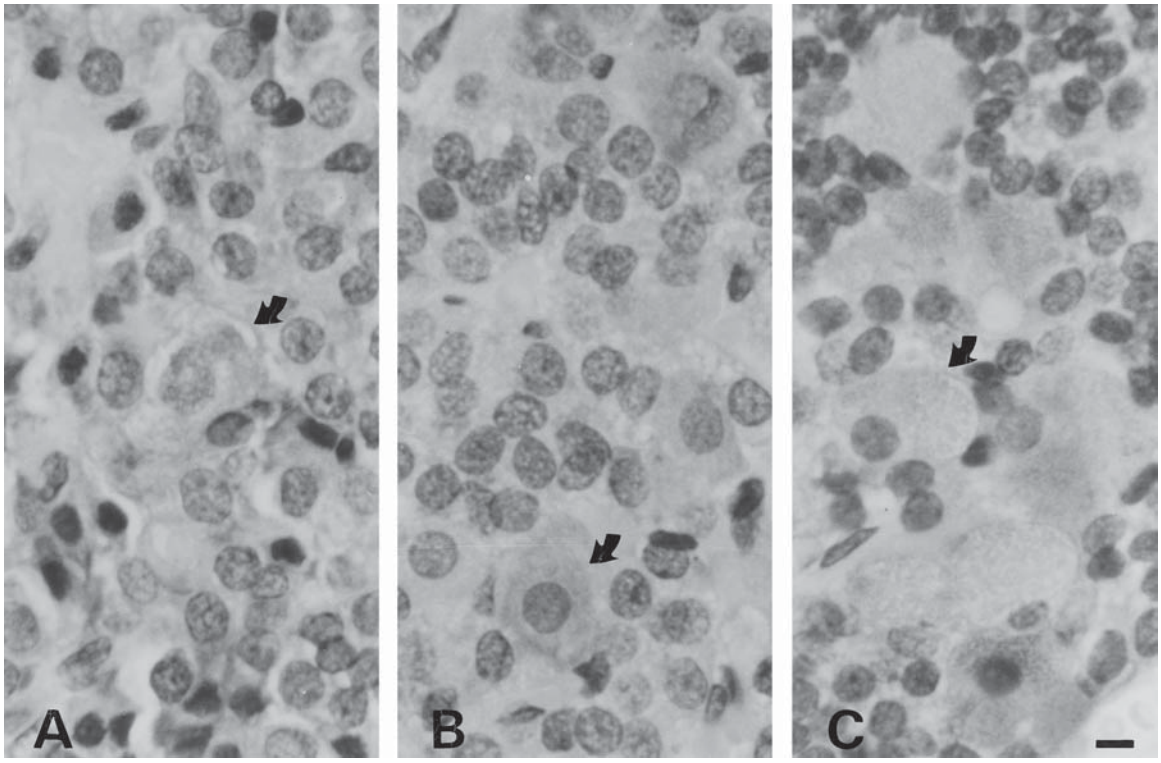

Fig. 2. Effect of thyroidectomy on NF-H immunoreactivity in adenohypophyses of rat. (**A**) Euthyroid controls, (**B**) thyroidectomized 20 d and (**C**) thyroidectomized 40 d. Arrows shows NF-H immunoreactivity in thyrotrophs. Nuclei were counterstained with hematoxilin. Magnification: ×400; bar = 10 μm.

showed centrally located nuclei surrounded by NF-H immunoreactivity. Immunostaining was weak in the vacuolated cytoplasm. In the Tx 20 d and Tx 40 d groups, the nuclei were eccentric and the cytoplasm was full of NF-H immunoreactive material. Immunoreactivity was prominent and the cell size was increased (Fig. 2). In rats thyroidectomized for 40 d and treated with T_4 for 20 d immunocytological changes were similar to those of control rats (Fig. 3). In rats treated for 20 d with T_4 initially after thyroidectomy, the thyrotrophs showed no changes.

Western Blotting Analysis

The NF-H antibody detected a band of 200 kDa in the immunoblots of anterior pituitary homogenates, being stronger in Tx 40 d rats and weaker in controls and T_4 treated rats (Fig. 4). Study of NF-H proteins by densitometry, revealed that the level of expression progressively increased in rats with thyroidectomy for 20 and 40 d (45.4 and 71.8 media of intensity as compared to controls). T_4 given immediately or 20 d after thyroidectomy caused no changes in NF-H levels (Fig. 4).

Discussion

The results showed that in euthyroid rats, thyrotrophs in anterior pituitary co-expressed NF-H and TSH confirming our previous findings *(12)*. We observed thyroidectomy cells 20 and 40 d after thyroidectomy; however, not all of them expressed NF-H. The number of thyroidectomy cells progressively increased being highest at 40 d after thyroidectomy, whereas no significant differences were observed in NF-H expression in adenohypophyses of rats at 20 or 40 d after thyroidectomy with or without T_4 treatment. The increased expression of NF-H in thyroidectomized rats may be due to intense TRH stimulation of thyrotrophs secondary to the absence of thyroid hormones. It is possible that some thyrotrophs are more sensitive to hypothyroidism and continue transforming to thyroidectomy cells without NF-H expression, suggesting the presence of two populations of thyrotrophs *(15)*.

T_4 administration prevented the development of thyroidectomy cells immunoreactive for NF-H in Tx 20 d rats and reduced their number in Tx 40 d rats. These findings may support the concept that the feedback effect of thyroid hormones may suppress proliferation of thyrotrophs *(3)*. Horvath et al. *(4)* demonstrated that the number of thyroidectomy cells decreased after discontinuation of PTU, similar to our results in thyroidectomized rats given T_4 replacement. However, in Tx 40 d + T_4 20 d rats, the number of thyrotrophs decreased but the decrease did not reach the control values, which may be due to the fact that more time

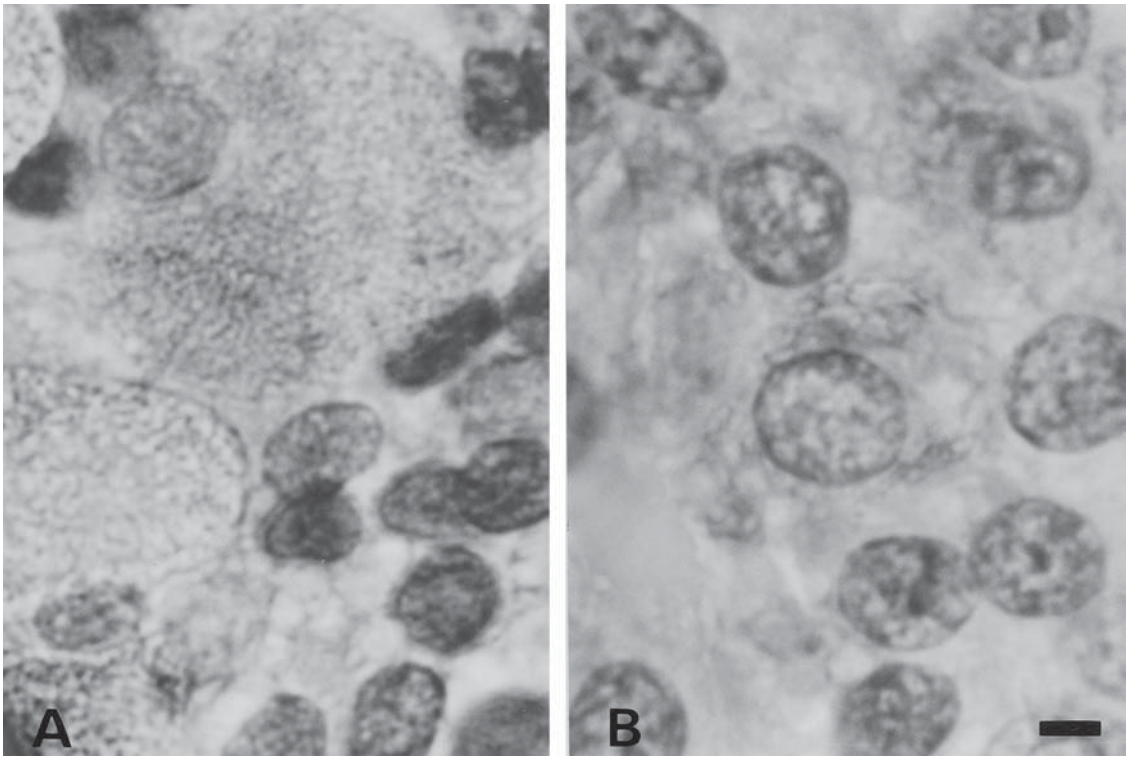

Fig. 3. Immunohistochemistry of NF-H in adenohypophyses of rat. (**A**) Adenohypophyses of thyroidectomized animals for 40 d showing thyrotrophs with eccentric nuclei and voluminous cytoplasm full of NF-H immunoreactive material. (**B**) Adenohypophyses of thyroidectomized rats for 40 d with treatment of T_4 20 d after surgery. The sections were counterstained with hematoxilin. Magnification: ×1000; bar = 10 μm.

of treatment is necessary for full normalization. Likewise, administration of T_4 in thyroidectomized animals reversed the cytological changes. In these rats the nuclei were centrally located, the voluminous and vacuolated cytoplasm was reduced and immunostaining for NF-H was weak.

In the pituitary, TRH, epidermal growth factor, insulin, and thyroid hormones are the obvious candidates to promote NF-H expression and it is conceivable that this promotion is mediated via genetic alterations.

The role of NFs on the endocrine activity of normal thyrotrophs as well as thyroidectomy cells is not clear. IFs normally occur in different cell types and are involved in the shaping and function of cytoskeleton. However, it has been suggested that the subunit proteins of IFs can act as signal transmitters from the plasma membrane to the nucleus since the subunit proteins can bind to nucleic acids *(14)*. Soluble IFs subunits may respond rapidly to such stimuli similar to heat shock proteins and growth factors *(16)*, which could act as stressors. It is possible that in the thyroidectomized rats, TRH stimulation acted as a stressor resulting in the formation of NFs in the thyrotrophs. Recent results showed that incubation with NF-H antibodies produced a significant inhibition of calcium-induced TSH release in digitonin-permeabilized adenohypophysial cells *(13)*. These findings may suggest the involvement of NF-H in this process.

The presence of NFs in pituitary adenomas is well documented *(8–11)*. According to our results, NF-H increases initially in hyperplasic cells, which may transform subsequently to adenoma cells. Asa & Ezzat *(3)* proposed a model of pituitary tumorigenesis where mutation occurs before the development of hyperplasia and precedes adenoma formation. It may well be that NF-H is involved in mutagenesis.

In conclusion, NF-H are expressed in pituitary thyrotrophs of the normal rat and also in the thyroidectomy cells of thyroidectomized rats. Administration of T_4 decreases NF-H expression in adenohypophyses of thyroidectomized rats.

Materials and Methods

Fifty female Wistar rats weighing 200–220 g were used. Forty animals were rendered hypothyroid by thyroparathyroidectomy under ether anesthesia. Ten nonoperated rats served as euthyroid controls. Animals were maintained on standard Purina chow and drinking water supplemented with 1% calcium lactate available *ad libitum* under a 12 h light:12 h darkness cycle and were treated according to the Institutional Normative on animal welfare. Every effort was made to minimize animal suffering. Rats were divided into the following five groups:

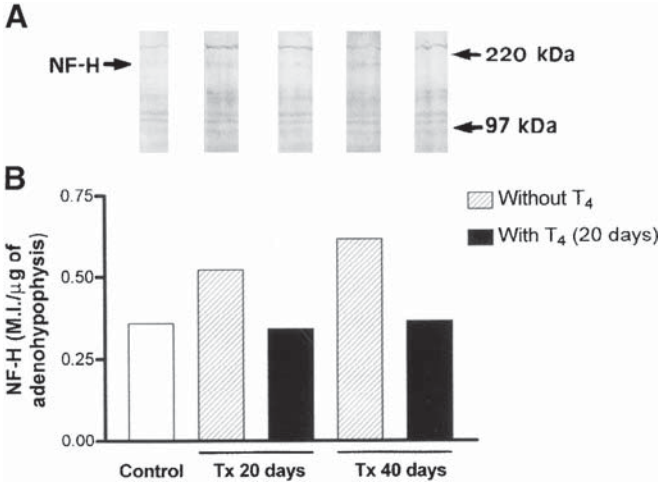

Fig. 4. Western blot analysis of NF-H in adenohypophyses of rat. (**A**) Homogenates from adenohypophyses (100 μg of protein/lane) of control, Tx 20 d and Tx 40 d with or without T_4 treatment were processed for SDS-PAGE. (**B**) Analysis by densitometry expressed as media of intensity (M.I.) obtained from Western blot results.

1. Nonoperated controls;
2. Thyroidectomized 20 d (Tx 20 d);
3. Thyroidectomized 20 d with replacement of T_4 (Sigma Chemical Co.; 3 μg/im every second day) (Tx 20 d + T_4 20 d);
4. Thyroidectomized 40 d (Tx 40 d);
5. Thyroidectomized 40 d with replacement of T_4 (3 μg/im every second day) starting at 20 d after surgery (Tx 40 d + T_4 20 d).

Groups were sacrificed by decapitation under deep sodium pentobarbital anesthesia (50-mg/kg ip) Blood was collected for plasma measurements of TSH and T_4 by the enzyme-linked immunosorbent assay. After removal of the neurointermediate lobe, the anterior pituitaries were weighed and used for immunohistochemical and Western blotting analysis.

Immunohistochemical Analysis

Five anterior pituitaries per group were fixed in 10% buffered formalin, dehydrated in graded ethanol and embedded in paraffin. Consecutive horizontal 5 μm-thick sections were prepared. For the immunohistochemical analysis of NF-H and TSH, the avidin-biotin-peroxidase complex method was applied *(17)* using a kit (Vectastin ABC kit; Dimension Laboratories Inc., CA). Primary antibodies raised against NFs of 200 kDa (rabbit polyclonal antineurofilaments; Sigma, St. Louis, MO) and rat TSHβ (kindly donated by Dr. Parlow; NIDDK, Bethesda, MD) were used at 1:200 and 1:300 dilutions, respectively and incubated at 4ºC overnight. The peroxidase complex was visualized by 0.05% diaminobenzidine and 0.01% H_2O_2. The nuclei were lightly counterstained with hematoxylin. The specificity of the immunoreaction was tested by:

1. Omitting the primary antibody
2. Replacing it with an equivalent concentration of nonspecific rabbit immunoglobulin G
3. Sections incubated with the primary antibody preabsorbed with neurofilaments obtained from spinal cord of rats.

No immunostaining was observed in these sections. In order to recognize that NF-H is present in thyrotrophs (immunoreactivity to TSH) and not in other pituitary cell types, in consecutive sections were immunostained for different hormones (LH, FSH, ACTH, GH and PRL).

The pituitaries were examined by light microscopy at a magnification of ×400 and ×1000 using an oil-immersion lens. The number of immunoreactive cells for NF-H and TSH was expressed in square millimeters per adenohypophysis using Woosley's method *(18)*.

Western Blotting Analysis

Five anterior pituitaries per group were homogenized in a manual tissue grinder in ice-cold buffer A containing 20 m*M* Tris-HCl, pH 7.4, 10 m*M* Cl_2Mg, 1 m*M* ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and 0.6 μ*M* aprotinin. The homogenate was incubated in this buffer at 4ºC for 20 min. The triton-insoluble cytoskeleton and associated proteins were isolated by centrifugation at 16,000*g* for 30 min. The pellet was solubilized in 50 μL of buffer A without Triton X-100 and the proteins were quantified by Bradford assay *(19)*. Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (7,5% SDS-PAGE) was carried out as described by Laemmli *(20)* using the miniprotean system (Bio-Rad, Hercules, CA) and running on 100 μg of proteins of each sample. After electrophoresis, gels were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Sigma) according to the procedure of Towbin et al. *(21)*. The membranes were blocked in a solution consisting of 3% bovine serum albumin in Tris buffer saline (TBS) (0.5 *M* NaCl, 20 m*M* Tris-HCl pH 7.5) for 1 h at room temperature and then incubated at 4ºC overnight with the rabbit polyclonal antineurofilament raised against 200 kDa (Sigma), diluted 1:1000 in blocking buffer. After several washes with TBS and Tween tris buffer saline (TTBS) (0.5 *M* NaCl, 20 m*M* Tris-HCl pH 7.5 with 0.05% Tween-20), the membranes were incubated for 2 h with alkaline phosphatase-conjugated secondary antibody (diluted 1:20,000 in blocking buffer), and after repeat washes, the alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma). 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 μg of adenohypophyses.

Results are expressed as the mean ± SEM. The differences between means were compared by the Tukey-Kramer

multiple comparison test. Values of $p < 0.05$ were considered statistically significant.

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References

1. Beck-Peccoz, P., Brucker-Davis, F., Persani, L., Smallridge, R., and Weintraub, B. D. (1996). *Endocr. Rev.* **17,** 610–638.
2. Vidal, S., Horvat, E., Kovacs, K., Cohen, S. M., Lloyd, R. V., and Scheithauer, B. W. (2000). *Virch. Arch.* **436,** 43–51.
3. Asa, S. L. and Ezzat, S. (1998). *Endocr. Rev.* **19,** 798–827.
4. Horvath, E., Lloyd, R. V., and Kavacs, K. (1990). *Lab. Invest.* **63,** 511–520.
5. Quintanar-Stephano, A., Valverde-R. C., and Kovacs, K. (1999). *Endocr. Pathol.* **10,** 335–341.
6. Julien, J. P. (1999). *Curr. Opin. Neurobiol.* **9,** 554–560.
7. Ogawa, A., Sugihara, S., Nakanishi, Y., et al. (1990). *Virch. Arch. B Cell. Pathol.* **58,** 331–340.
8. Höfler, H., Denk, H., and Walter, G. F. (1984). *Virch. Arch. [A]* **404,** 359–368.
9. Ogawa, A., Sugihara, S., Hasegawa, M., et al. (1990). *Virch. Arch. B Cell. Pathol.* **58,** 341–349.
10. Kasper, M. (1992). *Acta. Histochem. Suppl.* **42,** 221–223.
11. Mendez, A., Martin de las Mulas, J., Bautista, M. J., et al. (1998). *J. Comp. Pathol.* **118,** 29–40.
12. Salinas, E. and Quintanar, J. L. (2000). *Endocr. Pathol.* **11,** 195–200.
13. Quintanar, J. L. and Salinas, E. (2001). *Cell. Physiol. Biochem.* **11,** 27–32.
14. Ravindra, R. and Grosvenor, C. E. (1990). *Mol. Cell. Endocrinol.* **71,** 165–176.
15. Burrows, H. L., Douglas, K. R., Seasholtz, A. F., and Camper, S. A. (1999). *Trends Endocrinol. Metabol.* **10,** 343–352.
16. Houseweart, M. K. and Cleveland, D. W. (1998). *Curr. Opin. Cell. Biol.* **10,** 93–101.
17. Hsu, S. M., Raine, L., and Fanger, H. (1981). *Am. J. Clin. Pathol.* **75,** 816.
18. Woosley, J. T. (1991). *Arch. Pathol. Lab. Med.* **115,** 555–557.
19. Bradford, M. M. (1976). *Anal. Biochem.* **72,** 248–254.
20. Laemmli, U. K. (1970). *Nature* **227,** 680–685.
21. Towbin, H., Staehelin, J., and Gordon, J. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.