DISTRIBUTION OF THYROSTIMULIN IN THE RAT: AN IMMUNOHISTOCHEMICAL STUDY

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Objective. To identify the distribution of thyrostimulin, a heterodimer of glycoprotein hormone subunits (A2 and B5) by immunohistochemistry in the rat tissues using specific antipeptide antiserum which we recently produced.

Method. Anti-thyrostimulin antibody was raised in New Zealand white rabbits immunized with a conjugate of synthetic A2 or B5 with bovine serum albumin. Immunohistochemical analysis was performed by avidin-biotin complex method.

Results. Thyrostimulin immunoreactivity was visualized in the anterior pituitary, central nervous system, adrenal gland, stomach, duodenum, pancreas and testis. When using antiserum preincubated with synthetic peptides or rat pituitary homogenate which contains thyrostimulin peptide, no significant stain of the pituitary was detected.

Conclusion. These findings suggest that thyrostimulin is widely distributed and that the method used is valuable in studying the distribution of thyrostimulin in rats.

Key words: Thyrostimulin - Immunohistochemistry - Glycoprotein hormone subunits - A2 antibody - B5 antibody

Recently, NAKABAYASHI et al. (2002) described a novel heterodimeric glycoprotein hormone containing two unique subunits, which they termed A2 and B5, and this hormone was termed thyrostimulin by them. They identified A2 and B5 in a homology search of the human genome (GenBank). The same group of authors (HSU et al. 2002) also described orthologs of these subunits found in diverse species. A2 and B5 share only modest amino acid similarity with the related glycoprotein hormone subunit family members, containing all of the cysteine residues required to constitute the characteristic cysteine knot structure discovered in these and other growth factors. Thyrostimulin is synthesized in the pituitary and other peripheral tissues and it is suggested to act in a paracrine fashion at these sites (NAKABAYASHI et al. 2002; HSU et al. 2002). The finding prompted us to investigate the distribution of thyrostimulin in rats by immunohistochemistry using our newly produced specific antipeptide antiserum.

Materials and Methods

Animals. Male Wistar rats weighing 250-280 g were housed in a temperature (22 °C) and humidity (60 %) controlled room on 12 h illumination cycle. They were fed with a laboratory chow and water ad libitum.

Preparation of anti-thyrostimulin antiserum. Peptides corresponding to the following sequence of A2 or B5 were synthesized using a solid phase method and automated peptide synthesizer, followed by purification with HPLC: A2-38; VTVRSDRQGTCQGSH, A2-65; AFPSRYSVLVASGY, B5-86; YNETKQVTVKLPN, B5-100; APGVDPFYTYPVAI, according to the method previously described (HIROOKA et al. 1993). The peptides were conjugated on an equal weight basis to bovine serum albumin by the method previously described for anti-GHRH antibody (MITSUMA et al. 1983), using glutaraldehyde. New Zealand white rabbits were immunized with the emulsion of one mg of the conjugate in

one ml water in complete Freund's adjuvant (1:2, v/v) which was injected into the foot pad at intervals of three weeks. Blood was drawn one week after each injection. The presence of each antiserum was checked by imunoprecipitation method as reported elsewhere (Hirooka et al. 1992).

Preparation of tissue for thyrostimulin. The anterior pituitaries were dissected and a pool of these glands weighing 100 mg was homogenized in 5.0 ml of cold 0.32 M sucrose. The homogenate was centrifuged at 600 x g and the resulting supernatant was then centrifuged at 48000 x g for 20 min at 4 °C according to the reported method (Reubi et al. 1982). The pellet was resuspended in 0.32 M sucrose and used as thyrostimulin fraction.

Perfusion method and immunohistochemical method. The rats were anesthetized with sodium pentobarbital and transcardially perfused with 0.01 % glutaraldehyde and 4 % paraformaldehyde in Bouin's solution (pH 7.2). The brain, spinal cord, retina, pituitary gland, lung, heart, liver, kidney, thyroid gland, adrenal gland, pancreas, testis, stomach and duodenum were removed, postfixed for additional 24 hours at 4°C and cut into 4 μm slices using a viratome. Immunohistochemical treatment was performed by avidin-biotin compex (ABC) method, using Vecstatin kit (Vector Laboratories Inc., Burligam, CA). The primary antibody was used after dilution (1:50). To confirm the specificity of thyrostimulin antibody, the following methods were used: 1. omission of the primary antisera or the secondary antiserum in the peroxidase technique; 2. preabsorption of the antisera prior to the incubation of experimental tissues with anterior pituitary homogenate containing thyrostimulin (1.0 mg/ml antiserum). Specific immunohistochemical stain could not be seen in any of these control paradigms (Fig. 1); 3. serial dilution of primary antiserum, in which specific stain disappeared at 1:1000 dilution.

The brain nuclei were determined using the map by Pellegrino et al. (1969).

Results

Specific stain for A2-38 was found in the anterior pituitariy, central nervous system, stomach, adrenal gland and pancreas, but not in the heart, lung, liver, kidney, thyroid gland, posterior pituitary, duodenum and testis (Fig. 1-3, Tab.1). In the nervous system, significant stain revealed neural perikarya, axons and dendrites. For A2-65, specific staining was detected in the anterior pituitary, central nervous system, stomach, duodenum, adrenal gland, testis and pancreas, but not in the heart, lung, liver, kidney, thyroid gland and posterior pituitary (Fig. 1-3, Tab.1). Specific stain for B5-86 was observed in the anterior pituitary, central nervous system, stomach, duodenum, adrenal gland, testis and pancreas, but not in the heart, lung, liver, kidney, thyroid gland and posterior pituitary (Fig. 1-3, Tab. 1). As for B5-100, specific stain was shown in the anterior pituitary, central nervous system, duodenum and adrenal gland, but not in the heart, lung, liver, kidney, pancreas, thyroid gland, testis and posterior pituitary (Fig. 1-3, Tab.1).

Discussion

The distribution of thyrostimulin immunoreactivity in the rat body was estimated immunohistochemically. Anti-A2 or anti-B5 antibody was raised in New Zealand white rabbits by repeated injections of a conjugate of synthetic peptide to bovine serum albumin with complete Freund's adjuvant.

These antibodies were characterized by immunohistochemical method: thyrostimulin immunoreactivity was specially eliminated by the preincubation of antiserum with and excess amount of synthetic peptide or anterior pituitary homogenates containing thyrostimulin fractions. The significant stain was not detected without the antiserum and the dilution of antibody reduced the significant stain. These data indicated that the antiserum used in this study is specific and that this method can be utilized to detect the distribution of thyrostimulin in the rat body. The present study clearly demonstrated that thyrostimulin is widely distributed in rat body. Both A2 and B5 were shown in the anterior pituitary, but not in the posterior. Concerning extrapituitary region, there found in the central nervous system, stomach, adrenal gland, pancreas, duodenum and testis.

In the central nervous system, it stained the perikarya, dendrites and axons of the hypothalamus, cerebrum, cerebellum, midbrain, pons, medulla oblongata, spinal cord and retina. These data are partly comparable with previous reports (Nakabayashi et al. 2002; Hsu et al. 2002) in which thyrostimulin heterodimer is expressed in the brain, pituitary, thyroid, ovary and heart. We did not examined the ovary. Regarding the thyroid gland, A2 as well as B5 was not found in this study. One of the conceivable reason for the discrepancy between their finding and ours might be due to the difference of the antibody used. However, further studies

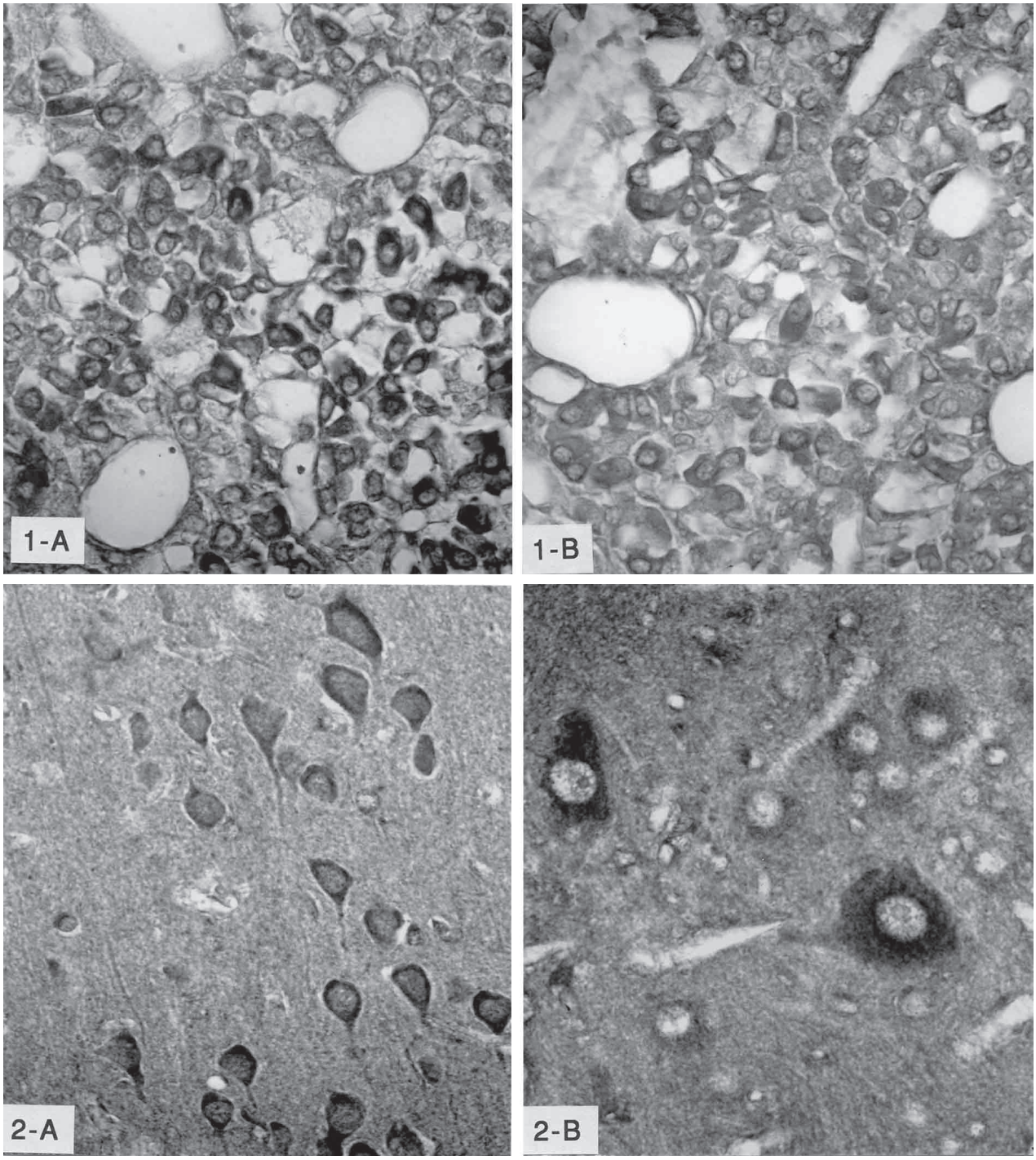

Fig. 1 A2-38 immunoreactivity in the anterior pituitary with (A) and without (B) anti-A2-38 antibody. Without anti-A2-38 antibody the staining completely disappeared.

Fig. 2 Demonstration of A2-38 immunoreactivity in the central nervous system: A- cerebrum, B- medulla oblongata.

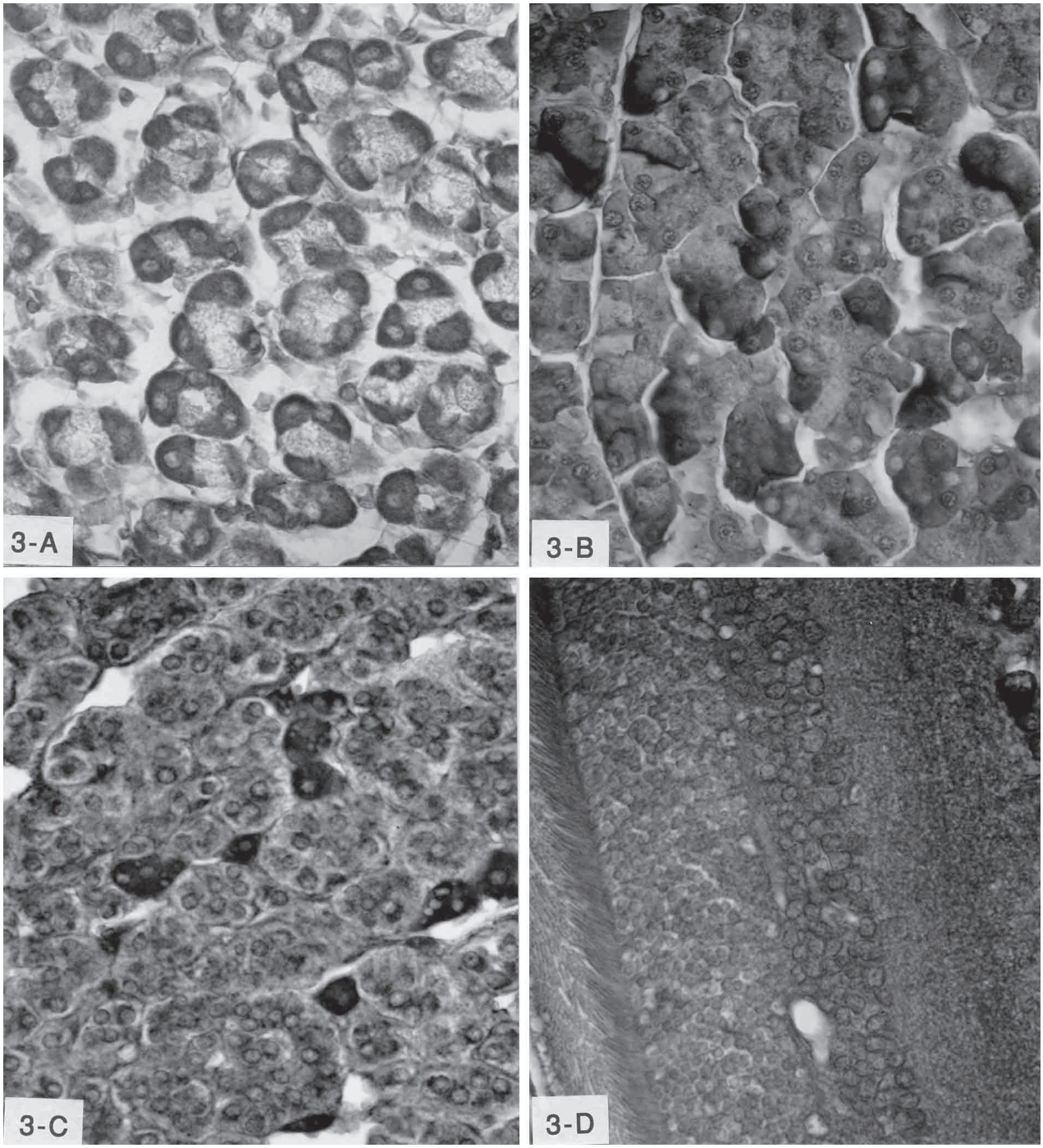

Fig. 3 Distribution of A2-38 immunoreactivity in gastric mucosa (A) , pancreas (B) , adrenal gland (C) and retina (D).

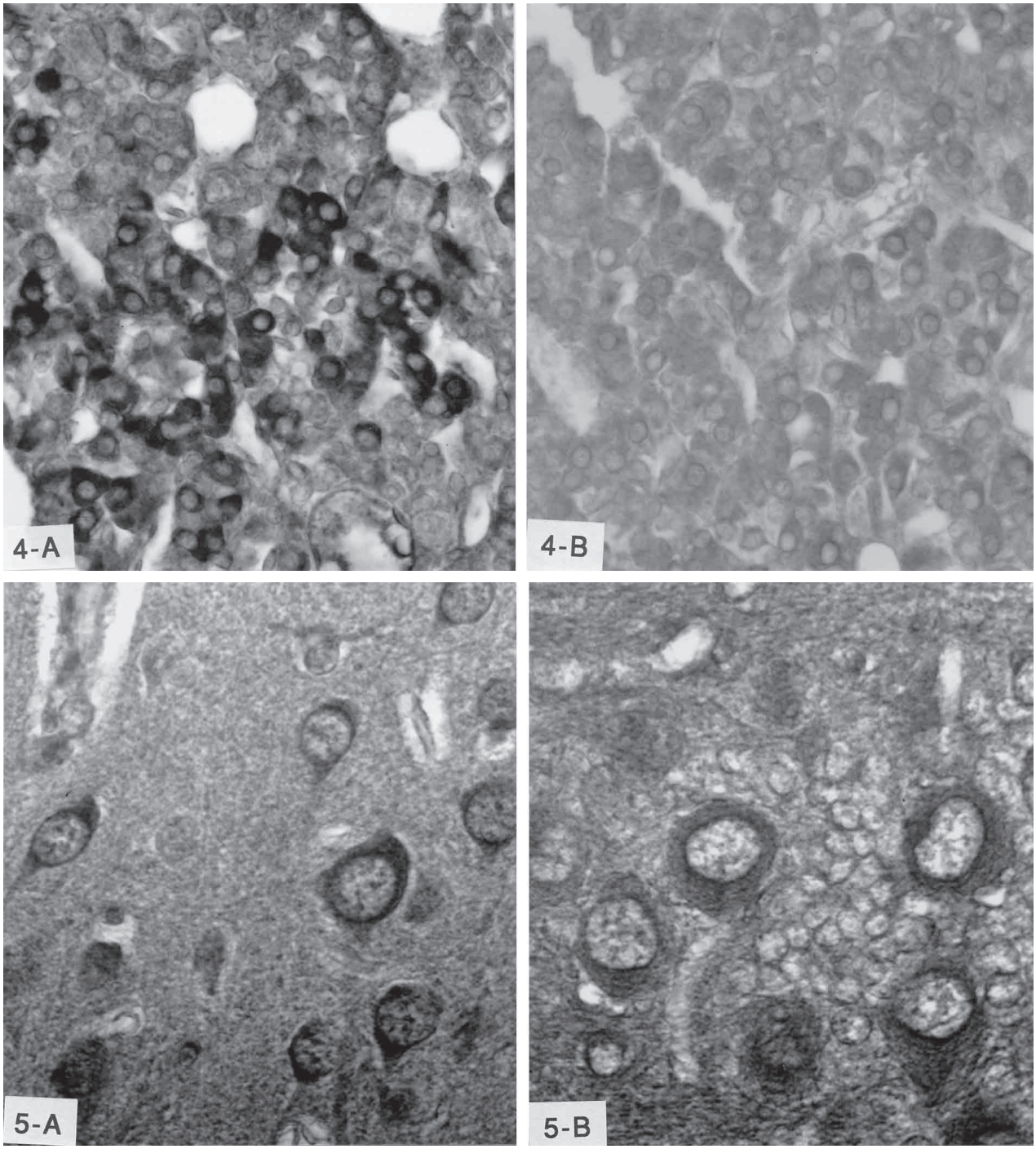

Fig. 4 A2-65 immunoreactivity in the anterior pituitary with (A) and without (B) anti-A2-65 antibody. Without anti-A2-65 antibody the staining completely disappeared.

Fig. 5 Demonstration of A2-65 immunoreactivity in the central nervous system: A- cereberum, B- cerebellum.

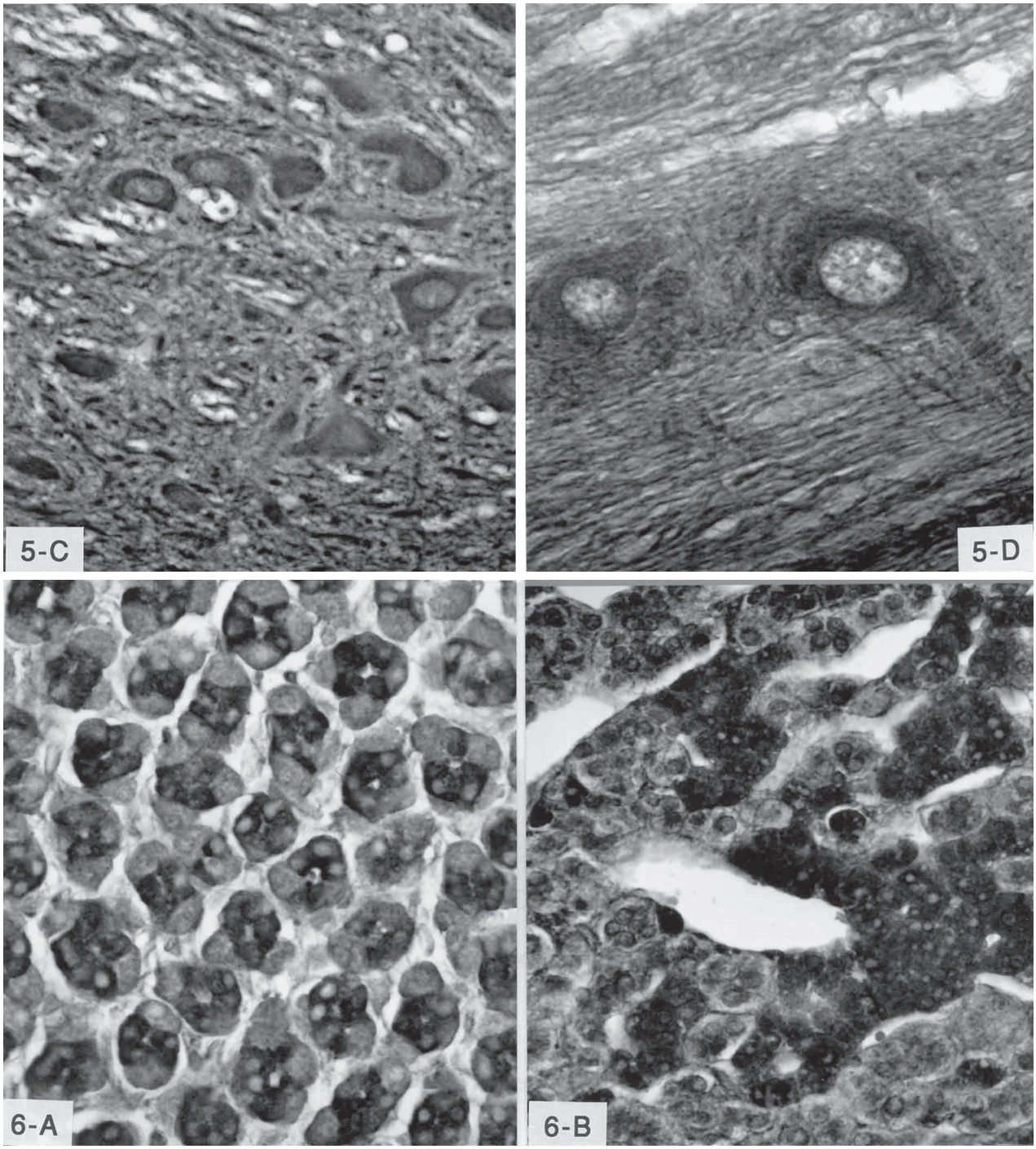

Fig. 5 Demonstration of A2-65 immunoreactivity in the central nervous system: C- medulla oblongata, D- spinal cord.

Fig. 6 Distribution of A2-65 immunoreactivity in gastric mucosa (A), adrenal gland (B).

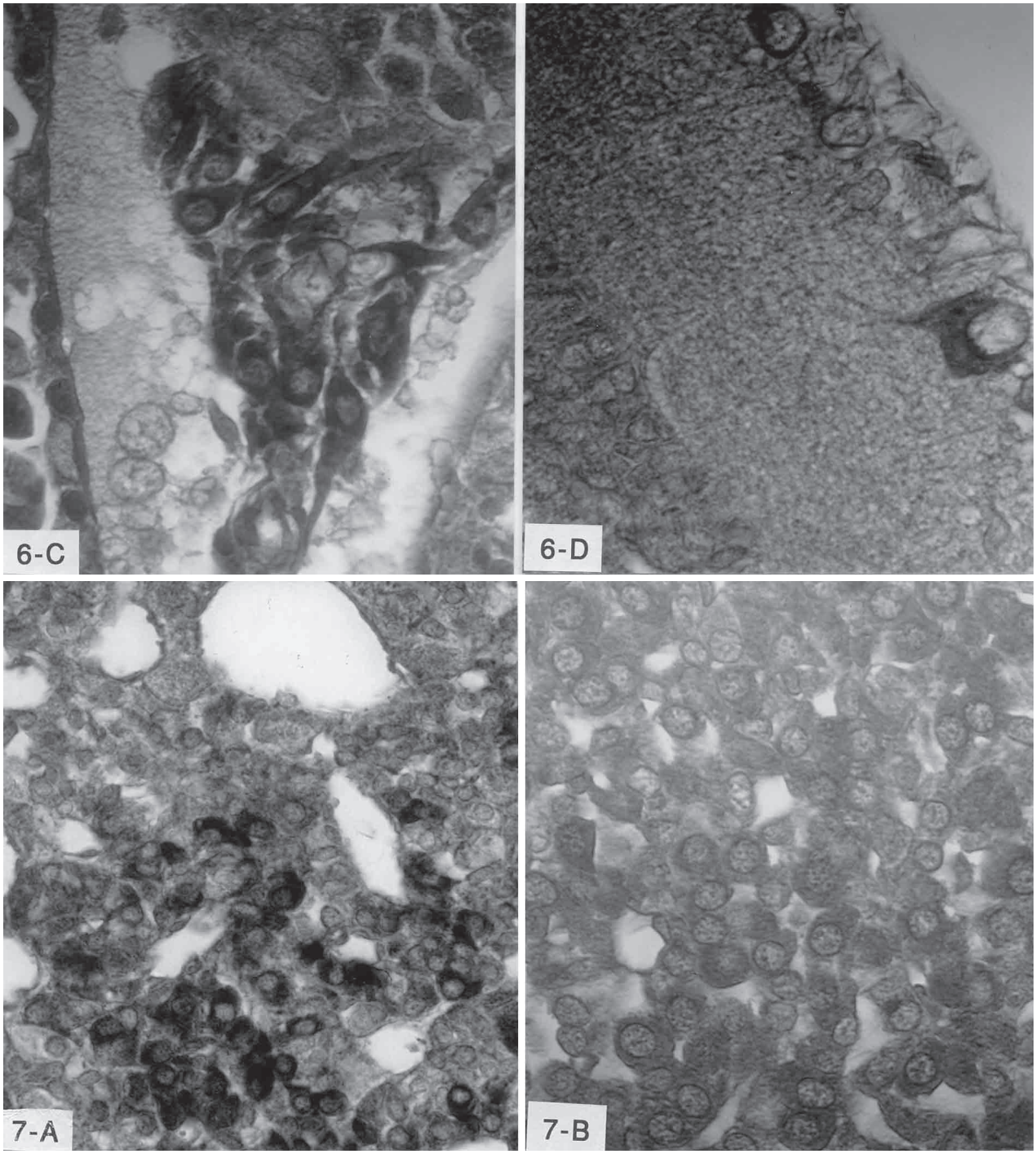

Fig. 6 Distribution of A2-65 immunoreactivity in testis (C) and retina (D).

Fig. 7 B5-86 immunoreactivity in the anterior pituitary with (A) and without (B) anti-B5-86 antibody. Without anti-B5-86 antibody the staining completely disappeared.

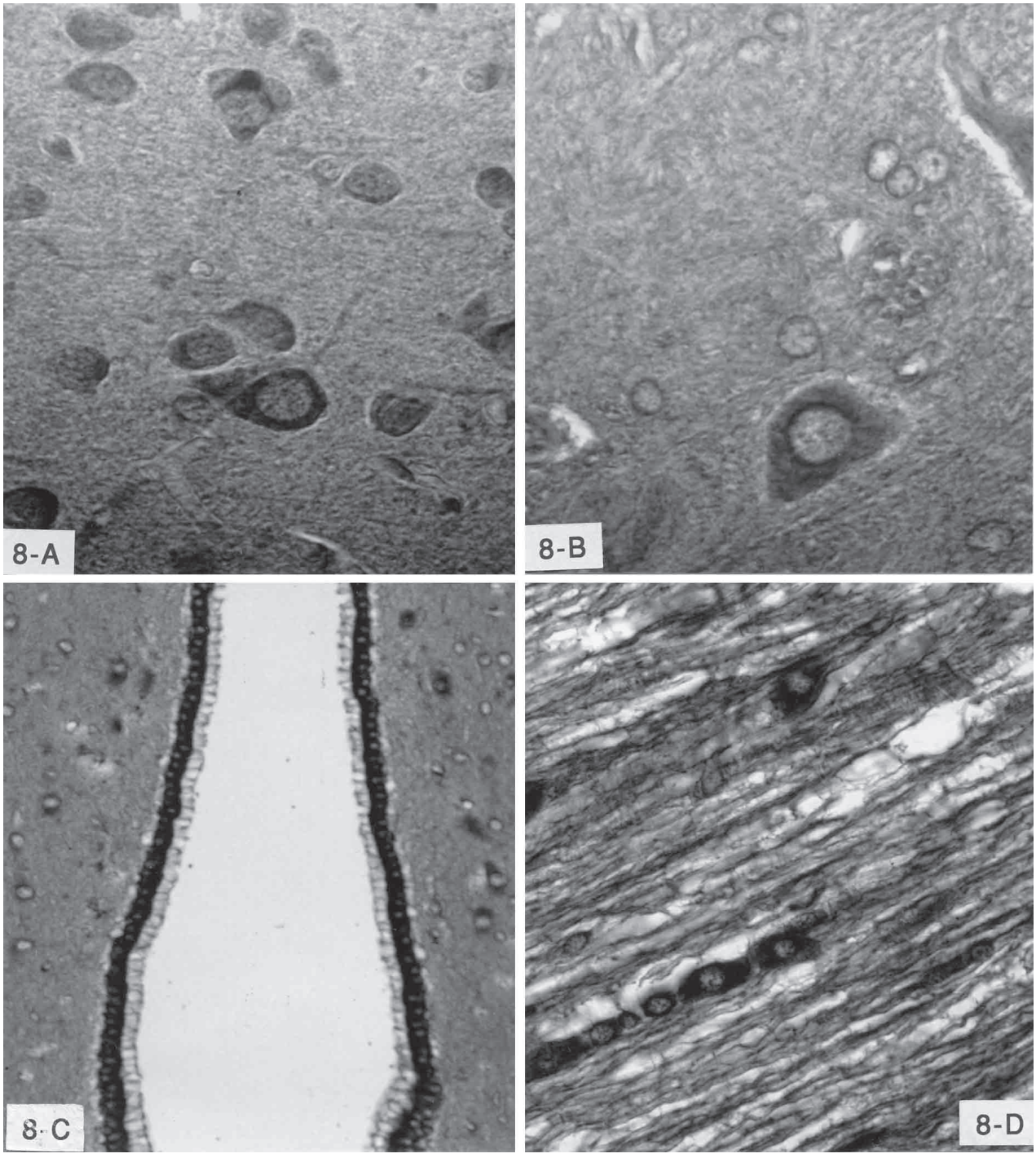

Fig. 8 Demonstration of B5-86 immunoreactivity in the central nervous system: A- cerebrum, B- medulla oblongata, C- hypothalamus, D- spinal cord.

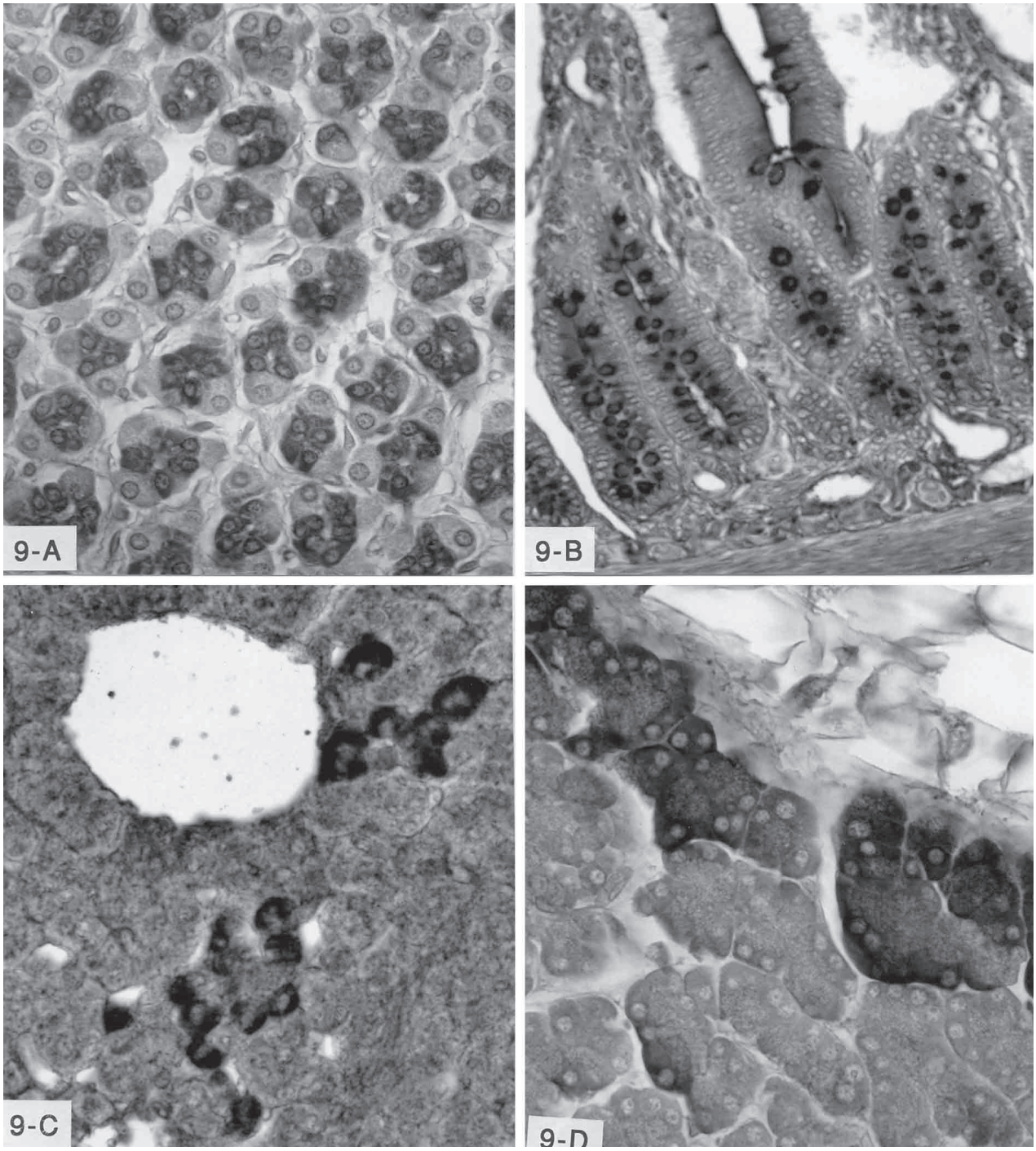

Fig. 9 Distribution of B5-86 immunoreactivity in gastric mucosa (A), duodenum (B), adrenal gland (C), pancreas (D).

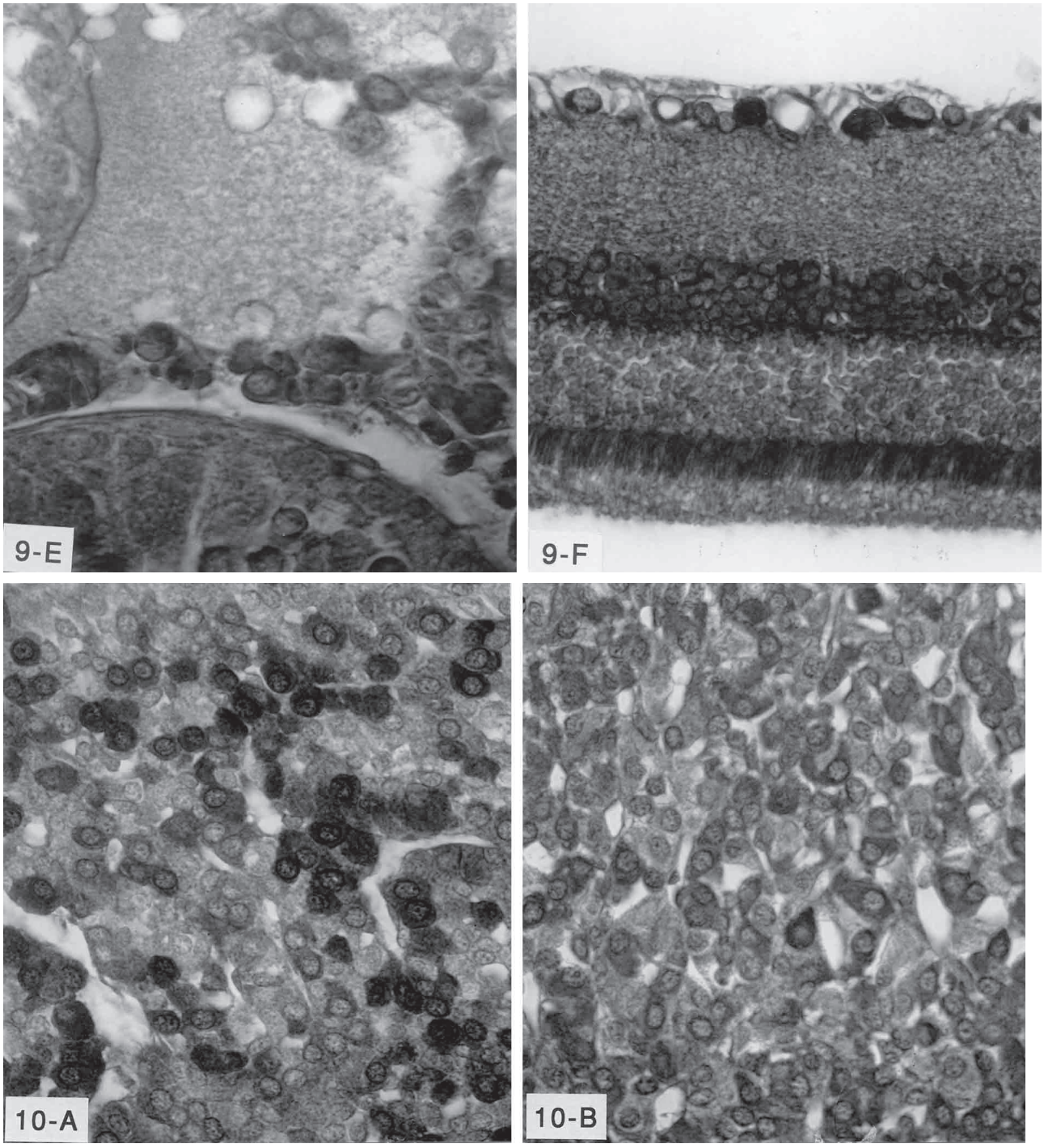

Fig. 9 Distribution of B5-86 immunoreactivity in testis (E) and retina (F).

Fig. 10 B5-100 immunorectivity in the anterior pituitary with (A) and without (B) anti-B5-100 antibody. Without anti-B5-100 antibody the staining completely disappeared.

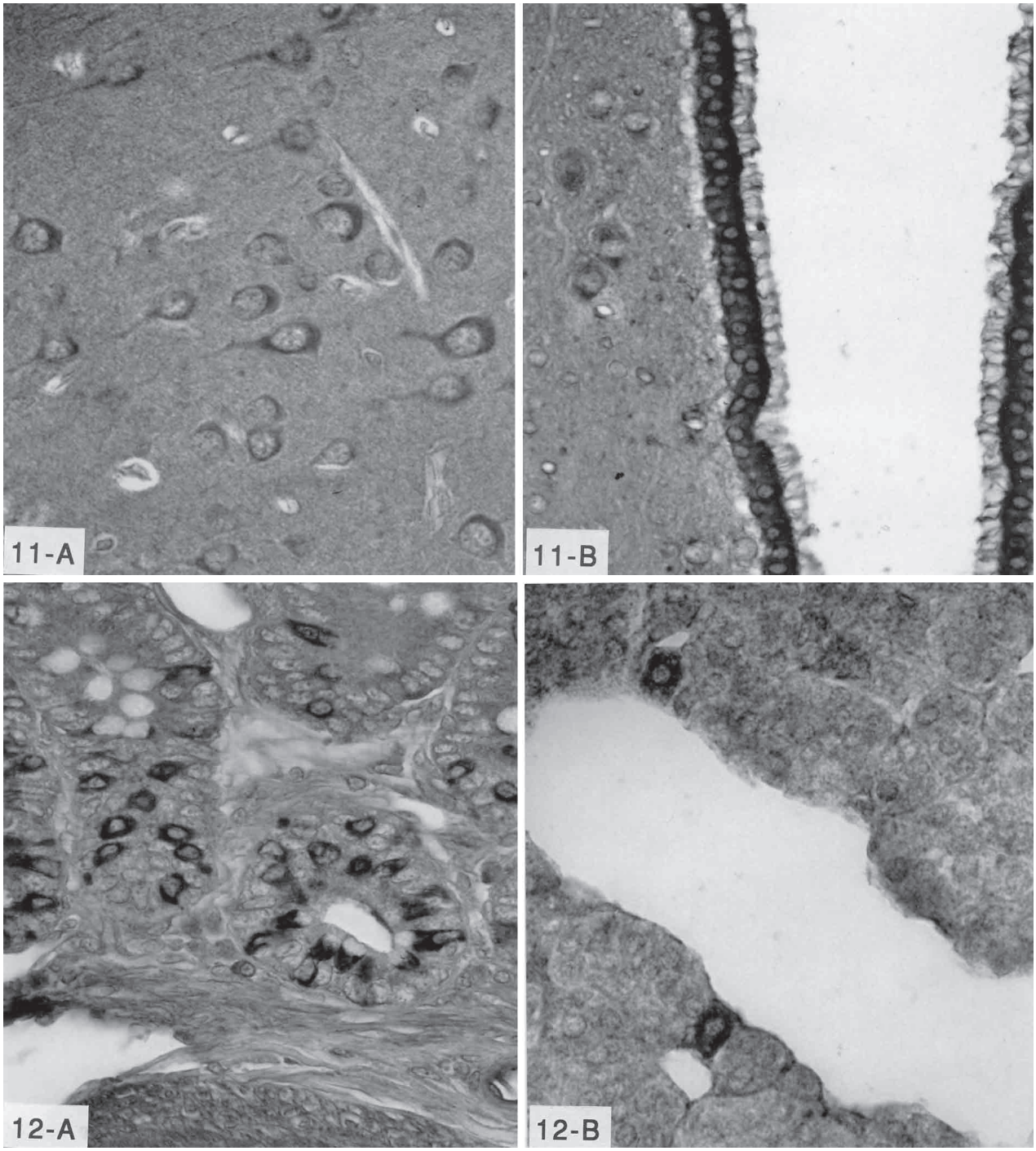

Fig. 11 Demonstration of B5-100 immunoreactivity in the central nervous system: A- cerebrum, B- hypothalamus.

Fig. 12 Distribution of B5-100 immunoreactivity in duodenum (A) and adrenal gland (B).

need to be done in order to elucidate this problem. It was reported that thyrostimulin was found to be capable of activating TSH receptor in vitro and in vivo (Nakabayashi et al. 2002) and the group also reported that thyrostimulin could act in a paracrine fashion at the extra-thyroid tissues. Although the expression of TSH receptor has been demonstrated in brain (Crisanti et al. 2001), orbital fibroblasts and heart (Bell et al. 2000), thymus (Spitzweg et al. 1999) and adipose tissues (Birnbaumer et al. 1969; Roselli-Rehfuss et al. 1992), further studies need to be done to clarify the mechanism of thyrostimulin signaling and also its exact role at the extra-thyroid tissues.

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