Expression of neuropeptides B and W and their receptors in endocrine glands of the rat

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Abstract. Neuropeptides B and W (NPB and NPW) have been identified as endogenous ligands of the G protein-coupled receptors (GPR) 7 and 8, which in humans are expressed in the hypothalamus and probably involved in the regulation of energy homeostasis and feeding behavior. GPR8 is absent in the rat, where the GPR8-like receptor (GPR8-LR) has been described. Reverse transcription-polymerase chain reaction detected the expression of NPB, NPW, GPR7 and GPR8-LR mRNAs in the hypothalamus, anterior pituitary, thyroid and parathyroid glands, pancreatic islets, adrenal glands, ovary and testis of the rat. Immunocytochemistry demonstrated the presence of NPB and NPW immunoreactivities in these same glands. Radioimmune assay showed that the bolus intraperitoneal injection of 2 nmol/100 g NPB or NPW raised the plasma levels of parathyroid hormone, corticosterone and testosterone. NPB also increased the blood concentration of thyroxine, and NPW that of ACTH and estradiol. Taken together, these findings allow us to suggest that NPB and NPW play a role in the autocrine-paracrine functional regulation of the endocrine system in the rat.

Introduction

Neuropeptides B and W (NPB and NPW) are 29- and 30-amino acid regulatory peptides, which have been recently identified as endogenous ligands of the G protein receptors (GPR) 7 and 8 (1,2). In rodents GPR8 is absent, its counterpart being described as GPR8-like receptor (GPR8-LR) (3). NPB, NPW and their receptors are highly expressed in the human and rodent central nervous system (especially hypothalamus) (1-8), and findings have been accumulated that these peptides are involved in the central regulation of feeding behavior and energy homeostasis (6,9-12).

Sparse and rather conflicting data are available on the expression of NPB, NPW and their receptors in the endocrine glands of the rat. To summarize, NPB and GPR7 mRNAs were identified in the pituitary, testis and ovary, but only GPR7 mRNA was detected in the thyroid, pancreas and adrenals (1). NPW-immunoreactivity (IR) was found in the larger diameter cells of the anterior pituitary (13). NPB, NPW, GPR7 and GPR8-LR mRNAs were demonstrated in both freshly dispersed and cultured adrenocortical cells (14). It, therefore, seemed worthwhile to investigate the expression of NPB, NPW and their receptors as mRNAs and proteins in the endocrine glands of the rat, as well as to ascertain whether these peptides affect *in vivo* their secretory activity.

Materials and methods

Animals. Adult male and female Wistar rats (200-250 g body weight) were kept under a 12:12-h light:dark cycle (illumination onset at 8.00 a.m.) at 23˚C, and maintained on a standard diet and tap water *ad libitum*. Rats were decapitated, and the hypothalamus, anterior pituitary, thyroid and parathyroid glands, pancreas, adrenal glands, ovaries and testes were promptly removed. Specimens were immediately placed in RNA later (Qiagen, Hilden, Germany) or fixed in Bouin's solution for immunocytochemistry (ICC). For reverse transcription (RT)-polymerase chain reaction (PCR) studies pancreatic islets were isolated by collagenase digestion (15). Groups of rats were given an intraperitoneal injection of 2 nmol/100 g body weight of NPB29 (rat) or NPW30 (human) (Phoenix Pharmaceuticals, Belmont, CA) dissolved in 0.2 ml 0.9% NaCl. The control group received the vehicle only. Animals were decapitated 60 min after the injection. The trunk blood was collected in the presence of EDTA (1 mg/ml), and plasma was separated and stored at -80˚C until radioimmune assay (RIA). The study protocol was approved by the local Ethics Committee for Biomedical Studies.

RT-PCR. Total RNA was extracted from 30 mg of tissues, and reverse transcribed to cDNA (16-18). PCR was performed in a Perkin-Elmer 480 DNA thermal cycler (Perkin-Elmer Life

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Key words: neuropeptide B, neuropeptide W, GPR7, GPR8-like receptor, hypothalamus, endocrine glands, rat

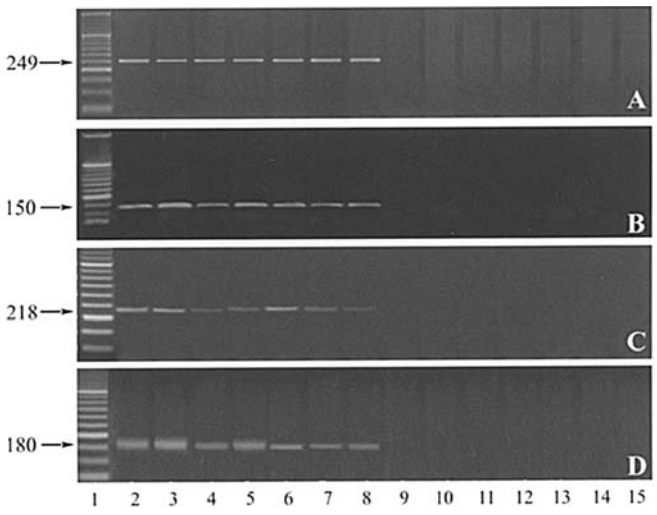

Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat NPB (A), NPW (B), GPR7 (C) and GPR8-LR (D) specific primers from an exemplary rat hypothalamus (2), anterior pituitary (3), thyroid gland (4), adrenal gland (5), testis (6), ovary (7) and pancreatic islets (8). Respective negative controls (no RT of the mRNA) are shown in lanes 9-14. Lane 1 was loaded with 200 ng of a DNA size marker (50 bp DNA Ladder; MBI Fermentas, Vilnius, Lithuania).

Sciences, Milan, Italy) (19-21), using the following program: predenaturation step at 94˚C for 30 sec, followed by 34 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 60 sec and extension at 68˚C for 120 sec. Primer sequence and the predicted size of amplicons were as follows: i) prepro (pp) NPB, sense (80-98) 5'-CGGGATCCCACCACTACTC-3' and antisense (305-324) 5'-GAAGACGTCCGCCTTACACT-3', 249 bp (NM 153293); ii) ppNPW, sense (489-510) 5'-ACCT GAGCAGTCGCTAAGCTTT-3' and antisense (619-638) 5'-CATCGGTTCTTGAGACGGTC-3', 150 bp (NM 153294); iii) GPR7, sense (660-679) 5'-CATCTGCGCCCTCTATATC A-3' and antisense (858-879) 5'-GAAGTAAGAGATGCCG ATGACC-3', 218 bp (AY 577901); and iv) GPR8-LR, sense (24-48) 5'-CAGCTCTCTTAGCCCAAGTGTAAGG-3' and antisense (283-303) 5'-GTGTCTTCCTCCCCCTGAATC-3', 180 bp (XM 578201). To rule out the possibility of amplifying genomic DNA, one PCR was performed without prior RT of the RNA. Detection of the PCR amplification product was performed by size fractionation on 2% agarose gel electrophoresis. The specificity of the PCR was further verified by sequencing analysis, as described previously (22).

ICC. Bouin-fixed tissues were processed for ICC, as detailed previously (23,24). Sections were incubated with the primary antibody (rabbit anti-NPB29 and anti-NPW23 antibodies, 1:100 dilution; Phoenix Pharmaceuticals) for 60 min at 4˚C. After 10-min washing in PBS, sections were incubated with the secondary antibody (Dako Strept AB Complex/HRP, 1:100 dilution; Dako, Glostrup, Denmark) for 60 min at 37˚C. Then peroxidase activity was detected using the DAB technique (Dako Liquid DAB Substrate-Chromogen System; Dako) and, when necessary, nuclei were counterstained with hematoxylin. Negative controls were carried out by similary treating adjacent sections and omitting the primary antibody, as well as using primary antibody preabsorbed with antigen excess.

RIA. ACTH, total-thyroxine (T_4), parathyroid hormone (PTH), insulin, corticosterone, testosterone and estradiol were measured using the following commercial kits: ACTH RIA kit (rat) (Phoenix Pharmaceuticals; sensitivity, 90 pg/ml; intra- and inter-assay CVs: 7% and 9%, respectively); T_4-RIA kit (Lynco, St. Charles, MO; sensitivity, 300 pg/ml; intra- and inter-assay CVs: 6% and 8%, respectively); PTH(1-34) RIA kit (Phoenix Pharmaceuticals; sensitivity, 220 pg/ml; intra- and inter-assay CVs: 5% and 7%, respectively); Insulin-RIA (Lynco; sensitivity, 100 pg/ml; intra- and interassay CVs: 5% and 8%, respectively); CRTX-RIA (Eurogenetix, Milan, Italy; sensitivity, 25 pg/ml; intra- and inter-assay CVs: 8% and 9%, respectively); Testosterone-RIA (Amersham Pharmacia, Aylesbury, UK; sensitivity, 5 pg/ml; intra- and inter-assay CVs: 7% and 9%, respectively); and Estradiol-RIA (Diagnostic Products, Los Angeles, CA; sensitivity, 8 pg/ml; intra- and inter-assay CVs: 6% and 8%, respectively). Data were expressed as means ± SEM (n=8), and their statistical comparison was made by ANOVA followed by the unpaired Student's t-test.

Results

RT-PCR allowed the detection of the mRNAs of ppNPB, ppNPW, GPR7 and GPR8-LR in the rat hypothalamus, anterior pituitary, thyroid and parathyroid glands, pancreatic islets, adrenal gland, testis and ovary (Fig. 1).

ICC demonstrated intense NPB-IR and NPW-IR in the perikarya of the magnocellular part of the hypothalamic paraventricular nucleus (PVN) (Fig. 2), as well as in the perikarya, axon hills and axons of supraoptic nucleus (SON) (Fig. 3). Nerve fibers of the outer and inner layers of the median eminence displayed moderate NPB-IR and NPW-IR, respectively (Fig. 4). Moreover, positivity for both peptides was found in the cerebellum, staining being more intense in the cytoplasm of the Purkynje cells (Fig. 5). In the anterior pituitary, NPB-IR and NPW-IR were more intense in basophils than in other cell types (Fig. 6). Particularly intense staining for NPB and NPW was found in parathyroids and the central portion of the thyroid gland, where NPB-IR was restricted to follicular cells and NPW-IR was present in both follicular and parafollicular cells (Fig. 7). All cell types of pancreatic islets displayed intense staining for both peptides, while acinar cells and epithelial cells of the excretory ducts exhibited moderate staining (Fig. 8). NPB-IR and NPW-IR were present in the entire adrenal cortex and, although less intense, in adrenal medulla, where the reaction was seen in the perinuclear region of chromaffin cells. NPW-positivity was also observed in medullary ganglion and SIF (small intensive fluorescent) serotonin-positive extra-ganglionic cells (Fig. 9). In the ovary, NPB and NPW immunostaining was observed in the thecal, granulosa and lutein cells, as well as in the oocyte cytoplasm (Fig. 10). In the testis, Leydig cells displayed intense positivity for both peptides. Immunostaining was also observed in the seminiferous tubules, NPW-IR being present in all generative-cell lineage and NPW-IR only in Sertoli cells, maturing spermatids and residual bodies (Fig. 11).

RIA. NPB acute administration evoked significant rises in the plasma concentration of T_4, PTH, corticosterone and

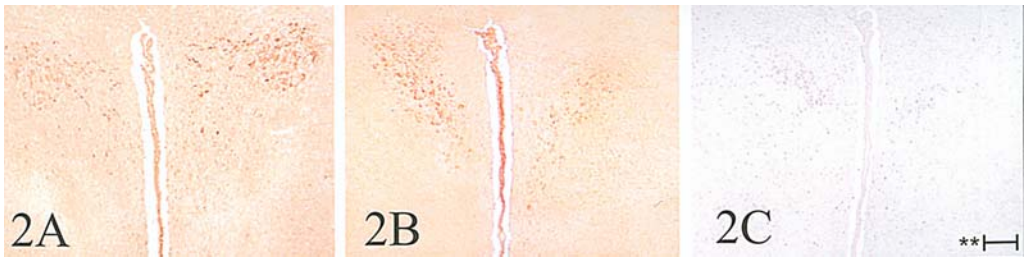

Figure 2. ICC localization of NPB-IR and NPW-IR in the rat hypothalamus. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, **100 μm.

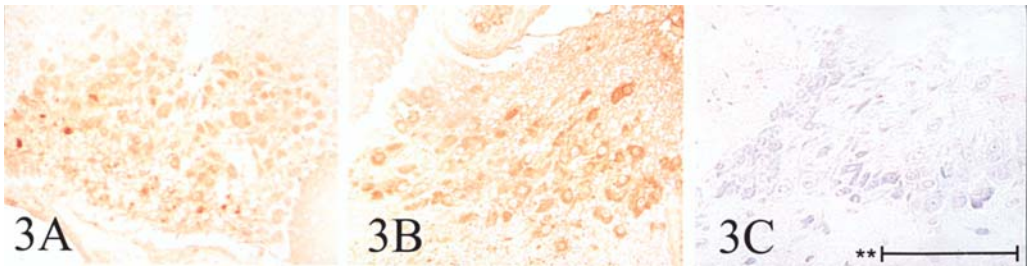

Figure 3. ICC localization of NPB-IR and NPW-IR in the rat supraoptic nucleus. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, **100 μm.

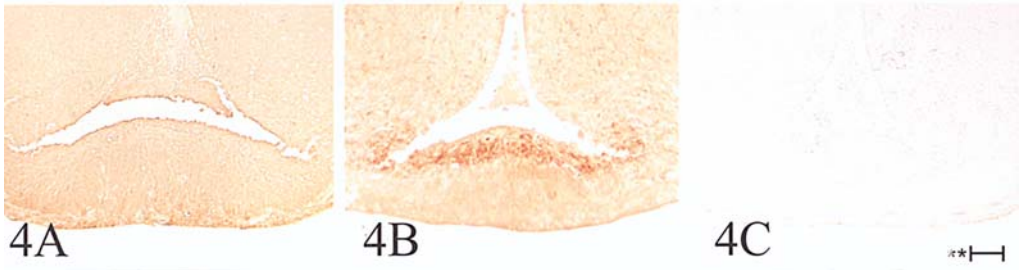

Figure 4. ICC localization of NPB-IR and NPW-IR in the rat median eminence. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, **100 μm.

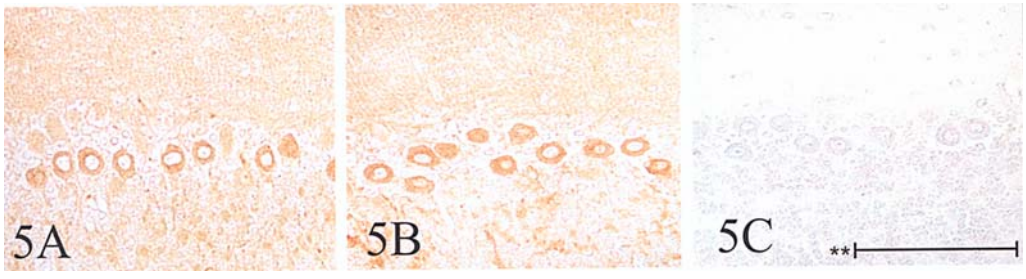

Figure 5. ICC localization of NPB-IR and NPW-IR in the rat cerebellar cortex. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, **100 μm.

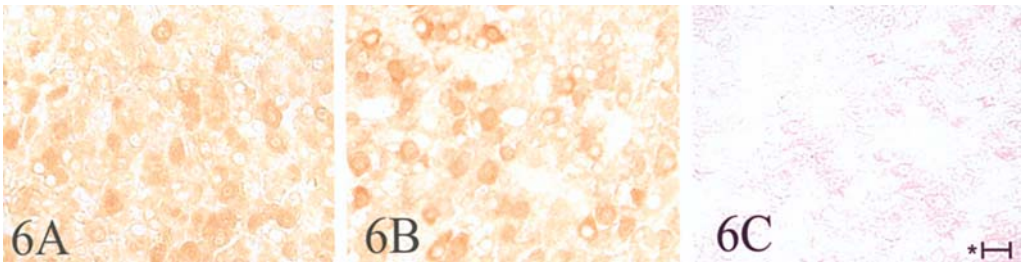

Figure 6. ICC localization of NPB-IR and NPW-IR in the rat anterior pituitary. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, *10 μm.

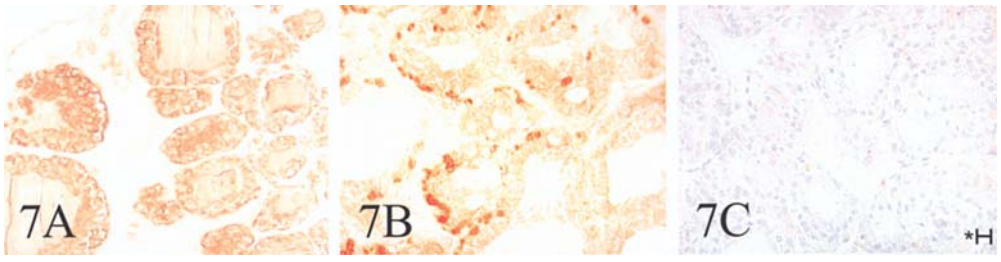

Figure 7. ICC localization of NPB-IR and NPW-IR in the rat thyroid and parathyroid glands. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, *10 μm.

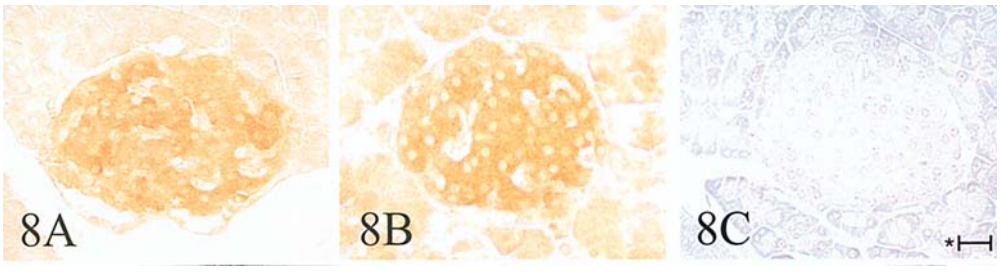

Figure 8. ICC localization of NPB-IR and NPW-IR in the rat pancreas. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, *10 μm.

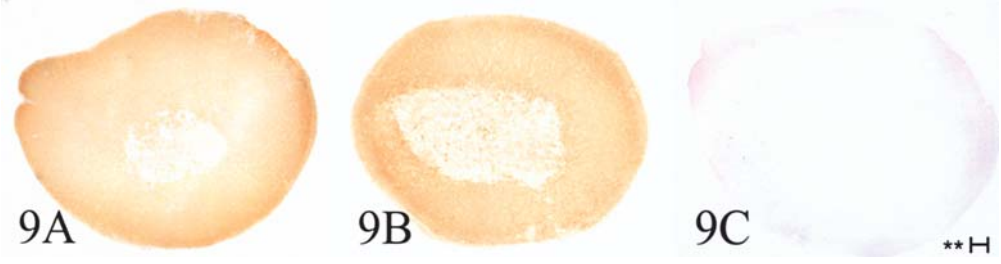

Figure 9. ICC localization of NPB-IR and NPW-IR in the rat adrenal gland. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, **100 μm.

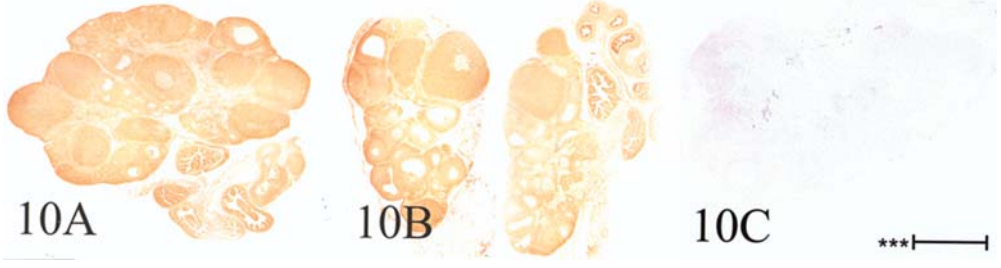

Figure 10. ICC localization of NPB-IR and NPW-IR in the rat ovary. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, ***1 mm.

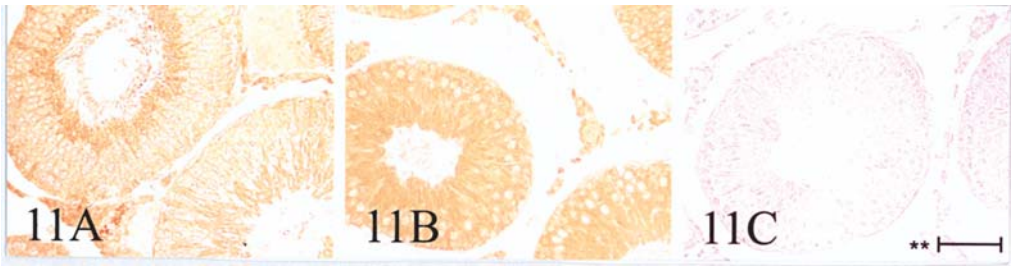

Figure 11. ICC localization of NPB-IR and NPW-IR in the rat testis. A, NPB-IR; B, NPW-IR; and C, control sections. Magnification, **100 μm.

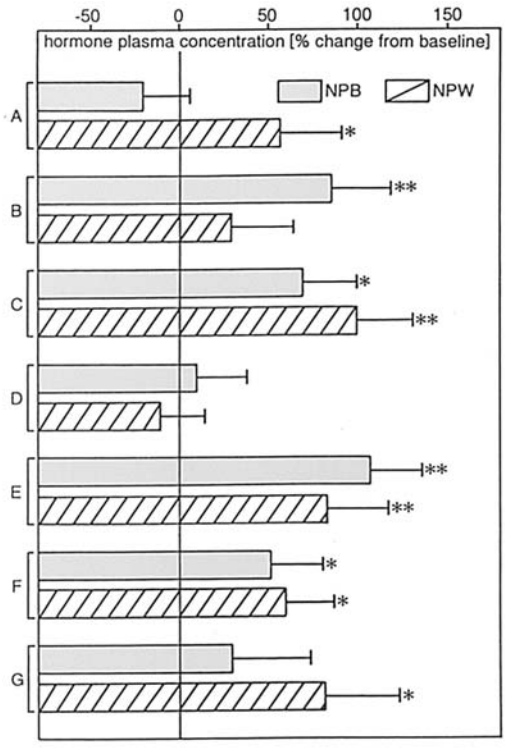

Figure 12. Acute effect of NPB and NPW bolus administration on the plasma concentrations of ACTH (A), total T_4 (B), PTH (C), insulin (D), corticosterone (E), testosterone (F) and estradiol (G). Values are expressed as percent change from baseline, and bars are means ± SEM (n=6). $^{*}P<0.05$ and $^{**}P<0.01$ from baseline (control value).

testosterone, and NPW caused increases in ACTH, PTH, corticosterone, testosterone and estradiol. Neither NPB nor NPW apparently affected insulin plasma levels (Fig. 12).

Discussion

Our present RT-PCR assays indicate that NPB, NPW, GPR7 and GPR8-LR mRNAs are expressed in the hypothalamus and endocrine glands of the rat. Moreover, ICC clearly shows that NPB and NPW are also present as proteins in the same tissues, thereby suggesting the possible involvement of these peptides in the autocrine-paracrine regulation of the endocrine-system function in this species.

NPB-IR and NPW-IR were found in the PVN, SOD and median eminence of the rat hypothalamus, as well as in the anterior pituitary basophils, which confirms earlier observations (13,25). These findings are in keeping with the contention that NPB and NPW may activate the central branch of the hypothalamic-pituitary-adrenal (HPA) axis, acting as potential stress mediators (9,11,26). According to the main role of NPW as stress mediator (26), our RIA findings clearly show a net *in vivo* acute stimulating effect of this peptide on ACTH and corticosterone secretion.

Abundant NPB-IR and NPW-IR were detected for the first time in the parathyroid and thyroid glands, especially in the follicular cells. The possibility that these peptides may control the secretory activity of these glands is supported by the demonstration that the acute administration of both NPB and NPW evoked a marked rise in the blood level of PTH and that of NPB in the plasma concentration of T_4. The presence of NPW-IR in the thyroid parafollicular cells could suggest the involvement of this peptide in the modulation of calcitonin release, and studies are under way to ascertain whether NPW and NPB play a role in the calcium homeostasis. The presence of NPB-IR and NPW-IR in the pancreatic islets would suggest their possible involvement in the regulation of insulin and/or glucagon secretion. However, our RIA data did not evidence any acute sizeable effect of these peptides on insulin blood concentration.

The expression of both NPB and NPW and their receptors in the adrenal cortex confirms earlier findings obtained in the rat (14) and human adrenocortical cells (17,27). These studies showed that both peptides stimulate *in vitro* glucocorticoid secretion from dispersed or cultured cells, and our present *in vivo* RIA observations agree with this contention. It may be conceived that *in vivo* NPB- and NPW-induced acute rise in the blood levels of corticosterone may ensue not only from the activation of the central branch of the HPA axis, but also from a direct secretagogue action on adrenocortical cells. The presence of NPB and NPW and their receptors in adrenal medulla could suggest the involvement of this system in the modulation of catecholamine release: an action connected with its possible role in stress responses (see above). However, the functional interrelationships between adrenal cortex and medulla are well established (reviewed in ref. 28): catecholamines are able to stimulate steroidogenesis, adrenocortical cells being provided with ß-adrenoceptors. Several regulatory peptides have been shown to enhance steroid secretion by eliciting the release of catecholamines from chromaffin cells, that in turn stimulate adrenocortical cells acting in a paracrine manner: VIP and PACAP (reviewed in ref. 29), neuropeptide-Y (reviewed in ref. 30), tachykinins (reviewed in ref. 31), endothelins (reviewed in ref. 32), adrenomedullin (reviewed in ref. 33) and ANP (34). The possibility that NPB and NPW may be included in this group of peptides, and that this effect may concur to the *in vivo* glucocorticoid secretagogue action of NPB and NPW is currently being explored in our laboratories.

The expression of NPB and NPW and their receptors has been found in the ovary and testis, especially in their steroid-secreting cells. This finding coupled with the observation that these peptides evoked a sizeable increase in the plasma concentrations of estradiol and testosterone, makes it likely that NPB and NPW may be involved in the regulation of gonadal-hormone secretion. It is also to be noted that the relevant expression of NPB and NPW in the testis seminiferous tubules could indicate their possible involvement in the regulation of spermatogenesis.

Our findings strongly suggest the possible role of NPB and NPW as endocrine-system regulators. However, the physiological and pathophysiological relevance of these effects remains to be addressed, and studies are under way that are aimed at ascertaining whether the immuno-blockade of the NPB and NPW system is able to affect the *in vitro* basal hormone secretion of rat endocrine glands.

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