Humoral and afferent neuronal regulation of parvicellular and magnocellular neurosecretory systems: immunocytochemical and in situ hybridization studies

PhD Thesis

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1. ABBREVIATIONS

ABC	- Avidin-biotin complex
ACTH	- Adrenocorticotropin
AN	- Arcuate nucleus of the hypothalamus
AP	- Alkaline phosphatase
BCIP/NBT	- 5-Bromo-4-chloro-3-indolyl-phosphate/4-Nitro blue tetrazolium
bp	- Base-pair
BSA	- Bovine serum albumin
BW	- Body weight
cDNA	- complementary deoxyribonucleic acid
CRF	- corticotrophic-releasing factor
CRH	- corticotropin-releasing hormone
cRNA	- complementary ribonucleic acid
DAB	- Diaminobenzidine tetrahydrochloride
dATP	- deoxyadenozin triphosphate
DBH	- Dopamine-beta-hydroxylase
DEPC	- Diethyl pyrocarbonate
DIG	- Digoxigenin
DTT	- Dithiothreitol
EDTA	- Ethylenediaminetetraacetic acid dihydrate, disodium salt
GH	- Growth hormone
GHRH	- Growth hormone-releasing hormone
ICC	- Immunocytochemistry
IP	- Intraperitoneal
IR	- Immunoreactive
ISH	- *In situ* hybridization
LH	- Luteinizing hormone
LHRH	- Luteinizing hormone-releasing hormone
ME	- Median eminence
MPOA	- Medial preoptic area
mRNA	- Messenger ribonucleic acid
Ni-DAB	- Nickel-diaminobenzidine
NSS	- Normal sheep serum
OT	- Oxytocin
OVLT	- Organum vasculosum of the lamina terminalis
PAP	- Peroxidase-antiperoxidase complex
PBS	- Phosphate buffered saline
PCR	- Polymerase chain reaction
pK	- Proteinase K
PNMT	- Phenylethanolamine-N-methyltransferase
POD	- Horse-radih peroxidase
PRH	- Prolactin-releasing hormone
PRL	- Prolactin
PVN	- Paraventricular nucleus of the hypothalamus
RF	- Releasing factor
RIF	- Release-inhibiting factor
RNase A	- Ribonuclease A
RT	- Room temperature
SDS	- Sodium dodecyl sulfate
SON	- Supraoptic nucleus of the hypothalamus
SSC	- Standard saline citrate solution
TH	- Tyrosine hydroxylase
TIDA	- Tuberoinfundibular dopaminergic
TRH	- Thyrotropin-releasing hormone
tRNA	- Transfer ribonucleic acid
UTP	- Uridin triphosphate
VP	- Arginin vasopressin

2. LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. Liposits Zs., **Hrabovszky E.** and Paull WK.: Catecholaminergic afferents to growth hormone-releasing hormone (GH-RH)-synthesizing neurons of the arcuate nucleus in the rat. **Biomedical Research 10, Supplement 3, 57-66, (1989)**

II. **Hrabovszky E.** and Liposits Zs.: Adrenergic innervation of dopamine neurons in the hypothalamic arcuate nucleus of the rat. **Neurosci. Lett. 182: 143-146 (1994)**

III. **Hrabovszky E.** and Liposits Zs.: Galanin-containing axons synapse on tyrosine hydroxylase-immunoreactive neurons in the hypothalamic arcuate nucleus of the rat. **Brain Res. 652: 44-55 (1994)**

IV. **Hrabovszky E.**, Vrontakis ME. and Petersen SL.: Triple-labeling method combining immunocytochemistry and in situ hybridization: Demonstration of overlap between Fos-immunoreactive and galanin mRNA-expressing subpopulations of luteinizing hormone-releasing hormone neurons in female rats. **J. Histochem. Cytochem. 43:363-370 (1995)**

V. Kovács M., Fáncsik A., **Hrabovszky E.,** Mező I., Teplán I. and Flerkó B.: Effects of continuous and repetitive administration of a potent analog of GH-RH(1-30)-NH2 on the GH release in rats treated with monosodium glutamate. **J. of Neuroendocrinol. 7:703-712 (1995)**

VI. **Hrabovszky E.**, Petersen SL., Kalló I. and Liposits Zs.: Combination of immunocytochemical (ICC) and in situ hybridization (ISH) techniques to study the molecular regulation of individual neurons in the rat brain. **Acta Histochemica et Cytochemica Vol. 29, Supplement:760-761 (1996)**

VII. **Hrabovszky E.**, Petersen SL., Kalló I. and Liposits Zs.: Synaptic regulation of tuberoinfundibular dopaminergic (TIDA) neurons by thyrotropin-releasing hormone (TRH) in the rat. Submitted to **Neuroendocrinology**

VIII. **Hrabovszky E.**, Kalló I., Hajszán T., Shughrue PJ., Merchenthaler I. and Liposits Zs.: Expression of estrogen receptor-β mRNA in oxytocin and vasopressin neurons of the rat supraoptic and paraventricular nuclei. **Endocrinology** (In press)

3. INTRODUCTION

3.1 Anatomical and neurochemical organization of the parvicellular neurosecretory system

Aschner reported in 1912 that hypothalamic lesions can result in gonadal atrophy, suggesting thereby an important role of the hypothalamus in the maintenance of normal anterior pituitary functions[2]. Other investigators including Dott[11], Mahoney and Sheehan[56], Harris[19], Hinsey[25] and Westman and Jacobsohn[92] were able to reproduce the phenomenon of gonadal atrophy via the interruption of connections between the hypothalamus and the hypophysis, by pituitary stalk trans-sections. In 1937 Haterius and Derbyshire[20] and Harris[19] showed that electrical stimulation of the hypothalamus can induce ovulation, corroborating the involvement of the hypothalamus in the control of gonadal functions. Dey in 1943 demonstrated[10] that guinea pigs are unable to ovulate if electrolytic lesions are placed at the posterior level of the optic chiasm of the hypothalamus and that the ovaries of the operated animals contain only follicles and no corpora lutea. In addition to the listed findings which indicated a fundamental role of the hypothalamus in the maintenance of normal gonadal functions, Cahane and Cahane reported[7] in 1938 that profound histological alterations also occur in the thyroid gland following destruction of the hypothalamic infundibular region.

Although the observations above indicated that the hypothalamus plays a crucial role in the regulation of normal anterior pituitary-related endocrine functions, the way the hypothalamus communicates with the anterior pituitary gland still remained obscure. Taking into account that **(i.)** nerve endings of hypothalamic origin are absent or very scarce in the anterior pituitary gland and that **(ii.)** the pituitary has a special kind of blood supply, termed *hypophysial portal circulatory system*, which is characterized by the intimate contact of blood vessels and hypothalamic nervous tissue, Harris[18] and Green and Harris[15] in 1947 proposed that the link between the hypothalamus and the anterior lobe might be neurovascular. The neurohumoral hypothesis gained a strong support by numerous subsequent observations that hypothalamic extracts exert direct effects on pituitary hormone secretion.

Such an action was first evidenced on adrenocorticotropin (ACTH) secretion and the active substance has been called corticotrophic releasing factor (CRF)[16, 71].

The basic organization of the hypothalamic area that regulates the adenohypophysis has been clarified by Hungarian neuroscientists. Halász et al. performed transplantation experiments with anterior pituitary tissue into different hypothalamic regions of the rat and concluded that only a very definite region of the hypothalamus is capable of maintaining the normal structure of pituitary implants[17]. This area was termed *hypophysiotropic* zone, and found to include mainly the mediobasal hypothalamus. The *parvicellular neurosecretory system* of the hypophysiotropic area which regulates anterior pituitary functions via secreting releasing- and release-inhibiting substances into the hypophysial portal circulation was defined and first described by Szentágothai et al.[83]. The axons emanating from the perikarya of small peptidergic and dopaminergic neurons in the arcuate (AN), periventricular and the parvicellular paraventricular (PVN) hypothalamic nuclei and in the medial preoptic area (MPOA) of the rat form the tuberoinfundibular tracts which terminate around the fenestrated capillaries of the median eminence (ME)-pituitary stalk complex. The neurosecretory products, isolated and structurally characterized so far, include growth hormone-releasing hormone (GHRH), dopamine, luteinizing hormone-releasing hormone (LHRH), thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH, identical to CRF) and somatostatin. These substances are transported via the long portal veins to the anterior pituitary gland where they bind to specific membrane receptors to modulate secretion of growth hormone (GH), prolactin (PRL), luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), ACTH, α-melanocyte stimulating hormone (α-MSH) and other troph hormones of the adenohypophysis[64]. In addition to be secreted as hypophysiotropic factors, most neurohormones have been shown to fulfill a neurotransmitter/neuromodulator function in the peripheral- as well as the central[46, 47] nervous system.

3.2 Anatomical and neurochemical organization of the magnocellular hypothalamo-neurohypophysial neurosecretory system

The phenomenon of neurosecretion by magnocellular neurons of the diencephalon was discovered and described by Scharrer and Scharrer in 1937[74]. Now little doubt can be raised against the fundamental correctness of the theory that the magnocellular neurosecretory system of the hypothalamus consists of large peptidergic neurons whose perikarya (about 25 μm in diameter in the rat) are located in the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei and several accessory magnocellular cell groups. These neurons synthesize arginine vasopressin (VP) and oxytocin (OT) and project through the supraoptico-hypophysial and paraventriculo-hypophysial tracts to the neural lobe of the pituitary gland where the neurosecretory substances, transported and stored in dense-core vesicles, are delivered to the bloodstream in response to various humoral and neuronal stimuli[64, 73]. While the main function attributed to VP is the central control of water and electrolyte balance[70], OT seems to be primarily involved in the regulation of reproductive events, including parturition[14], sexual[1, 8] and maternal[65] behaviour, and the milk ejection reflex[89]. In addition to innervate the neurohypophysis, paraventricular OT and VP neurons have been shown to project to the lower brain stem and the spinal cord to regulate autonomic responses[82].

3.3 Humoral and afferent neuronal regulation of the parvi- and magnocellular neurosecretory systems

The parvi- and magnocellular neurosecretory systems represent the final common pathways in the central regulation of pituitary functions, and neurohormone output from their neurons is determined by the dual control of *humoral* and *afferent neuronal* agents.

3.3.1 Humoral regulation of neurosecretory systems

Radioligand-binding[67, 69, 85], immunocytochemical[23, 24] and recently, *in situ* hybridization (ISH)[76-79, 85] studies have been used extensively to investigate potential feed-back sites of peripheral hormones to brain areas involved in the neuroendocrine regulation. The results of these experiments provided morphological evidences for the responsiveness of the endocrine hypothalamus to the feedback actions of glucocorticoids[54, 80], mineralocorticoids[38], estrogens[85], progesterone[90], androgens[5, 95] and thyroid hormones[42]. Neurons contributing with axon projections to the parvicellular

tuberoinfundibular and the magnocellular hypothalamo-neurohypophysial tracts either respond directly to the actions of peripheral hormones or the humoral effects are mediated to them via synaptic communication with hormone receptor-expressing interneurons. While CRH-IR neurons of the PVN can be regulated directly by glucocorticoids[54], estrogens seem to require cooperation of estrogen-receptive interneurons to influence LHRH neurons, which neither seem to accumulate estrogens[75], nor express estrogen receptor-immunoreactivity[22-24] in their nuclei. Similarly, an indirect action whereby estradiol regulates OT-ergic neurons of the SON has been proposed by Herbison et al.[21].

3.3.2 Regulation of neurosecretory systems by synaptic afferents

The neurons of the parvi- and magnocellular neurosecretory systems are regulated by synaptic afferents from hypothalamic as well as extrahypothalamic origins. Previous studies using dual-label immunocytochemistry (ICC) at the ultrastructural level[53] have partially elucidated the chemical identity of specific neuronal afferents to the hypothalamic hypophysiotropic neurons[26, 41, 44, 46-51].

4. SPECIFIC AIMS

The aim of the studies underlying this thesis was to provide new morphological data about the ***afferent connectivity*** as well as the ***humoral regulation*** of immunocytochemically characterized neurosecretory systems of the rat forebrain. To overcome some methodological limitations of classical immunocytochemical and ISH double-labeling techniques, we also attempted to develop new dual- and triple-labeling approaches with conjoint application of ICC and ISH to the same brain tissue sections. The specific aims of our studies are summarized below.

1. **To study the putative catecholaminergic afferent regulation of GHRH-synthesizing neurons by light- and electron microscopic dual-label ICC.**
2. **To provide light- and electron microscopic evidence for the involvement of adrenergic afferents in the regulation of the TIDA neuronal system.**
3. **To reveal galanin-IR synaptic afferents to TIDA neurons.**
4. **To provide a morphological support for the putative involvement of TRH-IR afferents and TRH receptors in the synaptic regulation of the TIDA neuronal system.**
5. **To develop and use a new technique combining ICC and ISH for the light microscopic analysis of galanin-IR afferents to parvicellular paraventricular neurons expressing prepro-TRH mRNA.**
6. **To develop a new methodological approach which enables the simultaneous use of ^{35}S-labeled oligodeoxynucleotide and digoxigenin-labeled cRNA probes for dual-label ISH experiments.**
7. **To develop and use a triple-labeling method for simultaneous detection of c-Fos- and LHRH immunoreactivities and prepro-galanin mRNA within the same neurons.**
8. **To compare the distribution of GHRH-IR neuronal elements in the medial basal hypothalamus of monosodium glutamate (MSG)-lesioned rats and study GH responses to challenges by a GHRH analog.**
9. **To demonstrate the putative coexpression of the recently-discovered estrogen receptor-β (ER-β) mRNA in OT and VP neurons of the PVN and SON.**

5. MATERIALS AND METHODS

5.1 Animals

Experimental animals used in these studies included male and female rats (200-250 g, bw) from either the Wistar or the Sprague-Dawley strains. The rats were kept under a controlled photoperiod (14light:10dark, lights on at 0500 h), with free access to standard rat chow pellets and tap water. Maintenance and handling of the animals were in accordance with the regulations and permission of the Ethic Board of the Albert Szent-Györgyi Medical University, Szeged, Hungary.

5.2 Surgical procedures

5.2.1 Colchicine administration

For the visualization of GHRH-synthesizing neuronal perikarya, the intracerebroventricular administration of the axonal transport inhibitor, colchicine was used[44]. Eighty μg/100g, bw colchicine, dissolved in 5 μl saline was injected into the lateral cerebral ventricle of the experimental animals via a stereotaxic approach, under Nembutal anesthesia (40 mg/kg, bw; ip). The animals were sacrificed 24-48 h later.

5.2.2 Exogenous estradiol and progesterone administration[32]

Bilateral ovariectomy was performed on female Sprague-Dawley rats (200-225g, bw). Seven days later, a pair of silastic capsules (3cm, each) containing 150 μg/ml estradiol in sesame oil were implanted subcutaneously into each animal. Surgeries were performed under ether anesthesia. Two days later at 9.00 am, the animals received a single subcutaneous injection of progesterone (5 mg; 50mg/ml in sesame oil). This pattern of steroid administration is known to mimic the proestrus surge of LH in the afternoon of the progesterone injection. The animals were anesthetized between 16.00 and 17.00 with Nembutal (40 mg/kg, bw; ip), and perfused transcardially with 4% paraformaldehyde in 500 mls of 0.1 M phosphate buffered saline (PBS, pH 7.4), as described below.

5.2.3 Tissue fixation by transcardiac perfusion of rats

To sacrifice, the animals were deeply anesthetized with Nembutal (40 mg/kg, bw; ip) and the thorax was opened with the aid of a pair of sharp scissors. A short cut was made on the right atrium to ensure outflow of the perfusate, and immediately after, the descending aorta was clamped to keep the fixative in the upper body-half. Then, the apex of the heart

was incised and a cannula was introduced and fixed into the ascending aorta. The blood was rinsed out with an initial 50 ml volume of PBS, then the perfusion was continued with 300-500 ml of fixative solution. For electron microscopic immunocytochemical studies, either a paraformaldehyde-based dual-pH fixative was used[4], or 1% glutaraldehyde was added to a 4% paraformaldehyde solution at pH 7.4[28, 29, 31]. For most light microscopic studies with either ICC or ISH, the perfusate consisted of phosphate-buffered (pH 7.4) 4% paraformaldehyde[27, 30, 32]. Following transcardiac perfusion, the brains were removed from the skull and immersed in 4% paraformaldehyde fixative for variable periods of time.

5.3 Preparation of sections

5.3.1 Section preparation on cryostat (Reichert-Jung)

Small tissue blocks were dissected out from the forebrains, postfixed in 4% paraformaldehyde for 2 h, then infiltrated with gradually increasing concentrations of sucrose through 30% at 4°C. The tissue pieces were snap-frozen on dry-ice and then, 20 μm-thick coronal sections were sliced on a cryostat. These sections were stored permanently in cryoprotectant solution at - 20 °C[55], and used for studies applying either ISH or combination of ICC and ISH[27, 30-32].

5.3.2 Section preparation on Vibratome (Technical Products International)

Ultrastructural ICC studies [27, 30, 32, 44] were conducted on sections prepared on a Vibratome.

5.3.3 Preparation of ultrathin sections for preembedding dual-label immuno-electron microscopic studies

Double-immunostained arcuate sections from male rats were also processed for electron microscopic studies[28, 29, 31]. They were osmicated, dehydrated and flat-embedded into Epon resin (LX-112; Ladd Research Industries, Burlington, VT), and then, semithin and ultrathin sections were cut on a Reichert ultratome. The ultrathin sections were mounted on Formvar-coated single-slot grids, contrasted with uranyl acetate and lead citrate and examined with a Tesla BS 500 transmission electron microscope.

5.4 Dual-label Immunocytochemistry

For preembedding dual-label ICC, the peroxidase-antiperoxidase complex (PAP) technique of Sternberger et al.[81] was used sequentially twice, for the detection of the two different antigens[53]. Following the development of the first signal, the brown DAB precipitate was silver-gold-intensified. Then the sections were further processed for the detection of the second antigen, using the unmodified DAB chromogen. The silver-gold intensification of the DAB immunostain[52] and the dual-label ICC protocol we used in our experiments have been developed and described in detail by Liposits et al.[53]. Our papers[28-31, 46] refer to these original publications.

5.5 Combination of Immunocytochemistry and in situ hybridization

5.5.1 Hybridization probes

Two types of "antisense" (complementary to the coding, "sense" mRNA sequence) hybridization probes were used. These included single-stranded synthetic oligodeoxynucleotide probes of a usual length of 48 bases, which were end-tailed by the terminal deoxynucleotidyl transferase enzyme reaction and ^{35}S-dATP substrate. The other approach for mRNA detection used cRNA probes transcribed *in vitro* from double-stranded DNA templates, using the T7, T3 or SP6 promoter of plasmid vectors and appropriate RNA polymerase enzymes. The label incorporated into these probes was digoxigenin for non-isotopic ISH or ^{35}S for the isotopic approach.

5.5.2 Preincubation, hybridization and posthybridization tissue processing steps

We have recently published a detailed tissue processing protocol for combined use of ICC and ISH in the same tissue sections[32]. In brief, immediately before hybridization, the sections were removed from the cryoprotectant solution[55] and the following sterile incubation steps were performed: Tissue permeabilization with a pK predigestion (1μg/ml; 30 min); 5 min fixation in 4% paraformaldehyde/0.1M PBS; 2 min rinse in 2Xstandard saline citrate solution (2XSSC; 1XSSC=0.15M NaCl-0.015M sodium citrate, pH 7.0; Sigma); 20 min acetylation in 0.25% acetic anhydride (Fisher Scientific)/0.1M triethanolamine(pH 8.0; Sigma)/ 0.9% NaCl; and 2 min rinse in 2XSSC.

For hybridization, the sections were tansferred with a bent syringe needle into microcentrifuge tubes containing the hybridization solution [50% formamide, 4XSSC, 10% dextran sulfate (500,000 MW; Sigma), 1X Denhardt's solution, 500μg/ml heparin sodium salt (Sigma), 0.5 mg/ml yeast tRNA (Boehringer Mannheim), 0.4 mg/ml sheared single-stranded salmon sperm DNA (Boehringer Mannheim), 0.1% sodium dodecyl sulfate (SDS), 0.1M DTT, and 20,000 cpm/μl of the hybridization probe.]. The tubes were sealed with Parafilm and placed in an incubator to hybridize for 16 h at 37 °C (for oligodeoxynucleotide probes) or 50 °C (for cRNA probes). The hybridized sections were rinsed in 1XSSC for 10 min, followed by stringent washes (total 2 h) in 50% formamide/2XSSC, at 40 °C (for oligodeoxynucleotide probes) or 50 °C (for cRNA probes). Following the use of cRNA probes, the introduction of an RNAse A digestion step (100μg/ml; 1 h) before 50% formamide/2XSSC rinses was necessary for sufficient control over background. Then the sections were transferred into 0.1M PBS and washed copiously before being processed for ICC.

5.5.3 Hybridization controls

1. For negative control, **A/** some sections were pre-digested for 30 min with RNAse A (100μg/ml at 37°C), then hybridized with the antisense probes. Absence of signal on predigested sections proved that binding of probes in our experiments was to tissue RNAs. **B/** Also, oligodeoxynucleotide and RNA probes with the "sense" sequence (mRNA-homologous) were labeled similarly to the antisense probes, and used for control hybridizations. Absence of signal following the use of sense-sequence hybridization probes indicated lack of non-specific interactions between the nucleic acid probe molecules and various tissue domains.

2. For positive control, the distribution pattern of the ISH signal was compared to the pattern of immunocytochemical signal distribution, whenever we possessed appropriate antibodies for ICC. The specificity of ER-β and galanin probes was confirmed by comparative use of two different antisense hybridization probes.

5.5.4 Immunocytochemistry

Posthybridization stringency treatments were followed by ICC. In our experience, sterility of antibody solutions was not required any more for hybrid stability, but presence of salt in all solutions was indispensable. Therefore, we kept concentration of sodium salts over 300mM in all incubation solutions.

Posthybridized sections were immersed in a 9:1 mixture of methanol:30% H_2O_2 for 5 min to inactivate endogenous peroxidases, permeabilized with 0.2 % Triton X-100 (in PBS) for 30 min, blocked with 2% bovine serum albumin (BSA) against non-specific antibody-binding for 20 min, and finally, placed in antibody solutions. Single- or double-labeling immunocytochemical procedures were performed as detailed elsewhere[32]. For specificity-testing of antibodies and further details of ICC, we refer to our original publications included in this thesis[27-32, 35, 44].

5.5.5 Detection of the hybridization signal

Hybridization signal was either detected with ICC (digoxigenin-labeled probes), or with autoradiography (isotopically labeled probes) as described in original publications[27, 30-32]. The immunocytochemical visualization of digoxigenin used a commercially available antibody directly conjugated to either horse-radish peroxidase (dig-POD; Boehringer-Mannheim), or alkaline phosphatase (dig-AP; Boehringer-Mannheim). The development of color reactions was performed as recommended by the manufacturer. For autoradiography, sections were first mounted onto double gelatin-coated microscopic slides, air-dried, then dehydrated in increasing concentrations of ethanol through 100%, and air-dried again. Dehydrated sections were dipped into nuclear-track emulsion (NTB-3; Kodak or LM-1; Amersham) in a dark-room. The slides were air-dried, then placed in light-tight slide-boxes with desiccant capsules and exposed at 4°C for variable periods of time. Then, the autoradiograms were developed with Dektol developer (Kodak) for 3 min, rinsed with distilled water briefly, then fixed with Kodak fixer for 5 min. Finally, the sections were dehydrated in ethanol dilutions, cleared with xylenes, and coverslipped with Permount to prepare for photography.

If either nickel-DAB (Ni-DAB) or BCIP/NBT chromogens were used for the immunocytochemical detection[32], we had to introduce an additional step prior to emulsion-dipping. In such cases the slides were covered by a thin layer of Parlodion (Mallinkrodt) film in an attempt to prevent interaction of chromogens with the emulsion. The Parlodion coat prevented fading of the histochemical stain and formation of chemographic artifacts on the emulsion[32, 66, 94].

6. RESULTS

6.1 Catecholaminergic afferents to growth hormone-releasing hormone-synthesizing neurons

a/ Following intracerebroventricular administration of colchicine to male Wistar rats, the hypothalamic AN exhibited numerous fusiform and multipolar GHRH-IR perikarya which were concentrated ventro-laterally in the nucleus. The same region also contained a dense network of noradrenergic and adrenergic fibers, IR to dopamine-β-hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT), respectively (**I.**, Figures 1c,d; p. 59).

b/ The use of immunocytochemical dual-labeling at the light microscopic level demonstrated juxtapositions between DBH-IR as well as PNMT-IR axons and GHRH-IR perikarya and dendrites. (**I.**, Figures 2a-f; p. 61).

c/ The ultrastructural analysis of double-labeled sections from the ventro-lateral part of the AN provided evidence for axo-dendritic (**I.**, Figure 3e; p. 62) and axo-somatic (**I.**, Figure 3f; p. 62) synaptic connections between PNMT-IR adrenergic axons and GHRH-IR neurons.

6.2 Adrenergic innervation of the tuberoinfundibular dopaminergic neuronal system

a/ The perikarya of TH-IR TIDA neurons in the AN were clustered in a dorso-medial and a ventro-lateral population. Adrenergic fibers exhibiting PNMT immunoreactivity were found to innervate abundantly both subdivisions of the AN (**II.**, Figures 1a,b; p. 144).

b/ Analysis of dual-labeled light microscopic preparations revealed PNMT-IR axons in contact with dopaminergic perikarya (**II.**, Figures 1c,e; p. 144) and dendrites (**II.**, Figure 1d; p. 144).

c/ Electron microscopic studies demonstrated asymmetrical (**II.**, Figure 1f; p. 144) as well as symmetrical (**II.**, Figure 1g; p. 144) synaptic specializations between the PNMT-IR axon terminals and the TH-IR neuronal profiles.

6.3 Galaninergic afferents to tuberoinfundibular dopaminergic neurons

a/ Galanin-immunoreactivity was confined to axons in the AN of rats that were not pretreated with colchicine (**III.**, Figure 1b; p. 51), but also appeared in numerous perikarya following an intracerebroventricular colchicine injection to the animals (**III.**, Figure 1c; p. 51). The untreated rat model was found suitable for the immunocytochemical visualization of TH-containing dopaminergic neurons (**III.**, Figure 1a; p. 51).

b/ In the untreated animals, both galanin-IR axons and TH-IR neurons were detected in the dorso-medial (**III.**, Figure 1d; p. 51) as well as the ventro-lateral (**III.**, Figure 1e; p. 51) subnuclei.

c/ Galanin-IR axons were frequently juxtaposed to dendrites (**III.**, Figure 1f; p. 51) and somata (**III.**, Figures 1g,h; p. 51) of dopaminergic neurons. Serial contacts (**III.**, Figure 1f; p. 51) as well as sole boutons (**III.**, Figure 1g; p. 51) could be revealed.

d/ Ultrastructural examination of dual-labeled sections detected axo-somatic synapses exhibiting either symmetrical (**III.**, Figures 2b,c; p. 52) or asymmetrical (**III.**, Figures 2d,e; p. 52) synaptic densities. Diaminobenzidine deposits were most abundant over the rough endoplasmic reticulum of TH-IR neurons (**III.**, Figures 2b,c; p. 52), whereas silver grains accumulated predominantly over the dense-core vesicles in galanin-IR axon terminals (**III.**, Figures 2c,e,f; p. 52).

e/ Asymmetrical galaninergic synapses were also revealed on dendrites of TH-IR neurons (**III.**, Figure 2f; p. 52).

6.4 Involvement of thyrotropin-releasing hormone-immunoreactive terminals and thyrotropin-releasing hormone receptors in the afferent regulation of tuberoinfundibular dopaminergic neurons

a/ By the application of light microscopic dual-label ICC, a dense plexus of TRH-IR axons was revealed in the dorso-medial part of the hypothalamic AN, where most TH-IR perikarya of the TIDA system can be localized (**VII.**, Figure a; p. 10).

b/ Dopaminergic neurons were embedded in the network of TRH-IR axons, which established contacts with dopaminergic cell bodies and dendrites (**VI.**, Figure d; p. 761 and **VII.**, Figures b-d; p. 10).

c/ Dual-label immuno-electron microscopic studies provided evidence for the synaptic communication between TRH-IR axons and TH-IR neuronal elements (**VII.**, Figure e; p. 10).

d/ Combined application of ICC and ISH to paraformaldehyde-fixed sections was found suitable for simultaneous detection of TH immunoreactivity and TRH receptor mRNA in the AN. The hybridization signal was visualized by autoradiography, following the use of a ^{35}S-labeled cRNA probe (**VI.**, Figure e; p. 761 and **VII.**, Figures f,g; p. 10).

e/ The autoradiographic signal (grain clusters) over TH-IR neurons appeared preferentially in the dorso-medial part of the AN (**VI.**, Figure e; p. 761 and **VII.**, Figure f; p. 10) and very few cases of colocalization were revealed laterally in the AN (**VII.**, Figure g; p. 10).

6.5 Combination of immunocytochemistry and in situ hybridization to demonstrate galanin-immunoreactive afferents to prepro-thyrotropin-releasing hormone mRNA-expressing neurons of the paraventricular hypothalamic nucleus

a/ Non-isotopic ISH with the use of a digoxigenin-labeled cRNA probe to prepro-TRH mRNA was found to represent a sensitive method for the visualization of diencephalic TRH neurons (**VI.**, Figures b,c; p. 761).

b/ The ISH method was found to be compatible with the subsequent immunocytochemical detection of galanin-IR axons in the hypothalamic PVN. The black staining in galanin-IR axons (visualized by silver-intensified DAB chromogen) gave sufficient color contrast with the purple of BCIP/NBT chromogen in prepro-TRH mRNA-expressing TRH neurons (**VI.**, Figures b,c; p. 761).

c/ The use of the combination method provided light microscopic evidence for the juxtaposition of galaninergic axons to TRH neurons in the PVN (**VI.**, Figure c; p. 761), as well as several other prosencephalic areas.

6.6 Simultaneous use of ^{35}S-labeled oligodeoxynucleotide and digoxigenin-labeled cRNA probes for dual-label *in situ* hybridization studies

a/ Overnight hybridization of tissue sections with a cocktail containing a ^{35}S-labeled oligodeoxynucleotide and a digoxigenin-labeled cRNA probe gave temperature-dependent results. A 37 °C hybridization temperature (other parameters also adjusted to the routine of oligodeoxynucleotide hybridizations) gave optimal autoradiographic signal strength with the oligodeoxynucleotide probe, but the signal was largely decreased with the digoxigenin-labeled cRNA probe.

b/ Increasing the temperature to 50 °C to favor cRNA hybridization conditions (with other parameters unaltered) ensured optimal signal strength with the cRNA probe without compromising the sensitivity of the oligodeoxynucleotide hybridization (**VI.**, Figure a; p. 761).

c/ Alternating use of the two different hybridization temperatures (37 °C and 50 °C) in a thermal cycler (30 min intervals, total of 20 hours), as well as various arbitrary time patterns of 37 °C and 50 °C temperatures resulted in maximal signal strengths with both probe components.

d/ Application of the new approach for simultaneous visualization of proopiomelanocortin neurons (using a digoxigenin-labeled cRNA probe and immunocytochemical detection) and galanin neurons (using an isotopic probe and autoradiographic detection) in the AN could not reveal colocalization of the two neuronal populations (**VI.**, Figure a; p. 761).

6.7 Triple-labeling for simultaneous detection of c-Fos- and luteinizing hormone-releasing hormone immunoreactivities and prepro-galanin mRNA

a/ We have developed a successful tissue processing protocol for combined application of ICC and ISH to the same tissue sections of perfused rat hypothalami. This method resulted in well-preserved and reproducible immunocytochemical and ISH signals. The regions of the organum vasculosum of the lamina terminalis (OVLT) and the MPOA contained many c-Fos-IR nuclei, LHRH-IR perikarya and galanin mRNA-expressing neurons (**IV.**, Figure 3; p. 367).

b/ It was found that preservation of Ni-DAB signal in c-Fos-IR nuclei during the triple-labeling procedure required the introduction of a slide-coating step with Parlodion. Otherwise, Ni-DAB was bleached out from the sections during the photographic development, and chemographic artifacts also occurred (**IV.**, Figure 3f-h; p. 367).

c/ Single-, double-, and triple-labeled LHRH neurons were revealed in the OVLT and MPOA regions.

d/ Quantitative analysis identified 66.2±2.5% of LHRH neurons that contained c-Fos-IR nuclei and 65,2±2.9% of LHRH neurons that coexpressed galanin mRNA in the OVLT and MPOA regions. Only 18.1%±1.9% of LHRH neurons contained none of these substances.

e/ A high correlation between coexpression of galanin mRNA and c-Fos immunoreactivity was evidenced (**IV.**, Figures 3i,j; p. 367): as many as 76.8±2.7% of LHRH neurons that were c-Fos-IR also coexpressed galanin mRNA and 73.6±3.2% of galanin mRNA-containing LHRH neurons contained IR c-Fos in their nuclei.

f/ In addition to triple-labeled neurons, c-Fos-IR but LHRH-negative neurons were often observed to express prepro-galanin mRNA in the ventro-lateral part of the preoptic area (**IV.**, Figure 3k; p. 367).

6.8 Distribution of growth hormone-releasing hormone-immunoreactive neuronal elements in the brain of monosodium glutamate-lesioned rats and growth hormone responses to continuous and repetitive administration of a growth hormone-releasing hormone analog

a/ Immunocytochemical mapping of GHRH-IR neuronal perikarya in the mediobasal hypothalami of control rats identified numerous GHRH-IR perikarya in the AN and a dense plexus of GHRH-IR axons in contact with blood vessels of the ME (**V.**, Figures 1a,b; p. 704).

b/ Neonatal administration of MSG resulted in complete disappearance of GHRH-IR perikarya from the AN and large reduction in numbers of the GHRH-IR axons in the ME. GHRH-IR perikarya could still be detected in several hypothalamic areas outside the AN (**V.**, Figures 1c,d; p. 704).

c/ Repetitive administration of GHRH analog (A-495) to MSG-treated rats by daily injections for two weeks caused significant increases in the growing rate of MSG-treated animals, and the body weight and length of these rats reached similar values to those of unlesioned controls. Continuous infusions of the GHRH analog, in turn, only moderately increased the growh rate of MSG-lesioned animals and a distinct backwardness could still be found (at 8 weeks age of the animals), as compared to the normal controls (**V.**, Figures 2,3; p. 705-706).

d/ Basal serum GH levels were only slightly increased by repetitive administration of the GHRH analog to MSG-treated rats, and remained unaltered following continuous infusions of A-495. However, the relative responses (ratio of the absolute GH responses to the basal GH concentrations) were equal in the MSG-treated and the untreated groups (**V.**, Figure 5; p. 708).

e/ *In vitro* studies of superfused pituitaries showed that the total GH secretion in response to challenges by A-495 was significantly higher if the GHRH analog was administered episodically, instead of continuously.

6.9 Coexpression of estrogen receptor-β mRNA in oxytocin and vasopressin neurons

a/ Single-labeling ISH studies using a mixture of two cRNA hybridization probes have revealed ER-β mRNA in the PVN and SON of the hypothalamus (**VIII.**, Figures 1a-c; p. 17), where ER-α was not detected previously.

b/ Dual-labeling experiments detected OT- (**VIII.**, Figures 2a,b; p. 18), as well as VP-IR (**VIII.**, Figure 2c; p. 18) neurons throughout the rostro-caudal extent of the PVN that exhibited autoradiographic hybridization signal for ER-β.

c/ The highest signal levels for ER-β mRNA were revealed in the posterior part of the nucleus, where 93% of OT neurons also expressed ER-β mRNA (**VIII.**, Figure 2a; p. 18).

d/ The SON contained somewhat stronger hybridization signal in its ventral part. Here ER-β mRNA could be detected within VP-IR neurons (**VIII.**, Figure 2e; p. 18). In addition, variable levels of hybridization signal also occurred over OT-IR supraoptic neurons (**VIII.**, Figure 2f; p. 18).

7. DISCUSSION

7.1 Catecholaminergic afferents to growth hormone-releasing hormone-synthesizing neurons

The GH secretion by the anterior pituitary gland is under the control of a dual hypothalamic mechanism which utilizes the inhibitory somatostatin and the stimulatory GHRH hypophysiotropic neuronal systems as "final common pathways"[91]. The involvement of norepinephrine and epinephrine in the regulation of GH secretion has been clearly documented[12, 58, 59], however, the site(s) of action of central catecholamines remained unclear. Because administration of the α2 adrenergic receptor agonist, clonidine to experimental animals increased GH secretion following the suppression of endogenous somatostatin with anti-somatostatin antibodies[12], and to the opposite, the effects of clonidine on GH secretion were absent following passive immunization of rats with anti-GHRH antiserum[59], we proposed that the adrenergic effects on GH secretion are mediated, at least in part, by the hypophysiotropic GHRH neuronal system. Our findings that axons IR for the marker enzymes of catecholamines, DBH and PNMT form dense plexuses within the AN where most hypophysiotropic GHRH neurons occur, suggested that a monosynaptic pathway might account for the adrenergic effects upon GHRH neurons. This hypothesis was verified by the electron microscopic observation of synaptic contacts between PNMT-IR adrenergic axons and GHRH-IR perikarya and dendrites of the AN. Liposits et al. have recently demonstrated that somatostatinergic neurons of the anterior periventricular nucleus are also innervated by the adrenergic neuronal system[45], therefore, central catecholamines can also influence GH secretion via somatostatin, in addition to GHRH neurons. The receptorial mechanism underlying the synaptic communication between adrenergic axons and GHRH neurons is presently unclear, although the results of binding studies with α2 adrenergic receptor radioligand[93] as well as the pharmacological effects of clonidine on GH secretion[12, 59] suggest that the α2 adrenergic receptor is involved in this mechanism.

In summary, the observation of synaptic communication between adrenergic axons and GHRH-IR neuronal elements in the AN indicates that the central adrenergic neurons influence GH secretion, at least in part, via the hypophysiotropic GHRH neuronal system.

7.2 Adrenergic innervation of the tuberoinfundibular dopaminergic neuronal system

The TIDA neuronal system represents the final common pathway in the central inhibitory control of PRL secretion[3]. It has been well documented that the PRL output from lactotrophs is largely influenced by systemic[36, 39, 43] as well as intracerebroventricular[88] administration of adrenergic drugs. Local injections of α2- and β-adrenergic agents into the mediobasal hypothalamus where neuronal perikarya of the TIDA system are located were shown to increase plasma PRL levels, indicating that the TIDA system might account for the mediation of adrenergic effects upon the lactotrophs. We hypothesized that a direct synaptic communication exists between the adrenergic and the TIDA neurons. To find a morphological support for this concept, we performed preembedding dual-label immunocytochemical studies, and provided light- and electron microscopic evidences for the synaptic interaction of PNMT-IR (adrenergic) axons and TH-IR (dopaminergic) neurons.

Our observations give a firm support to the idea that central catecholaminergic pathways can influence PRL secretion by establishing synaptic contacts with neurons of the TIDA system.

7.3 Galaninergic afferents to tuberoinfundibular dopaminergic neurons

The involvement of the 29-amino acid gut-brain peptide galanin in the regulation of various pituitary functions has been well estabished[34, 58, 63, 72]. Because intracerebroventricularly administered galanin was found to increases PRL secretion[34, 58, 63], furthermore, a similar effect was absent in hypophysial cultures *in vitro*[63], we proposed that galanin exerts a synaptic effect upon the TIDA system, instead of directly regulating pituitary lactotrophs. To provide a morphological support for our hypothesis, we used preembedding dual-label ICC and revealed both axo-somatic and axo-dendritic synaptic contacts between galaninergic axons and the TIDA neurons.

Our data indicate that galaninergic pathways regulate PRL secretion via a synaptic communication with TIDA neurons.

7.4 Involvement of thyrotropin-releasing hormone-immunoreactive terminals and thyrotropin-releasing hormone receptors in the afferent regulation of tuberoinfundibular dopaminergic neurons

While the function of the TIDA neuronal system as a final common inhibitory pathway in the central regulation of PRL secretion has been widely accepted, the chemical identity of PRL-releasing hormone (PRH) is still controversial[3]. Thyrotropin releasing hormone (TRH) which is one of the PRH candidates, is capable of stimulating pituitary lactotrophs via a direct action[33, 84]. In addition to fulfill a hypophysiotropic function, TRH also represents an important synaptic transmitter/modulator substance in the brain. Several lines of evidence suggested that the suckling-induced PRL secretion is accompanied by both an increased release of TRH and a decreased output of dopamine into the hypophysial portal vasculature. Furthermore, a transient dopamine antagonism was found to be necessary to sensitize lactotrophs to the stimulatory action of TRH[9, 13, 68]. To clarify the central mechanism underlying the reciprocal regulation of dopamine- and TRH secretion, we proposed that an intrahypothalamic neuronal communication might exist between the hypophysiotropic TRH and TIDA neuronal systems. To elucidate this putative interaction, two strategies were used: (i) We performed dual-label immunocytochemical studies at the light- and electron microscopic levels and revealed synaptic contacts between TRH-IR axon terminals and TH-IR neurons of the AN. (ii) To reveal the receptorial mechanism of the synaptic transmission, we combined ICC and ISH and demonstrated the coexpression of TRH receptor mRNA in a subpopulation of TIDA neurons. The putative source of origin of TRH-IR axons innervating the AN was clarified earlier by Brownstein et al. who demonstrated a 65% depletion of the TRH content in the AN following lesions of the hypothalamic PVN[6]. Because the PVN also represents the main source of TRH axons in the ME[6], we have a good reason to believe that the same paraventricular TRH neurons that innervate the ME also regulate synaptically the TIDA system. If this is the case, TRH-IR fibers synapsing with TH-IR arcuate neurons either represent "en-passant" contacts or axon collaterals of the hypophysiotropic TRH fibers, with the final destination in the palisade zone of the ME. At present, the precise role of the intrahypothalamic link we described between TRH neurons and the TIDA systems is not fully understood. Also, the location of the TRH receptor protein within TIDA neurons is uncertain.

In addition to mediate synaptic effects to TIDA neurons at the synapses we described, TRH receptor can hypothetically occur on dopaminergic terminals in the ME to be involved in paracrine regulatory mechanism.

Our results demonstrate that the TRH-containing neuronal system regulates TIDA neurons via a synaptic pathway. Furthermore, the detection of TRH receptor mRNA-coexpression in TIDA neurons reveals receptorial mechanism of the synaptic transmission.

7.5 Combination of immunocytochemistry and *in situ* hybridization to demonstrate galanin-immunoreactive afferents to prepro-thyrotropin-releasing hormone mRNA-expressing neurons of the paraventricular hypothalamic nucleus

The use of dual-label immunocytochemical technique for innervation studies is frequently compromised by moderate levels of the neuropeptide antigen in the postsynaptic structure. To overcome this limitation, the concentration of neuropeptides in perikarya can be increased by pretreatment of the experimental animals with the axoplasmatic transport inhibitor substance, colchicine[44, 47-49, 51]. Because colchicine administration to animals, in itself has multiple disadvantages (e.g., it causes pain to the animals, further, it results in an unwanted perikaryal staining during the detection of the first antigen, etc.), we attempted to detect the postsynaptic neuronal element by means of non-isotopic ISH histochemistry, in lieu of ICC. Using a modification of a tissue-processing paradigm we developed for conjoint application of ICC and ISH[32], we demonstrated that the combination technique is suitable for simultaneous detection of galanin-IR axons and prepro-TRH mRNA-containing perikarya in the hypothalamic PVN.

Dual-labeling studies provided light microscopic evidence for neuronal contacts between galanin-IR axons (immunostained by silver-gold-intensified DAB) and paraventricular neurons expressing prepro-TRH mRNA (visualized by non-isotopic ISH and BCIP/NBT chromogens)[30].

In conclusion, the ICC-ISH combination method was found to be a suitable new approach to reveal neuronal contacts between two chemically characterized neurotransmitter systems. In addition, we provided light microscopic evidence for the afferent regulation of TRH neurons by galanin-containing neuronal pathways, which still awaits ultrastructural confirmation.

7.6 Simultaneous use of ^{35}S-labeled oligodeoxynucleotide and digoxigenin-labeled cRNA probes for dual-label *in situ* hybridization studies

Dual-label ISH studies aimed at colocalizing the mRNAs of two different neuronal compounds either utilize two oligodeoxynucleotide-[86] or two cRNA[66, 94] probes combined together within the same hybridization buffer. Both of these approaches have specific advantages. On the one hand, the use of cRNA hybridization probes ensures a maximal detection sensitivity for the hybridization, which is especially advantageous when detecting unabundant mRNAs. Synthetic oligodeoxynucleotide probes, in turn, although provide much lower sensitivity than cRNAs, can be easily designed to hybridize specifically to a single mRNA target sequence, and to distinguish even between structurally very similar mRNAs derived from the same gene family. A further advantage of using oligodeoxynucleotide probes is easier control over background labeling, which represents a frequent problem when "sticky" cRNA probes are utilized. To simultaneously benefit from the advantages of the two probe types, we tried to develop a dual-label ISH technique which enables combined application of *a ^{35}S-labeled oligodeoxynucleotide* and *a digoxigenin-labeled cRNA* probe in the same dual-label ISH. Briefly, our results showed that either proper choice of a single hybridization temperature, or alternatively, periodic application of two different hybridization temperatures can yield optimal detection sensitivity for the two hybridization components[30].

We believe that this method will be especially valuable in staining a maximal number of neuronal perikarya of a neuropeptide system by non-radioactive ISH (with a cRNA probe), and to colocalize with these neurons a specific member of a larger neuropeptide receptor family (by using a specific oligodeoxynucleotide probe).

7.7 Triple-labeling for simultaneous detection of c-Fos- and luteinizing hormone-releasing hormone immunoreactivities and prepro-galanin mRNA

Several lines of evidence have suggested that LHRH-IR neurons can be subdivided into functionally distinct populations. One subpopulation characterized by the coexpression of IR c-Fos during the surge-release of LH upon proestrus[40] seems to play a crucial role in the regulation of the ovulation. Therefore, the identification of the receptor or regulatory peptide complement in c-Fos-IR, LHRH neurons is critical in order to understand the mechanisms of the LH surge and the subsequent ovulation. Consequently, we tried to develop a triple-labeling technique which would enable the colocalization of a receptor or regulatory peptide mRNA in the active subpopulations of LHRH-IR neurons, characterized by the coexpression of nuclear c-Fos immunoreactivity. The prepro-galanin mRNA we chose to colocalize with c-Fos- and LHRH-immunoreactivities was identified earlier in a subset of LHRH-IR neurons[57]. The results of our triple-labeling experiments demonstrated that the tissue processing protocol we chose was compatible with various immunocytochemical and ISH detections. Furthermore, with this technique we were able to colocalize prepro-galanin mRNA in 76% of LHRH neurons that also expressed c-Fos immunoreactivity during the estrogen- and progesterone-induced surge-release of LH[32].

The observation of this high correlation strongly suggests that the galanin content of activated LHRH neurons has an important, albeit presently unknown function in the regulation of the preovulatory surge-release of LH. In addition, the triple-labeling method can gain wider applications in colocalization studies of functionally heterogenous neuronal systems which expresses nuclear c-Fos immunoreactivity upon activation.

7.8 Distribution of growth hormone-releasing hormone-immunoreactive neuronal elements in the brain of monosodium glutamate-lesioned rats and growth hormone responses to continuous and repetitive administration of a growth hormone-releasing hormone analog

Parenteral administration of MSG to neonatal rats induces local brain lesions, most prominently in the AN of the hypothalamus where the cell bodies of most GHRH-synthesizing neurons are located[62]. Because one half or more of GH deficiency cases are caused by hypothalamic dysfunctions, MSG-treated rats have been used extensively as a GHRH deficiency model[60]. In a series of experiments we have used MSG-treated animals to assess the effects of a potent GHRH analog on the GH secretory responses and the gain of body weight[35]. To supplement these data, we have also mapped the *immunocytochemical distribution* of GHRH-IR neuronal elements. The largely reduced numbers of GHRH-IR axons in the ME, together with the absence of GHRH-IR neurons in the AN were in keeping with the slow growing rate of MSG-lesioned animals. The scattered GHRH-IR axons maintained in the ME might originate outside the mediobasal hypothalamus, e.g. in the lateral hypothalamus, where GHRH-IR neurons remained detectable in our study. The results of our *in vivo* and *in vitro* experiments harmoniously indicated that pituitary cells respond with much higher GH pulses to the repetitive, as compared to the continuous administration of the GHRH analog, and that the growing rate of MSG-lesioned and GHRH-substituted animals depends on the presence and amplitude of the GHRH-stimulated secretory pulses of GH, rather then the basal GH concentrations.

In conclusion, evidence have been provided that the attenuated growth rate of MSG-lesioned rats can be fully restored with the chronic and repetitive administration of A-495, a new potent GHRH analog.

7.9 Coexpression of estrogen receptor-β mRNA in oxytocin and vasopressin neurons

The regulatory actions of estradiol upon magnocellular OT and VP neurons of the PVN and the SON are well documented[61, 87], but whether they are exerted directly or mediated by estrogen-sensitive interneurons has long been a question of debate. Previous immunocytochemical and ISH studies detected low levels or absence of the classical form of estrogen receptor (ER-α) in the PVN and the SON of the rat[21, 22, 76, 78, 79]. Recently, a second form of ER, termed ER-β has been cloned from the rat prostate[37], and its wide

distribution in the hypothalamus[77] and brain[78] of the rat has been evidenced. Our experiments using combined ICC and ISH were undertaken to examine the putative coexpression of ER-β mRNA in OT- and VP-IR neurons of the rat hypothalamus. The results of double-labeling studies have established the coexpression of ER-β mRNA in a large percent of OT- and VP-IR magnocellular neurons. Dual-labeled OT and VP neurons were observed in both the PVN and the SON and the highest levels of hybridization signal were detected in the posterior portion of the PVN where ER-β mRNA was colocalized within 91% of OT-IR neurons[27].

Our data provide a strong support for the concept that the functions of OT and VP neurons are regulated directly by estradiol, and the genomic effects of estrogen in these neurons are mediated by the recently discovered β form of ER.

8. SUMMARY OF THE MAIN ACHIEVEMENTS

8.1 We have performed immunocytochemical as well as pharmacological studies of the hypophysiotropic ***GHRH*** neuronal system. We have used preembedding dual-label ICC, and demonstrated ***synaptic contacts between PNMT-IR, adrenergic axons and GHRH-IR neurons*** of the hypothalamic AN. These findings elucidated the mechanism of the previously known adrenergic actions upon GH secretion**(I).** In addition, we have used MSG-pretreated rats as a GHRH deficiency model, for studying GH responses to continuous and repetitive administration of A-495, a potent GHRH analog. We have evidenced that the attenuated growth rate of MSG-lesioned rats is reflected by ***a largely restricted distribution of GHRH-containing neurons*** in the medial basal hypothalamus, and that the ***growth responses of MSG-lesioned animals can be fully restored with the chronic and repetitive administration of the GHRH analog*****(V).**

8.2 We have studied ***the afferent connectivity of the TIDA neuronal system***, which plays a pivotal role in the central inhibitory regulation of prolactin secretion. We have provided light- and electron microscopic evidences that ***adrenaline*****(II)**, ***galanin*****(III)** and ***TRH*****(VI, VII)** are involved in the synaptic regulation of TIDA neurons. These observations contribute to our knowledge about the neuronal circuitry which regulates PRL secretion via synaptic interaction with neurons of the TIDA system.

8.3 We have developed a tissue-processing protocol for ***conjoint use of various immunocytochemical and ISH techniques*** in the same tissue sections.

8.3.1 We have modified this method for ***innervation studies***, and revealed ***galanin-IR axon juxtapositions to prepro-TRH mRNA-containing neuronal perikarya.*** The light microscopic observation of contacts indicates that galaninergic pathways might regulate TRH functions via synaptic channels**(VI).**

8.3.2 Further, we have adapted the hybridization protocol for ***simultaneous use of oligodeoxynucleotide and cRNA hybridization probes in dual-label ISH experiments*****(VI)**. We have found that appropriate choice of a single hybridization temperature, or alternatively, cyclic application of two different hybridization temperatures (other parameters of hybridization unchanged) can result in maximal signal strengths for both the ^{35}S-labeled oligodeoxynucleotide and the digoxigenin-labeled cRNA hybridization probes**(VI).**

8.3.3 We have used the protocol of ***combined ICC and ISH*** for ***colocalization studies of neurotransmitter receptors in neurosecretory systems.*** In these studies we have ***detected TRH receptor mRNA in a subpopulation of TH-IR, TIDA neurons*****(VI, VII)**, revealing the putative receptorial mechanism of the synaptic interaction we have described between TRH-IR axons and the TIDA neurons**(VI).**

8.3.4 Combined ICC and ISH was also applied to ***hormone receptor-colocalization studies***. We have investigated the possibility that the feedback action of estrogen upon magnocellular OT and VP neurons is mediated by the recently discovered β subtype of estrogen receptor ***(ER-β).*** In contrast to the currently-accepted concept that estrogen regulates magnocellular neurons via estrogen-sensitive interneurons, ***we have demonstrated that the effects of estrogen on the magnocellular neurosecretory systems are direct, and mediated by ER-β which is coexpressed in a large percent of paraventricular and supraoptic OT and VP neurons.***

8.4 We have developed and used a ***triple-labeling method*** for studying receptor- or neuropeptide coexpression in distinct subpopulations of functionally heterogenous neurotransmitter systems. In our studies we have examined the putative coexpression of prepro-galanin mRNA in the subset of LHRH-IR neurons which exhibits ***c-Fos-IR nucleus,*** as a marker of LHRH neuronal activity during the estrogen- and progesterone-induced surge-release of LH. The results of triple-labeling experiments demonstrated that ***prepro-galanin mRNA is expressed in 76% of LHRH neurons that also contain c-Fos-IR nucleus during the estrogen- and progesterone-induced surge of LH.***

The observation of such a high correlation suggests that the galanin content of activated LHRH neurons has an important, albeit presently unknown function in the regulation of the preovulatory surge-release of LH. We propose that the triple-labeling method can gain a wider applications in colocalization studies of functionally heterogenous neuronal systems**(IV).**

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11. ANNEX: FULL PAPERS PUBLISHED IN INTERNATIONAL JOURNALS REFERENCED BY SCIENCE CITATION INDEX

I.

Biomedical Research 10, Supplement 3, 57-66, 1989

CATECHOLAMINERGIC AFFERENTS TO GROWTH HORMONE-RELEASING HORMONE (GH-RH)-SYNTHESIZING NEURONS OF THE ARCUATE NUCLEUS IN THE RAT

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ABSTRACT

Growth hormone-releasing hormone (GH-RH; somatocrinin) is one of the main diencephalic factors that regulates growth hormone (GH) production of the anterior pituitary gland. In order to elucidate the nature of the specific afferent systems to GH-RH-synthesizing neurons, immunocytochemical double labelling studies were carried out in the arcuate nucleus (ARC), the main source of hypophysiotrophic GH-RH. The possible interrelationship between GH-RH-producing neurons and catecholaminergic axons was studied, using dopamine-β-hydroxylase (DBH) and phenylethanolamine-*N*-methyltransferase (PNMT) immunolabelling for tracing the catecholaminergic system. At the light microscopic level, the dual antigen localization revealed a similar pattern of distribution of catecholaminergic fibers and GH-RH-immunoreactive (IR) neurons in the ARC. It also demonstrated that DBH- and PNMT-IR axon varicosities were in juxtaposition with GH-RH-synthesizing neurons. The simultaneous detection of PNMT- and GH-RH-immunoreactivities at the electron microscopic level, demonstrated axo-dendritic and axo-somatic synaptic specializations between these systems. These morphological findings indicate that the hormone production and/or release of hypophysiotrophic GH-RH-synthesizing neurons can be influenced by catecholaminergic fibers ascending from the brainstem via synaptic mechanisms.

Peptides possessing a stimulatory action upon the growth hormone (GH) production and release from anterior pituitary cells have recently been isolated from a human pancreatic tumor (16, 39) and the rat hypothalamus (41). Growth hormone-releasing hormone (GH-RH) appears to be produced by neurons locating mainly in two well-defined regions of the diencephalon, the arcuate nucleus (ARC) and the parvocellular division of the hypothalamic paraventricular nucleus (PVN) (3, 6, 7, 11, 21, 22, 33, 34, 40). Axons arising from these GH-RH-producing neurons terminate in

Dedicated to Professor Yutata Sano, on the occasion of his retirement.

the median eminence-pituitary stalk complex (3, 33, 34) and are also distributed to certain diencephalic and telencephalic nuclei (40). Therefore, at least two functions might be attributed to GH-RH. First, it plays a critical role, as a hypophysiotrophic hormone, in the control of growth hormone secretion, and second, the hormone might be utilized as a neurotransmitter or neuromodulator substance in influencing the function of other neuronal circuits. This latter assumption is supported by recent data (19) that have demonstrated the presence of GH-RH in presynaptic neuronal structures.

From a neuroendocrine point of view, it has been challenging to examine the neuronal systems that are capable of influencing the activity of hypophysiotrophic GH-RH-neurons. Substance-P (12), methionine-enkephalin (12), somatostatin (12, 20, 28) and GH-RH (19)-containing axons have been demonstrated to form synapses with neurons which synthesize growth hormone releasing hormone. This observation supports previous pharmacological data (9, 10, 23).

Catecholamines have been shown to alter growth hormone secretion (for reviews, see 14, 32), however, to date, the site(s) of action for monoamines have not been mapped completely. In addition to their direct influence upon GH-synthesizing cells of the adenohypophysis, the catecholaminergic modulation might also be mediated via somatostatin and GH-RH-synthesizing neuronal systems located within the hypothalamus. Recent pharmacological studies support the view that an alpha-adrenergic receptor mechanism stimulates growth hormone release via a GH-RH dependent action (13, 35).

To characterize this interrelationship, catecholaminergic fibers and growth hormone-releasing hormone producing neurons were simultaneously analyzed in the ARC by means of a recently developed immunocytochemical double labelling technique at the light and electron microscopic levels. A preliminary report of these findings has appeared recently in abstract form (29).

MATERIALS AND METHODS

Animals

Adult, colchicine pretreated male rats (n = 20) were used in this study. A single dose of colchicine (80 μg/100 g, b.w.; dissolved in 5 μl of saline) was injected into the central part of the lateral cerebral ventricle, under Nembutal (40 mg/kg; i.p.) anaesthesia. The animals were allowed to survive for a period of 30–36 h.

Fixation and Section Preparation

Re-anaesthetized (Nembutal) animals were perfused through the ascending aorta, first with 30–50 ml of 0.1 M phosphate buffered saline (PBS; pH 7.4) and then with 400–500 ml of a 4% paraformaldehyde solution, used at variable pH (6.5 and 11) according to the concept of Berod *et al.* (2). Satisfactory visualization of GH-RH antigen required the addition of glutaraldehyde (0.02%) to the alkaline component of the fixative. Finally, the animals were perfused with the high pH fixative solution without glutaraldehyde. The same glutaraldehyde free solution was used for overnight postfixation. Thereafter, the brains were removed from their skull and 30 μm thick coronal sections were cut on a Lancer vibratome.

Immunocytochemical Labelling

Detection of single tissue antigens

Preembedding immunocytochemical techniques were employed for the detection of growth hormone-releasing hormone containing neurons and catecholaminergic axons. Both the traditional peroxidase anti-peroxidase complex (PAP)-3,3′-diaminobenzidine (DAB) technique of Sternberger *et al.* (42) and a modified version of this procedure (26), that amplifies the final DAB product by silver-gold intensification (15) were used. The rat hypothalamic GH-RH antiserum (R567) was raised in rabbit and used at 1:5,000 working dilution. The details of the production and characterization of this antiserum have been published elsewhere (34). Antibodies raised against bovine dopamine-β-hydroxylase (DBH) (8) and rat phenylethanolamine-*N*-methyltrans-

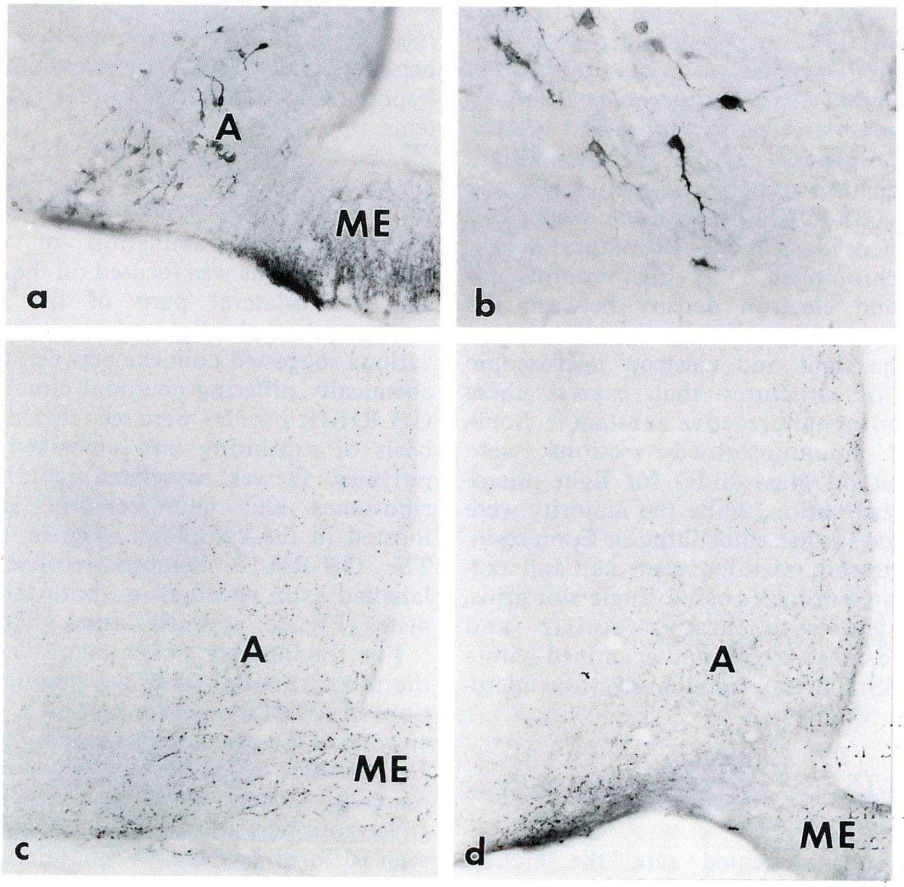

Fig. 1 Immunocytochemical detection of growth hormone-releasing hormone (GH-RH)-immunoreactive (IR) (a and b) and catecholamine-containing (c and d) structures in the arcuate nucleus (A). ME, median eminence. a, b: Non-intensified DAB-chromogen. c, d: Silver-gold postintensified DAB-chromogen. a: GH-RH-synthesizing neurons residing in the arcuate nucleus of the hypothalamus. The median eminence contains intensely stained GH-RH-IR axons. ×56 b: GH-RH-synthesizing neurons shown at higher power. Note the well-developed dendritic spines. ×110 c: Dopamine-β-hydroxylase-IR axons distributed in the arcuate region and median eminence. ×42 d: Adrenergic, phenylethanolamine-*N*-methyltransferase-containing axons located in the arcuate nucleus. ×42

ferase (PNMT) (4) were utilized to demonstrate noradrenergic and adrenergic axons. Working dilutions of the anti-DBH and anti-PNMT sera were 1:10,000 and 1:3,000, respectively. All antibodies were diluted in PBS containing 1% normal goat serum and 0.1% sodium azide. The sections were incubated in the primary antibodies for 36–48 h and then for 1–2 h in both the bridging (goat, anti-rabbit IgG, 1: 200, Jackson Immunoresearch Laboratories, Inc.) and PAP-complex (Jackson Immunoresearch Laboratories, Inc.). The antigen-antibody sequences were visualized according to the method of Streit and Reubi (43). The laboratory protocol of the silver-gold intensification of the DAB reaction product for electron microscopic purposes has previously been reported (26).

Sequential detection of two tissue antigens
In order to elucidate the relationship of either DBH- or PNMT-immunoreactive (IR) axons, with GH-RH-producing neu-

rons in the arcuate nucleus, a recently developed dual antigen localization technique (27) was applied. First, either DBH-IR or PNMT-IR fibers were visualized by the black, silver-gold intensified (SGI)-DAB-end-product. After the complete intensification of labelled monoaminergic axons, GH-RH-containing neuronal elements were identified by the natural brown DAB chromogen. The high contrast of color and electron density between the chromogens allows a clear distinction, at both the light and electron microscopic levels, of structures that express these different immunoreactive substances. Some of the immunostained sections were mounted on glass slides for light microscopic evaluation, while the majority were processed for flat embedding in Epon resin. The ultrathin sections were cut and collected on Formvar coated single slot grids, contrasted with uranyl acetate and Reynolds' lead citrate and examined with a Tesla BS 500 transmission electron microscope.

RESULTS

Light Microscopy

In colchicine treated rats, the arcuate nucleus exhibited numerous fusiform and multipolar GH-RH synthesizing neurons (Fig. 1a, b). The median eminence also contained a substantial number of GH-RH-IR fibers (Fig. 1a). The cells were concentrated ventro-laterally within the nucleus. The same region of the arcuate nucleus was seen to receive both DBH- (Fig. 1c) and PNMT- (Fig. 1d) containing axons.

In the double labelled sections, the black silver-gold intensified DAB-end-product labelled DBH- or PNMT-IR axons, while GH-RH-producing neurons were labelled by brown non-intensified DAB chromogen (Fig. 2). Low power micrographs (Fig. 2a, b) revealed congruency in the distribution of catecholaminergic fibers and GH-RH-IR neurons. The labelled cells were surrounded by fibers expressing DBH- (Fig. 2c) and PNMT- (Fig. 2d) immunoreactivity. High power micrographs of GH-RH neurons demonstrated that these cells were in close juxtaposition with both DBH- (Fig. 2e) and PNMT- (Fig. 2f) containing axons, suggesting the possible occurrence of interactions between the central catecholamine and hypothalamic GH-RH-synthesizing systems.

Electron Microscopy

Ultrastructural examination of double labelled sections was focused on the ventral and ventro-lateral parts of the arcuate nucleus where the light microscopic observations suggested contacts between the two chemically differing neuronal circuits. The GH-RH-IR profiles were recognized on the basis of exhibiting non-intensified DAB-polymer. It was associated mainly with ribosomes and neurosecretory granules located in the cytoplasm (Fig. 3a, c, e, f). The GH-RH-IR neurons received non-labelled axon terminals on both their dendrites (Fig. 3c) and cell bodies (Fig. 3f).

For tracing catecholaminergic axons at the ultrastructural level, the immunodetection of PNMT was chosen as a marker enzyme of the central adrenergic system (17, 18). Fibers expressing PNMT immunoreactivity were selectively labelled by silver-gold particles (Fig. 3b) and they were seen to form asymmetric synaptic specializations (Fig. 3d) with neuronal elements of the arcuate nucleus. As far as the chemical nature of structures being post-synaptic to these adrenergic, PNMT-immunopositive axons was concerned, GH-RH-immunoreactivity was detected in some of the profiles. PNMT-IR axons were observed to establish synaptic connections with dendrites (Fig. 3e) and cell bodies (Fig. 3f) of GH-RH-producing neurons.

DISCUSSION

It has been well established that the growth hormone production by the anterior pituitary gland is under the influence of a dual hypothalamic regulatory mechanism (47) that utilizes somatostatin (5) and GH-RH (16, 39, 41) as neuromessengers. A peculiar feature of this regulation has been the physiological interrelationship (30, 38, 47) and the recently published anatomical link (12, 20, 28), at the level of the hypothal-

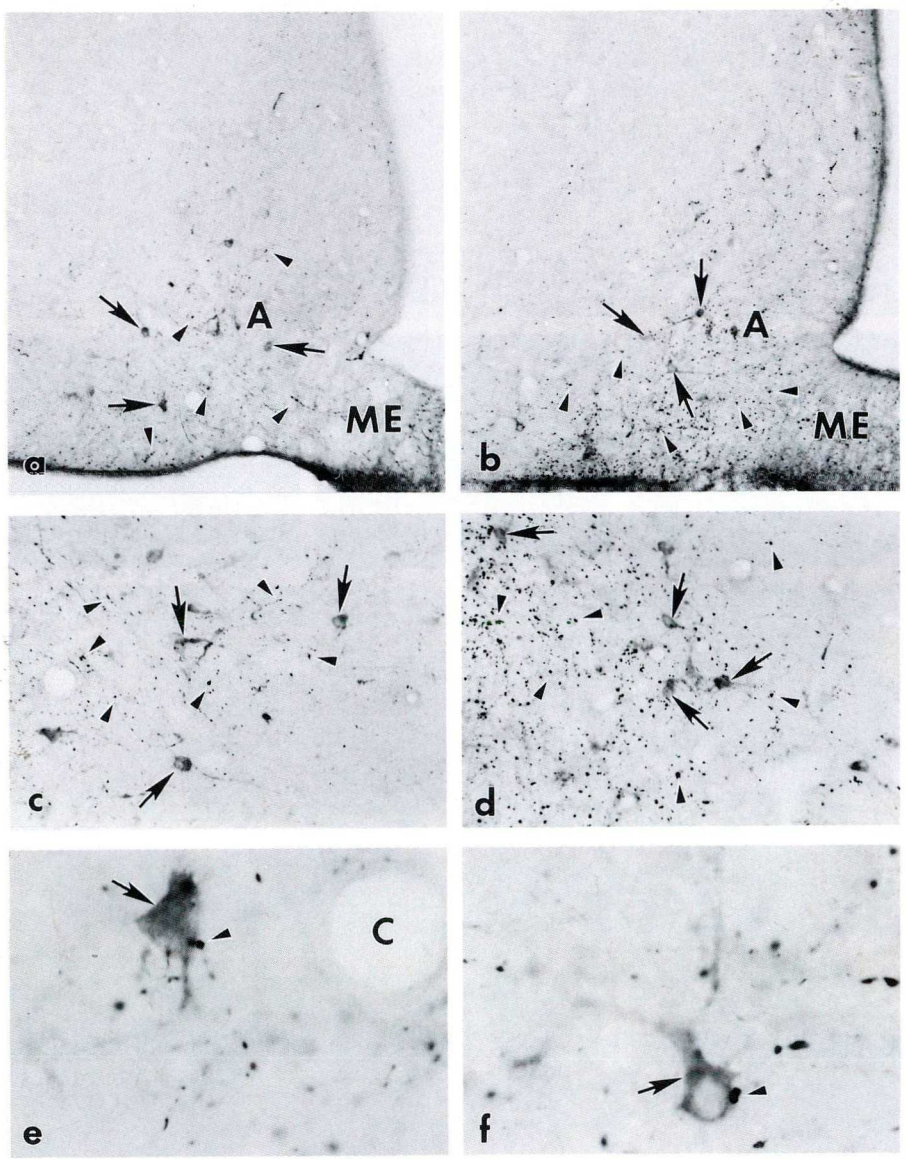

Fig. 2 Simultaneous immunohistological demonstration of catecholaminergic axons and growth hormone-releasing hormone (GH-RH)-synthesizing neurons in the arcuate nucleus (A) of colchicine treated rats. ME, median eminence. a, c, e: Co-distribution of dopamine-β-hydroxylase (DBH)- and GH-RH-immunoreactive (IR) elements. b, d, f: Co-visualized phenylethanolamine-*N*-methyltransferase (PNMT)- and GH-RH-IR neuronal structures. a: Low power micrograph demonstrates co-distribution of DBH- (arrowheads) and GH-RH- (arrows) containing profiles. $\times 42$ b: GH-RH-synthesizing neurons (arrows) located among PNMT-IR axons (arrowheads). $\times 42$ c and d: Demonstration of the relationship of DBH- (c) and PNMT- (d) IR axons (arrowheads) with GH-RH-producing neurons (arrows). Silver-gold intensified DAB endproduct labels the catecholaminergic axons, while the less intensely stained GH-RH-IR cells are detected by non-intensified DAB-chromogen. $\times 70$; $\times 70$ e: DBH-IR axon (arrowhead) juxtaposed to a proximal dendritic shaft of a GH-RH-synthesizing neuron (arrow). $\times 280$ f: Adrenergic, PNMT-containing axon-varicosity (arrowhead) contacts the perikaryon of a GH-RH-IR neuron (arrow). $\times 280$

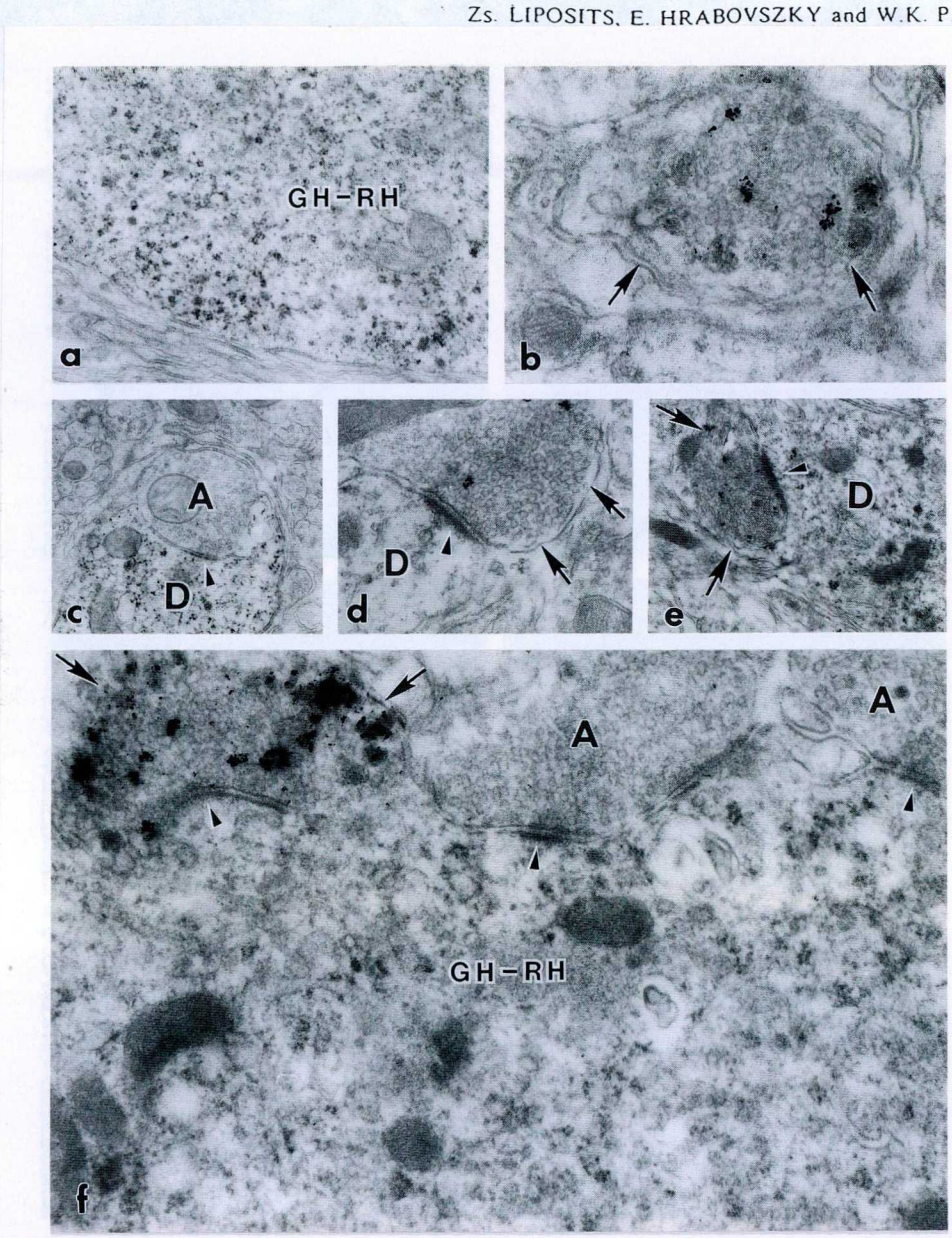

Fig. 3. Ultrastructural analysis of phenylethanolamine-*N*-methyltransferase (PNMT)- and growth hormone-releasing hormone (GH-RH)-immunoreactive (IR) structures in the arcuate nucleus of a colchicine treated rat. a: Cytoplasmic detail from a GH-RH-synthesizing neuron labelled by non-intensified DAB-chromogen. ×14,300. b: High power micrograph of a cross sectioned PNMT-IR axon, tagged selectively by silver-gold particles. ×34,800. c: Dendrite (D) immunoreactive for GH-RH, receiving (arrowhead) a non-labelled axon terminal (A). ×4,750. d: PNMT-IR axon (arrows) synapsing (arrowhead) on a non-identified dendrite (D). ×4,550. e: Axo-dendritic communication (arrowhead) between an adrenergic, PNMT-containing axon-terminal and a GH-RH-IR dendritic process (D). ×4,550. f: PNMT-immunoreactive axon (arrows) establishes a synaptic connection (arrowhead) with a growth hormone-releasing hormone-synthesizing perikaryon (GH-RH). Note the different properties of the pre- and postsynaptic immunolabels. Non-labelled axon terminals (A) also communicate with the releasing hormone producing neuron. ×14,200.

amus, between the somatostatin and GH-RH systems. The assumed interaction of the periventriculo-infundibular, inhibitory (1, 25) and tubero-infundibular, stimulatory (34, 40) 'final common pathways' of growth hormone regulation establishes an intra-hypothalamic loop that can be balanced by diverse neuronal circuits of the brain impinging on the somatostatin and somatocrinin synthesizing hypophysiotrophic neurons.

In relation to the hypothalamic GH-RH-system, several substances including catecholamines, serotonin, acetylcholine, substance-P, somatostatin, endorphins (9, 10, 23, 31) have been implicated to function as neurotransmitters or neuromodulators. The aim of the present work was to study the morphological correlates of the previously proposed catecholamine-GH-RH interaction (13, 35, 44) by means of immunocytochemical double labelling technique. The distribution pattern of GH-RH-synthesizing neurons and catecholaminergic axons found in the arcuate nucleus is in agreement with previous morphological data (3, 6, 7, 34, 40). The overlapping of GH-RH-synthesizing neurons with DBH- and PNMT-containing axons, respectively, indicates that the central noradrenergic and adrenergic pathways are in an optimal anatomical position to interact with GH-RH-producing cells. The ultrastructural analysis of juxtaposed PNMT-IR axons and GH-RH-synthesizing neurons revealed synaptic specializations. These synapses, formed by PNMT-containing axon terminals on the cell bodies and dendrites of GH-RH-IR neurons, are indicative of a direct monosynaptic input from central adrenergic neurons (17, 18, 24, 45) to cells of the arcuate nucleus which synthesize GH-RH. Moderate noradrenaline (37) and adrenaline (46) levels have been previously reported in the arcuate nucleus. This suggests that its neurons might be influenced by catecholamines.

The administration of selective epinephrine synthesis inhibitors results in the suppression of pulsatile GH-secretion, that can be eliminated by the administration of clonidine, an alpha-receptor activating compound (44). Clonidine treatment has also been reported to increase GH-secretion in animals that were injected with anti-somatostatin serum (13). These observations suggest a GH-RH neuronal system mediated action. After passive immunization with anti-hp-GR-RH-serum, an i.v. injection of clonidine failed to stimulate GH release in the unanaesthetized rat (35), indicating that alpha-adrenergic mechanisms are mediated through the stimulation of endogenous GH-RH release.

In summary, the present findings indicate that PNMT-IR axons synapsing on GH-RH-synthesizing neurons in the arcuate nucleus might represent an anatomical route for the adrenergic regulation of this hypophysiotrophic system.

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Adrenergic innervation of dopamine neurons in the hypothalamic arcuate nucleus of the rat

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Abstract

Tuberoinfundibular dopaminergic (TIDA) neurons, which represent the final common pathway in the inhibitory neuronal control of prolactin (PRL) secretion, are regulated by synaptic input from various transmitter systems. Because adrenergic receptors at hypothalamic sites were implicated in the central regulation of lactotrophs, we hypothesized that a synaptic communication might exist between adrenergic pathways ascending from the brain stem and the TIDA system. Polyclonal antisera directed towards phenylethanolamine *N*-methyltransferase (PNMT) and tyrosine hydroxylase (TH), biosynthetic enzymes of catecholamines, were used for the simultaneous immunocytochemical detection of adrenergic fibers and TIDA neurons, respectively, in Vibratome sections of the rat hypothalamus. By the light microscopic evaluation of double-immunostained sections, PNMT-immunoreactive (IR) axon varicosities were localized in juxtaposition to TH-IR cell bodies and dendrites in the arcuate nucleus (AN) which contains perikarya and dendrites of TIDA neurons. The ultrastructural analysis of contacts provided firm evidence for the occurrence of synaptic interactions between the adrenergic and TIDA neuronal systems. These morphological findings show that adrenergic neurons are involved in the afferent regulation of the TIDA system and indicate a putative pathway of central adrenergic effects upon PRL secretion.

Key words: Arcuate nucleus; Catecholamine; Electron microscopy; Hypothalamus; Immunocytochemistry; Phenylethanolamine *N*-methyltransferase; Synapse; Tyrosine hydroxylase

Prolactin (PRL) secretion by the anterior pituitary gland is influenced by systemic [7–9] and i.c.v. administration [18] of adrenergic agents. The elevation of plasma PRL by local injection of α2- and β-adrenergic agonists into the mediobasal hypothalamus (MBH) and α2-adrenergic agonists into the preoptic-anterior hypothalamus strongly suggests that at least two hypothalamic sites participate in the adrenergic stimulation of lactotrophs [6]. One of these areas, the MBH, contains the tuberoinfundibular dopaminergic (TIDA) system [2] which represents the final common pathway in the inhibitory neuronal control of PRL secretion [1]. Therefore, we proposed that the adrenergic mechanisms regulating PRL secretion act, at least in part, via modulation of TIDA neuronal activity.

The aim of this study was to provide a morphological support for the synaptic regulation of TIDA neurons by central adrenergic neuronal pathways [5]. The hypothalamic arcuate nucleus (AN), which contains the perikarya and dendrites of TIDA neurons [2], was analysed by preembedding double-labeling immunocytochemistry [13].

Five male Wistar rats (200–250 g) were deeply anesthetized with Nembutal (60 mg/kg i.p.) and perfused through the ascending aorta with 4% paraformaldehyde-1% glutaraldehyde in 300 ml of a 0.1 M phosphate-buffered saline solution (PBS; pH 7.4). The brains were immersed in the same fixative for 2 h and then 25-μm-thick coronal sections containing the AN were cut from the hypothalami on a Vibratome (Technical Products International, St Louis, MO). Residual aldehydes were

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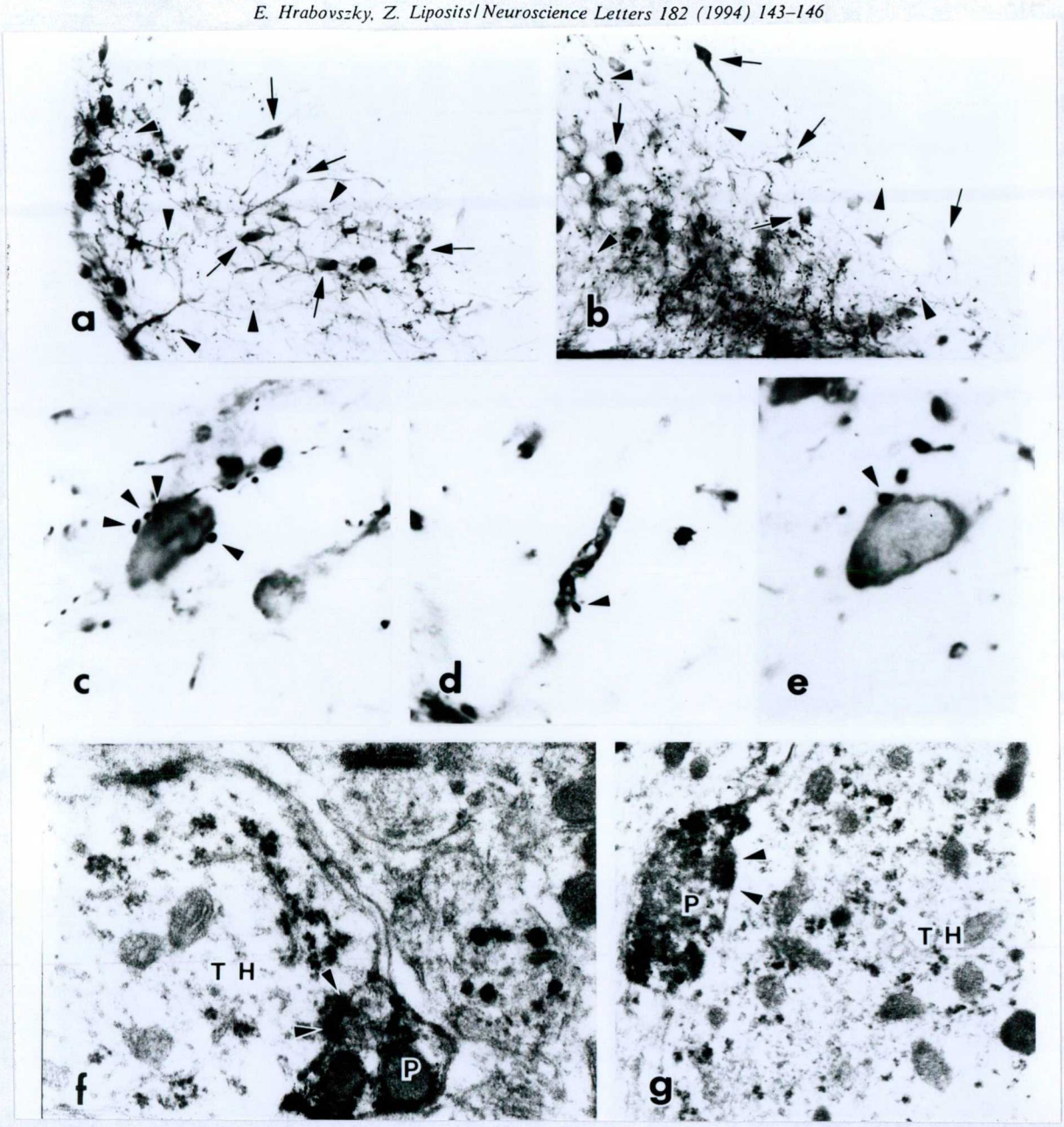

Fig. 1. Simultaneous detection of PNMT- and TH-IR neuronal elements in the hypothalamic AN of the rat. a–e: light microscopic demonstration of PNMT-IR, adrenergic and TH-IR, dopaminergic neuronal structures. f,g: electron micrographs of adrenergic synapses on dopaminergic neurons. a,b: overlapping distribution of silver-gold-stained PNMT-IR axon varicosities (arrowheads) and DAB-labeled, TH-IR neurons (arrows) in the dorso-medial (a) and ventro-lateral (b) subdivisions of the nucleus. $\times_{a,b}250$. c,d: axo-somatic (c) and axo-dendritic (d) juxtapositions (arrowheads) between adrenergic fibers and dopaminergic neurons, representing potential sites of neuronal interactions. $\times_{c,d}1450$. e: light microscopic demonstration of an axo-somatic juxtaposition (arrowhead) in a semithin section. ×2000. f: synaptic communication (arrowheads) between PNMT-IR axon (P) and TH-expressing dendrite (TH). ×36,000. g: synaptic interaction of PNMT-IR terminal (P) and TH-immunopositive perikaryon (TH). Arrowheads point to a symmetrical synapse. ×23,000.

inactivated with a 1% sodium borohydride ($NaBH_4$; Sigma, St Louis, MO) treatment for 30 min, followed first, by permeabilization of sections with 0.2% Triton X-100 (Sigma) for 20 min and then blockage of non-specific antibody binding with 2% normal sheep serum (NSS) for 10 min. Adrenergic fibers were characterized by their phenylethanolamine *N*-methyltransferase (PNMT) content. This enzyme catalyzes the conversion of noradrenaline to adrenaline, therefore, it occurs in adrenergic but not in noradrenergic or dopaminergic neuronal structures. For the immunocytochemical detection of PNMT, the peroxidase–antiperoxidase complex (PAP) technique [16] and 3,3′-diaminobenzidine (DAB) chromogen were used, with the subsequent silver-gold intensification of the DAB precipitate [12]. Then, dopaminergic neurons were demonstrated by means of

the PAP-DAB method, on the basis of immunoreactivity for the dopaminergic marker enzyme, tyrosine hydroxylase (TH). The rabbit, anti-rat PNMT [3] and the rabbit, anti-bovine TH (#1012, Eugene Tech International, Allendale, NJ) primary antisera were diluted to 1:3000 and 1:1000, respectively, and the sections incubated at 4°C for 48 h.

While some double-immunostained sections were mounted on glass slides and coverslipped for light microscopic evaluation, most were osmicated, dehydrated and flat-embedded into Epon resin (LX-112; Ladd Research Industries, Burlington, VT). The resin blocks were further processed with an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria). Some were serially cut at 2 μm for light microscopic studies, others were designated for ultrastructural analysis. The ultrathin sections were collected on Formvar-coated, single-slot grids, contrasted with uranyl acetate and lead citrate and examined with a Tesla BS 500 transmission electron microscope.

In light microscopic preparations, the silver-gold-labeled PNMT-IR axons appeared in black which contrasted with the brown (gray, in black-and-white micrographs) DAB-stained TH-containing structures. PNMT-IR fibers had a wide distribution throughout the AN, whereas this entire area was devoid of PNMT-IR cell bodies. TH-IR neurons of the AN formed two distinct subpopulations in the dorso-medial and ventro-lateral parts of the nucleus, and they intermingled with beaded PNMT-IR axons (Figs. 1a,b). At higher power, appositions of PNMT-positive fibers to TH-containing perikarya and dendrites were revealed (Figs. 1c–e). Because of the neurochemical diversity of TH-IR neurons in the dorso-medial and ventro-lateral portions of the AN [14,15], both regions were separately analysed by electron microscopy. At the ultrastructural level, PNMT-containing axons were recognized by the accumulation of highly electron-dense, silver-gold grains over both dense core and clear vesicles. In contrast, the deposition of DAB in TH-IR neuronal elements resulted in a medium degree of electron density. The examination of ultrathin sections revealed PNMT-IR axon terminals which were engaged in synaptic contacts with both TH-IR dendrites (Fig. 1f) and cell bodies (Fig. 1g). Synapses between PNMT-containing terminals and TH-labeled neurons were detected in both the dorso-medial and ventro-lateral subdivisions of the AN.

Our morphological findings strongly indicate a physiological adrenergic/noradrenergic involvement in the afferent neuronal regulation of the TIDA system. The type of adrenergic receptors underlying this synaptic interaction might be $\alpha 2$, based on the abundance of p-[^{3}H]aminoclonidine-binding sites in the AN [19] and the observation of reduced TIDA neuronal activity following $\alpha 2$-adrenergic receptor stimulation with clonidine [4]. Because the major function attributed to TIDA neurons is the inhibitory control of PRL secretion [1], we propose that adrenergic input to the TIDA system might modulate this process.

The choice of male animals in these studies was made to eliminate estrogen-dependent variations in the staining pattern of antigens in the course of the estrus cycle. However, in preliminary light microscopic studies, we verified the existence of PNMT-IR axon juxtapositions to TIDA neurons of the female rats as well. Possible sexual dimorphism and other quantitative aspects of the explored synaptic interaction were not addressed in this study.

It remains to be determined whether central noradrenergic pathways also contribute to the afferent regulation of TIDA neurons. Unfortunately, commonly used immunocytochemical markers of the noradrenergic system, dopamine β-hydroxylase or noradrenaline itself also occur in adrenergic, in addition to noradrenergic, neurons.

TH-IR nerve cells in the ventro-lateral part of the AN, which are chemically distinct from the dorso-medial TH-expressing neuronal population, are thought to be partially identical with growth hormone-releasing hormone (GH-RH)-synthesizing neurons [14]. Our present findings on their adrenergic regulation is in harmony with the earlier observation of PNMT-IR synapses on GH-RH-IR arcuate neurons [10,11].

In conclusion, we explored synaptic communication between adrenergic axons and TIDA neurons in the hypothalamic AN. These results support the concept that adrenaline and/or intermediates of its biosynthesis modulate the operation of TIDA neurons via synaptic channels.

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BRAIN RESEARCH

Research report

Galanin-containing axons synapse on tyrosine hydroxylase-immunoreactive neurons in the hypothalamic arcuate nucleus of the rat

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Abstract

Prolactin (PRL) secretion by the anterior pituitary gland is dependent upon the tonic inhibitory influence of the tuberoinfundibular dopaminergic (TIDA) neuronal system. TIDA neurons, in turn, are regulated by various afferent neuronal systems. To support the concept that the recently-discovered neuropeptide, galanin (GAL), is one of the neurotransmitter/neuromodulator substances which might synaptically regulate the function of the TIDA system, immunocytochemical double-labeling studies were carried out in the hypothalamic arcuate nucleus (AN) of the male rat. The analysis of light microscopic preparations revealed the overlapping of GALergic and dopaminergic (detected by tyrosine hydroxylase immunoreactivity) neuronal elements in both the dorsomedial and ventrolateral parts of the AN. TH-containing perikarya and dendrites were contacted by varicose GAL-IR axons in these regions. The electron microscopic studies of ultrathin sections demonstrated axosomatic and axodendritic synapses between GALergic axons and TH-IR neurons. These findings support the view that GAL may modulate PRL release, acting as a neurotransmitter/neuromodulator in synaptic afferents to the TIDA system.

Key words: Arcuate nucleus; Dopamine; Double-labeling; Electron microscopy; Galanin; Hypothalamus; Immunocytochemistry; Interaction; TIDA; Tyrosine hydroxylase

1. Introduction

The biologically active, 29-amino acid peptide, galanin (GAL), was originally isolated from porcine intestinal extracts [39], and later its ubiquitous presence in both the peripheral and central nervous systems was established [5,8,25,32,35,36]. Within the brain, the hypothalamus is especially rich in GAL immunoreactivity [5,8,25,32,35,36], GAL mRNA [3,12] and GAL-binding sites [3,24,27,29,34]. The presence of the peptide and its mRNA in neurons of the supraoptic, paraventricular, periventricular, arcuate and medial preoptic nuclei and the wide distribution of GAL-containing axons and GAL receptor sites within the hypothalamus are of importance from a neuroendocrine point of view. Several lines of consistent data demonstrate that the secretion of prolactin (PRL) [14,23,31], growth hormone [31] and luteinizing hormone [33] can be influenced by GAL. In addition to its central effects, GAL might also act at the pituitary level and alter anterior pituitary cell functions. This hypophysiotropic role of the peptide has been concluded from (i) the presence of a dense network of GAL-immunoreactive (IR) fibers within the external layer of the median eminence (ME) [5,21,25,32,35], (ii) the higher concentration of the peptide in hypophysial portal than in peripheral blood [19,21], and (iii) the labeling of GAL neurons from the ME with the retrograde tracer, Fluoro-Gold [28].

Galanin, when administered to rats intracerebroventricularly (i.c.v.), produces increased plasma levels of PRL [14,23,31]. Because isolated pituitaries are not affected by GAL treatment [31], and systemic administration of the peptide does not modify PRL levels [14,31], a central, instead of hypophyseal site of action

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upon the lactotrophs has been proposed. Melander et al. [23] have found that the elevation of plasma PRL by i.c.v. injection of GAL is associated with reduced PRL release inhibiting factor, dopamine (DA) content of the ME. Taken together, these data suggest that GAL acts upon the tuberoinfundibular dopaminergic (TIDA) neuronal system, and the inhibition of DA release into the portal blood may account, at least in part, for the GAL-induced rise in PRL secretion.

Because the major source for DA-containing terminals of the ME is the hypothalamic arcuate nucleus (AN) [2], we hypothesized a direct GAL-DA interaction within this region. To provide morphological evidence for the synaptic regulation of TIDA neurons by GALergic axons, immunocytochemical double-labeling studies were conducted at the light and electron microscopic levels.

2. Materials and methods

2.1. Animals

Adult, male Wistar rats ($n = 10$) weighing 250–300 g were maintained under standard environmental conditions. Two of them received a single i.c.v. injection of colchicine (80 μg, dissolved in 5 μl of saline) 36 h before sacrifice; whereas the rest remained untreated. While the former model was used for the detection of GAL in cell bodies, the immunostaining of GAL-containing fibers, DAergic neurons and immunocytochemical double-labelings were performed without colchicine-pretreatment of the animals.

2.2. Fixation and tissue preparation

Rats were anesthetized with Nembutal (40 mg/kg; ip) and perfused through the ascending aorta. A short initial rinse with 0.1 M phosphate-buffered saline (PBS, pH 7.4) was used to remove the blood from the vasculature. Subsequently, the animals were fixed with 500 ml of a mixture containing 1% paraformaldehyde and 1% glutaraldehyde in PBS, at a slow flow-rate (60 drops/min). The brains were removed and immersed in the same fixative for 2 h. Thereafter, the hypothalami were dissected and 25 μm thick, coronal sections were cut on a Vibratome (Technical Products International, St. Louis, MO, USA).

2.3. Immunocytochemical labeling

Prior to incubation in the primary antibodies, the residual glutaraldehyde was neutralized in the sections by a 30 min treatment in 1% sodium borohydride ($NaBH_4$, Sigma Chemical Co., St. Louis, MO, USA) solution under constant gentle agitation. Sections were subsequently permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 20 min and treated with 2% normal sheep serum (NSS) in PBS for 10 min to prevent non-specific antibody binding.

2.4. Detection of single tissue antigens

For the immunostaining of DAergic neurons, Sternberger's [37] peroxidase-antiperoxidase complex (PAP) technique and the 3,3'-diaminobenzidine (DAB) chromogen were used. The primary antiserum was raised in rabbit against tyrosine hydroxylase (TH) (#1012, Eugene Tech International, Allendale, NJ, USA), the rate-limiting enzyme of DA synthesis, and used at 1:1000 working dilution.

The immunocytochemical detection of GAL-containing structures utilized the combination of the PAP-DAB procedure and subsequent silver-gold intensification of the DAB end-product [9]. The anti-porcine GAL antibody (RAS7153N, Peninsula Laboratories, Belmont, CA, USA) was generated in rabbit, and diluted to 1:4000. Following 36 h incubation in the primary antisera, the sections were placed in the bridging antibody (sheep, anti-rabbit IgG, 1:500, Arnel Products Co., New York, NY, USA), and after in the PAP complex (rabbit, 1:500, Arnel) for 1 h in each. All antibodies were dissolved in PBS-1% NSS, and the incubations were followed by 30 min rinses in several changes of PBS. The antigen-antibody complexes were visualized according to the method of Streit and Reubi [38]. The silver-gold intensification of the DAB endproduct was carried out as adapted to electron microscopic studies by Liposits et al. [16].

2.5. Sequential detection of two tissue antigens

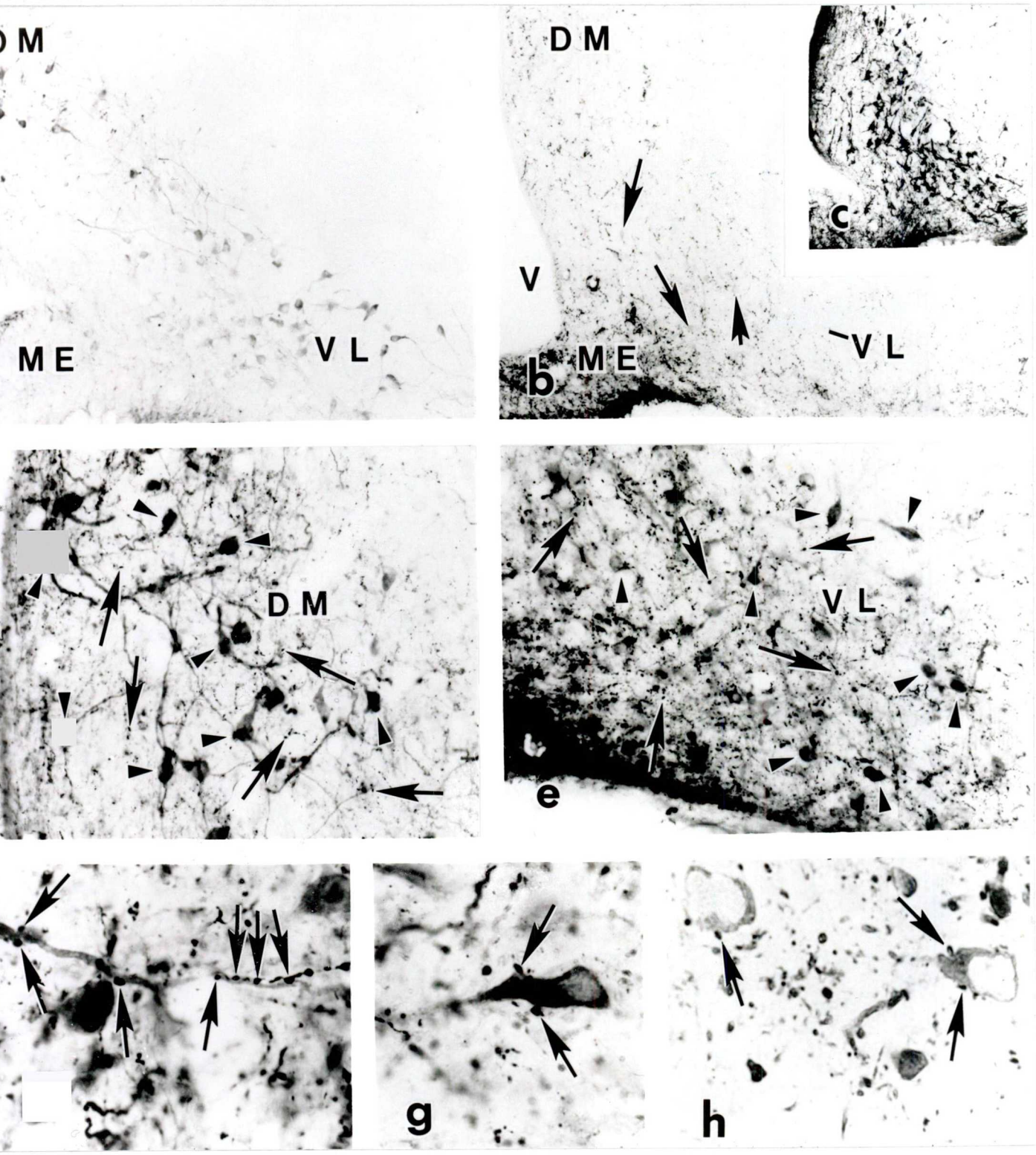
To visualize both the TH- and the GAL-IR antigenic sites within the same sections, a dual antigen localization technique [17] was used. First, GALergic elements were immunostained by the black, silver-gold-intensified DAB endproduct, followed by the detection of TH-containing structures using the non-intensified, brown DAB chromogen. Some of the immunostained sections were mounted on glass slides for light microscopic evaluation; whereas the majority was processed further for electron microscopic flat embedding in Epon resin (LX-112, Ladd Research Industries, Burlington, VT, USA). Because of the marked contrast between the two reaction products in color and electron-density, a clear distinction of GAL- and TH-containing neuronal elements was achieved at both the light and electron microscopic levels. Semithin and series of ultrathin sections were cut from the resin blocks on an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria). Ribbons of ultrathin sections were collected on Formvar-coated, single-slot grids, contrasted with uranyl acetate and Reynolds' lead citrate and examined with a Tesla BS 500 transmission electron microscope.

3. Results

3.1. Light microscopy

The distribution of TH immunoreactivity in the AN substantiated previous immunocytochemical data [4,10,41]. A prominent accumulation of the DAB chromogen was observed in dendrites and small perikarya of the dorsomedial TH-IR cell group; whereas immunopositive neurons in the ventrolateral portion of the AN were larger and expressed lesser amounts of the enzyme (Fig. 1a). GALergic elements were stained by the black, silver-gold-intensified DAB chromogen. The AN of colchicine-untreated animals contained numerous GAL-IR axons, but only some poorly labeled cell bodies could be visualized (Fig. 1b). In contrast, a substantial number of neurons contained detectable levels of the peptide in the AN of colchicine-administered rats (Fig. 1c).

Double-labeling immunocytochemistry revealed the overlapping distribution of GAL-and TH-IR antigenic sites in the AN. The silver-gold-stained, GALergic



1. Immunocytochemical detection of tyrosine hydroxylase (TH)- and galanin (GAL)-containing neuronal structures in the arcuate nucleus (N) of the rat. ME, median eminence; V, 3rd ventricle; DM, dorsomedial portion of the AN; VL, ventrolateral subdivision of the AN. For the demonstration of TH-immunoreactive (IR) neurons, the peroxidase-antiperoxidase complex (PAP) technique and 3,3′-diaminobenzidine (DAB) chromogen were used; whereas GAL-IR elements were stained by the silver-gold-intensified DAB chromogen. a: Small, heavily labeled TH-IR neurons in the dorsomedial subunit and larger less intensely-stained cells in the ventrolateral part of the arcuate area. ×130. b: In intact animals, GAL immunoreactivity is confined to axons, arrows point to some lightly-stained cell bodies. ×130. c: Colchicine administration resulted in intense GAL immunostaining in numerous perikarya and dendrites of the AN. ×70. d–h: Simultaneous demonstration of GAL- and TH-IR structures in the AN. Both the dorsomedial (d) and the ventrolateral (e) subdivisions of the AN exhibit overlapping in the distribution of neuronal elements expressing GAL (arrows) and TH (arrowheads) immunoreactivities. (d, e) ×270. High power micrographs of axodendritic (f) and axosomatic (g) juxtapositions (arrows). Such relationships between GALergic axons and TH-immunopositive neurons are characteristic of both portions of the AN. (f, g) ×830. h: Semithin section from the dorsomedial part of the AN shows GAL-IR fibers contacting (arrows) TH-IR perikarya. ×1,100.

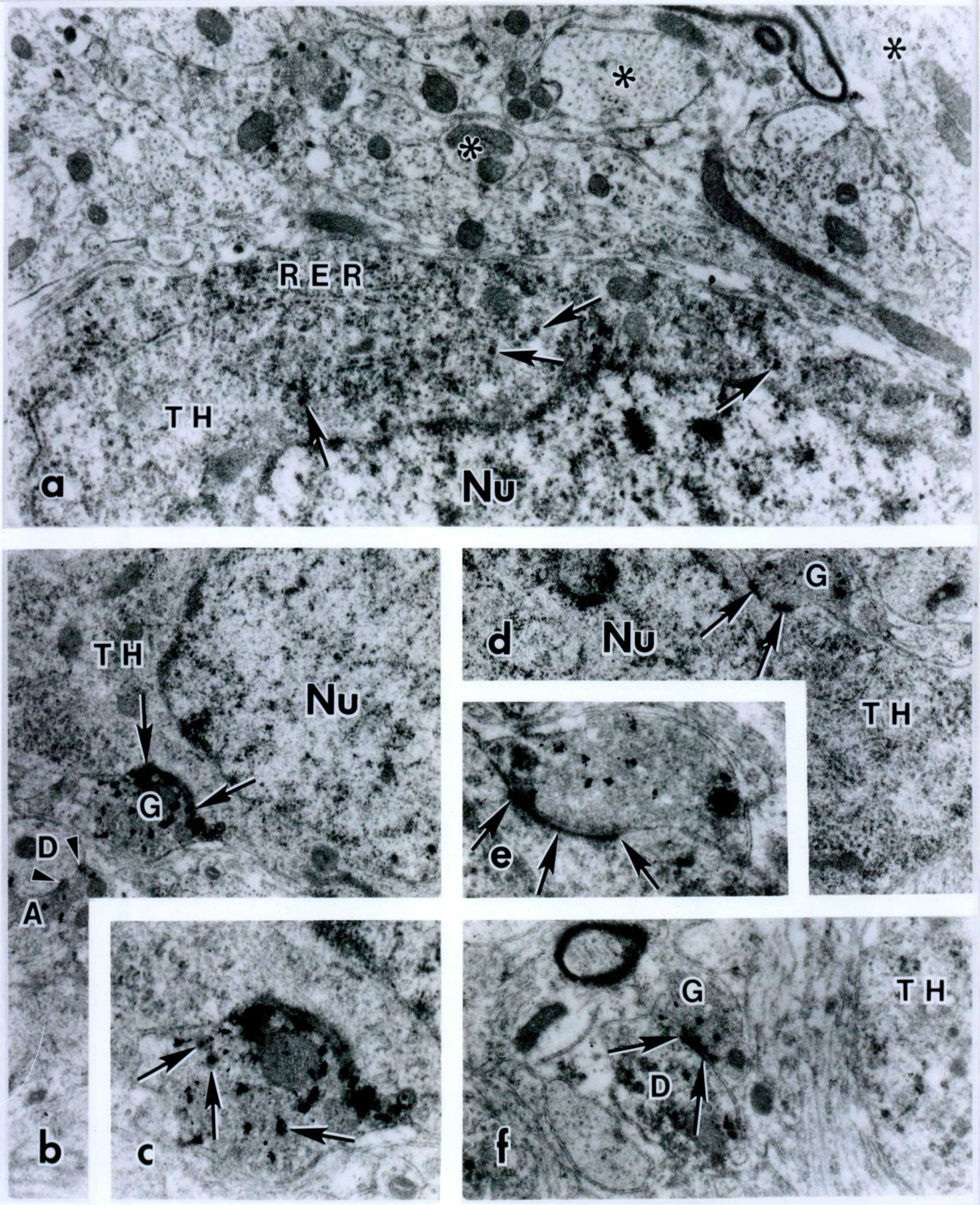


Fig. 2. Ultrastructural demonstration of tyrosine hydroxylase (TH)- and galanin (GAL)-immunoreactive (IR) neuronal elements in the arcuate nucleus. Nu, nucleus. a: TH-IR perikaryon (TH) accumulating large amounts of non-intensified diaminobenzidine (DAB) chromogen over the rough endoplasmic reticulum (RER). Arrows point to labeled dense-core vesicles. *, immunonegative profiles. ×15,000. b: GAL-IR axon terminal (G) containing silver-gold particles establishes synaptic contact (arrows) with a TH-IR perikaryon (TH). Another GAL-IR axon (A) synapses (arrowheads) on an immunonegative dendrite (D). ×16,500. c: High power micrograph of the synapsing profiles shown in b demonstrates the symmetrical character of the synapse and the predominant accumulation of metallic grains over dense-core vesicles (arrows). ×31,000. d, e: By the evaluation of series of ultrathin sections, GAL-IR axons (G) were also found to form asymmetric synapses (arrows) with TH-IR neurons (TH). d and e show the same synapsing profiles on consecutive ultrathin sections. Note the clear synaptic cleft and the predominance of the post-synaptic density (arrows) in e. (d) ×13,000; (e) ×28,000. f: GAL-IR axon (G) establishes synaptic contact (arrows) with a DAB-tagged, TH-IR dendrite (D). Note the immunostained TH-IR cytoplasmic detail (TH). ×13,500.

axons contacted the DAB-labeled TH-IR perikarya and dendrites both in the dorsomedial (Fig. 1d) and ventrolateral (Fig. 1e) subdivisions of the nucleus. At higher power, double-labeled axodendritic (Fig. 1f) and axosomatic (Fig. 1g) juxtapositions were detected throughout the AN. The close relationship between GAL- and TH-IR neuronal structures was also apparent in semithin sections (Fig. 1h).

3.2. Electron microscopy

At the ultrastructural level, the DAB deposits produced a medium degree of electron density in TH-IR neuronal structures, as compared to the surrounding non-labeled profiles. Abundant chromogen was observed over the rough endoplasmic reticulum and dense-core vesicles of labeled perikarya (Fig. 2a). In GAL-IR axons, the highly electron-dense silver-gold grains were associated primarily with dense-core vesicles (Figs. 2c, e). By the serial evaluation of double-immunostained ultrathin sections (Figs. 2d, e), GAL-IR fibers were revealed to establish axosomatic (Figs. 2b–e) and axodendritic (Fig. 2f) synaptic contacts with TH-IR neurons. Both the symmetrical (Figs. 2b, c) and the asymmetrical (Figs. 2d–f) forms of synaptic specializations occurred.

4. Discussion

The A12 catecholaminergic cell group of the hypothalamic AN [6] is largely involved in the central inhibitory control of PRL secretion by the anterior pituitary gland. A subpopulation of these neurons, termed TIDA, send short axons to the external layer of the ME and release the putative PRL release inhibiting factor, DA, into the primary capillaries of the hypophysial portal system [2]. The function of TIDA neurons is influenced by multiple humoral and neuronal mechanisms [1], the latter represented by axonal inputs from various neuronal sources. On the basis of ultrastructural observations, DA [13], serotonin [40], γ-aminobutyric acid (GABA) [15] and endogenous opioids [11] are assumed to act as neurotransmitters/neuromodulators in these afferent synaptic connections.

In the present study, we provided morphological evidence for the GALergic regulation of TIDA neurons. In pharmacological experiments, GAL was found to increase the secretion of PRL when administered i.c.v. to rats [14,23,31]. Because the peptide did not exert the same effect on isolated pituitaries [31], and its systemic injection did not modify the release of PRL [14,31] it was concluded that the GAL-induced rise of PRL was mediated through the central nervous system. Earlier studies aimed at identifying neuronal circuits that participate in the stimulation of lactotrophs by GAL yielded discordant results. Koshiyama et al. demonstrated that vasoactive intestinal polypeptide (VIP), a putative PRL releasing factor, is involved in PRL rise by GAL, since passive immunization of rats with anti-VIP antibody significantly suppressed PRL response to i.c.v. administration of GAL [14]. The mediation of the GALergic action by the TIDA system was suggested by Melander et al., who reported a 24% reduction of the catecholamine stores in the medial palisade zone of the ME 20 min after i.c.v. GAL injection, an effect associated with an unequivocal increase in PRL secretion [23].

Our present immunocytochemical findings show that GALergic axons innervate TH-containing neurons in the AN of the rat. Because TH-IR neurons in the anterior AN project to the posterior pituitary, instead of the ME [2], we preferentially analyzed more caudal arcuate regions, neurons of which represent presumably the TIDA system. On the basis of the earlier suggestion that TH-IR neurons located in the ventrolateral part of the AN might represent a non-DAergic subpopulation [22,30], we performed a separate immunocytochemical analysis of the dorsomedial and ventrolateral portions of the AN. By the light microscopic application of a double-labeling immunocytochemical technique, we demonstrated GAL-IR axon varicosities in close appositions to TH-IR cell bodies and dendrites in both subdivisions of the nucleus. At the ultrastructural level, the double-labeled juxtaposed profiles showed patterns of synaptic communication and displayed symmetrical and asymmetrical specializations. These observations support the concept that GAL modulates the operation of TIDA neurons via synaptic mechanisms.

However, additional site/s for GAL to interact with the TIDA system cannot be ruled out. Nordström et al. [29] have recently shown that the high potassium-evoked [^{3}H]DA release from medio-basal hypothalamic fragments, including the ME, is inhibited by GAL. Moreover, the distribution of [^{125}I]GAL binding sites and that of TH-IR axons overlaps in the medial palisade zone of the ME [29]. Consequently, these authors propose that GAL might exert a paracrine inhibitory effect on DAergic terminals at the level of the ME. Based on the co-localization of GAL and TH within the same arcuate neurons [7,26,29], it is assumed that a part of TIDA neurons secrete both GAL and DA into the portal blood. Thus, the idea that the synthesis and/or release of DA by these particular neurons might depend partially upon an autocrine control by GAL can also be challenged. The similar location of GAL binding sites and GAL- and TH-immunoreactivities in the external zone of the ME [29] supports this concept.

The physiological action of GAL upon TIDA neurons is strongly supported by recent findings, i.e., the

blockade of endogenous GAL by passive immunization is capable of blunting the proestrous afternoon PRL surge without affecting the time of its onset [20]. Whether, and to what extent, the assumed synaptic, paracrine and autocrine GALergic control mechanisms might be involved in the regulation of DA synthesis and/or release requires clarification.

The source of GALergic axons synapsing in the AN is still unknown. López et al. have provided pharmacological and electron microscopic immunocytochemical data suggesting that a local GALergic circuit exists within the AN [18]. Consequently, we propose that TIDA neurons might be innervated, at least in part, by arcuate GAL neurons. If this is the case, it remains to be determined whether these GALergic interneurons represent a population separate from the hypophysiotropic GAL cell group of the AN [28].

In summary, we demonstrated synaptic connections between GAL-IR axons and TH-IR neurons in the AN of the rat hypothalamus. These results strongly indicate a role for GAL as a possible synaptic modulator of TIDA neurons.

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Original Article

Triple-labeling Method Combining Immunocytochemistry and In Situ Hybridization Histochemistry: Demonstration of Overlap Between Fos-immunoreactive and Galanin mRNA-expressing Subpopulations of Luteinizing Hormone-releasing Hormone Neurons in Female Rats[1]

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We describe a sensitive technique combining dual-label immunocytochemistry (ICC) with isotopic in situ hybridization histochemistry (ISHH). We developed this technique to characterize the receptor and/or peptide content of phenotypically identified neurons that express cell markers of neuronal activity (immediate early gene products) after physiological or pharmacological perturbation. Tissue was fixed by perfusion with 4% paraformaldehyde in PBS, sucrose-infiltrated, and cryosectioned. Sections were stored in cryoprotectant or immediately hybridized. After stringent hybridization wash procedures, Fos and luteinizing hormone-releasing hormone (LHRH) neurons were visualized sequentially using immunocytochemistry. Finally, galanin mRNA was detected autoradiographically. We applied the technique to study of subpopulations of LHRH-containing neurons. Results of this study indicate that a majority of the LHRH neurons activated during the luteinizing hormone (LH) surge (as indicated by presence of nuclear Fos staining) also express mRNA encoding galanin. However, there is not a complete overlap between the subpopulation of LHRH neurons that express Fos and that which expresses galanin mRNA. (*J Histochem Cytochem 43:363–370, 1995*)

KEY WORDS: Immunocytochemistry; Immunohistochemistry; In situ hybridization histochemistry; Triple-labeling; Luteinizing hormone-releasing hormone; Galanin; Fos; mRNA; Immediate early genes.

ntroduction

Neuroanatomic and neurochemical studies of peptidergic neuronal ystems have been facilitated by the development of sensitive dual-abel immunocytochemical (ICC) and in situ hybridization (ISHH) nethods. Studies with these methods have demonstrated that ypothalamic neurons expressing a common neuroactive substance an be divided further into subpopulations on the basis of differ-nces in coexpressed peptide or receptor complement. However, he functional significance of neuronal subpopulations defined on he basis of these chemical differences has been difficult to deter-nine without a method for detecting changes in the activity of in-ividual neurons. Abundant data now indicate that many neu-ronal systems express immunoreactivity for immediate-early gene (IEG) products soon after activation (22,26,27,29). The goal of the present studies was to develop a method with which we could further characterize responsive (IEG-positive) neuronal subpopulations based on their receptor and/or peptide content.

In these studies we focused on the luteinizing hormone-releasing hormone (LHRH) neuronal system of the hypothalamus, the final common pathway by which estrogen, through modulation of various neuropeptide, neurotransmitter, and amino acid systems, regulates luteinizing hormone (LH) secretion. Functional diversity of the LHRH system has been implied by studies demonstrating that not all LHRH neurons project to the median eminence, where they would presumably play a role in LH release (8,10,21,30). Furthermore, some but not all LHRH neurons coexpress Fos during the LH surge (5,6,12–14), indicating that only a subpopulation of neurons is activated during the surge. Finally, functional diversity is implied by ICC studies using different antibodies that recognize high molecular weight or fully processed forms of LHRH (4,9). The results of these studies indicate that there are subgroups of LHRH

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neurons that may vary in their rates of biosynthesis, processing, transport, or release under different steroid conditions (4).

In addition to their functional diversity, neurochemical diversity of LHRH neurons has been demonstrated by studies of receptor complement (25) and peptide co-localization (2,19,20,34). Of particular interest is a subpopulation of LHRH neurons that coexpresses the peptide galanin (2,19,20). Although little is known about the physiological significance of this coexpression, there is abundant evidence that galanin plays an important role in LH secretion (15,16,18,28). However, until now there was no direct evidence that the LHRH neurons containing galanin comprise a subpopulation that is active during periods of LH hypersecretion. Therefore, in the present studies our goal was to determine whether the galanin-containing subpopulation of LHRH neurons also expresses Fos during the LH surge. To accomplish this goal, we developed a sensitive triple-label technique which combines dual-label ISHH with ICC or dual-label ICC with isotopic ISHH. A preliminary report of our results has been published in abstract form (7)

Materials and Methods

Animals

Female Sprague-Dawley rats (Charles River, Wilmington, ME; n = 20, 200–225 g) were maintained under a controlled photoperiod (14L:10D, lights on at 0500 hr). The animals were ovariectomized, and steroid treated as described previously (23). Briefly, 1 week after ovariectomy (Day 0), each was implanted SC with two silastic capsules containing estradiol in sesame oil (3 cm; 150 μg/ml). Surgeries were performed under methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) anesthesia. On the morning of Day 2 (0900 hr), animals were injected SC with 5 mg progesterone (50 mg/ml diluted in sesame oil).

To verify and establish the time of peak LH release induced by this method, some animals (n = 6) were treated as described above, but on Day 1 were fitted with right atrial cannulae. On Day 2, serial blood samples were drawn hourly from 1100 through 1800 hr and serum was frozen for subsequent determination of LH concentrations by radioimmunoassay (23)

Animals used for various ISHH and immunocytochemical analyses were anesthetized with 87 mg/kg ketamine (Sigma, St Louis, MO) and 10 mg/kg xylazine (Sigma) and were sacrificed by transcardiac perfusion between 1530 and 1700 hr on Day 2. A short initial flush with 0.1 M PBS, pH 7.4, was followed by fixation with 400 ml of a freshly made 4% paraformaldehyde solution in PBS. After perfusion, brains were removed and small tissue blocks were dissected out. For post-fixation, the tissue blocks were immersed in phosphate-buffered 4% paraformaldehyde solution for 1 hr. Infiltration of the tissue pieces with gradually increasing sucrose concentrations through 30% was performed in diethyl pyrocarbonate (DEPC, 0.1%; Sigma)-treated, and subsequently autoclaved PBS solutions containing molecular biology grade sucrose (Fisher Scientific, Pittsburgh, PA) and sodium azide (0.1% Fisher Scientific) preservative. After the hypothalami were soaked, they were snap-frozen on dry ice and 16-μm thick coronal sections were cut on a cryostat (Reichart-Jung; Nussloch, Germany) from the region of the organum vasculosum of the lamina terminalis (OVLT) through the rostral medial preoptic area (MPOA; A7.4:0-A6.8:0) (11), where LHRH neurons were previously co-localized with Fos (5,13,14) and galanin (2,19,20) immunoreactivities. Sections were thaw-mounted onto a sterile glass hook and transferred immediately into pre-chilled cryoprotectant solution for longer storage at −20°C as described previously (17). The cryoprotectant was made by dissolving 300 g molecular biological grade sucrose (Sigma) in 500 ml of 0.1 M DEPC-treated PBS, then adding 300 ml ethylene glycol (Fisher Scientific) and bringing the volume of the solution to 1 liter with DEPC-treated water.

LH Radioimmunoassay

Serum samples were assayed in quadruplicate for LH by methods described previously (23). All samples were analyzed in the same assay. NIAMDD rat LH RP-2 was used as the reference material and iodinated rat LH (Hazelton Laboratories; Vienna, VA) as competitor. The CSU-120 antibody (provided by Dr. Gordon Niswender, Ft Collins, CO) was used at a final concentration of 1:120,000. In this assay, the least detectable amount of LH was 0.04 ng (the lowest amount of LH that significantly displaced iodinated LH from the antibody) and the intra-assay coefficient of variation was 5.6%.

Triple-labeling Procedure

The main steps of the triple-labeling procedure used are shown in Figure 1 and presented in detail below. To prevent degradation of tissue mRNAs, all solutions (except for the fixative and the cryoprotectant) used for incubations before ISHH were treated with DEPC and autoclaved. All metal and glassware was baked at 180°C overnight before use.

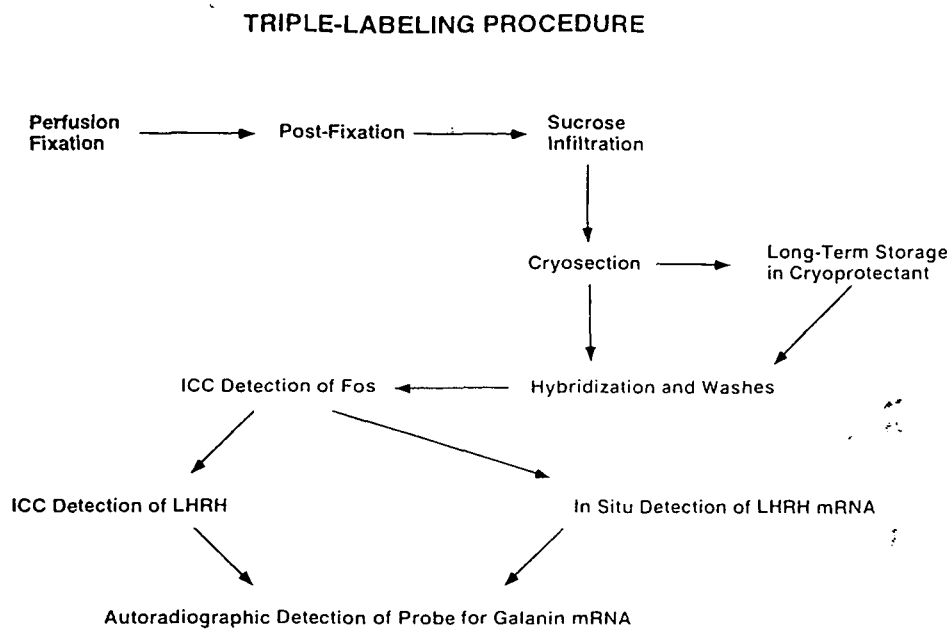
In Situ Hybridization Histochemistry

Preparation of Anti-sense RNA Probes. Anti-sense cRNA probes labeled with digoxigenin or ^{35}S were prepared by in vitro transcription as described previously (24). Briefly, a 330 BP BamHI–HindIII fragment of the rat LHRH cDNA, corresponding to exons I–IV of LHRH mRNA, was subcloned into a modified pSP65 plasmid containing a T7 promoter (1) (provided by Dr. John Adelman, Vollum Institute, Portland, OR). The plasmid was linearized with HindIII and transcribed in the presence of digoxigenin-11-UTP (Boehringer Mannheim). The template for in vitro synthesis of anti-sense pre-pro-galanin probe was prepared by cloning a 678 BP fragment of the rat pre-pro-galanin cDNA (35) into the EcoRI site of the Bluescript M13+ vector. The vector was linearized with HindIII and transcribed in the presence of [^{35}S]-UTP. The specificity of this probe was confirmed by demonstrating that the cRNA probe for galanin mRNA used in this study labels the same cells on adjacent sections as a previously described 44 base oligodeoxynucleic acid probe synthesized to detect galanin mRNA (complementary to bases 228–271 of rat galanin cDNA) (36). In addition, we found good correlation between the neuroanatomic localization of galanin peptide as described in previous studies (19,20) and the localization of galanin mRNA detected in the present study.

Preparation of Oligodeoxynucleotide Probe to Galanin. As an alternative to the cRNA probe described above, a synthetic 44 base oligodeoxynucleotide complementary to bases 228–271 of the rat pre-pro-galanin cDNA (36) was used to detect galanin mRNA. The probe was 3'-end-tailed with [^{35}S]-dATP (New England Nuclear; Boston, MA) using terminal deoxynucleotidyl transferase (Gibco BRL; Gaithersburg, MD) as described previously (23). In triple-labeling studies, detection of galanin mRNA with this probe was combined with ICC detection of Fos and LHRH.

Pre-incubation, Hybridization, and Washes. For pre-incubation, sections were removed from cryoprotectant solution by straining through sterile stainless steel fine-meshed sieves. The sieves were then placed in glass Pyrex custard dishes containing 4% paraformaldehyde–0.1 M PBS solution for 5 min. After this post-fixation, the sieves were lifted and the fixative was replaced with 2 × standard saline citrate solution (2 × SSC. 1 × SSC = 0.15 M NaCl–0.015 M sodium citrate, pH 7.0; Sigma) for a short (2-min) rinse. Tissues were acetylated with 0.25% acetic anhydride (Fisher Scientific) in 0.1 M triethanolamine–0.9% NaCl (pH 8.0; Sigma) for 10 min, followed by a 2-min rinse in 2 × SSC. Then the sections were incubated in 50% formamide–4 × SSC containing 0.1% sodium dodecyl sulfate (SDS, Boehringer Mannheim) and 25 mM dithiothreitol (DTT; Sigma) at 52°C for 30 min and stored in this solution until hybridized. Hybridization was performed in sterile 250-μl microcentrifuge tubes containing the hybridization buffer [50% formamide, 4 × SSC, 10% dextran sulfate (500,000 MW; Sigma), 1 × Denhardt's solution, 500 μg/ml heparin sodium salt

gure 1. Flow chart of the procedure used r triple labeling.



gma), 0.5 mg/ml yeast tRNA (Boehringer Mannheim), and 0.4 mg/ml ared single-stranded salmon sperm DNA (Boehringer Mannheim)], 0.1% S, 0.1 M DTT, and both the digoxigenin-labeled LHRH (1:25 final dilun) and ^{35}S-labeled galanin probes (20,000 cpm/µl). The sections were nsferred from the sieves into the tubes with a sterile, bent syringe nee:. The tubes were filled to the edge with the hybridization solution, sealed th Parafilm to exclude air, and incubated at 52°C overnight. Alternaely, if the 44 base oligodeoxynucleotide probe was used to detect galanin NA, hybridization was performed in the same buffer but at 37°C overght.

After hybridization, sections were transferred into glass cylinders covd at one end with fine plastic meshwork for easier handling. The cylinders re placed in plastic tea strainers and post-hybridization treatments were rformed by placing strainers in Pyrex custard dishes containing solutions. ese treatments for tissues hybridized with the cRNA probes included: ee 5-min rinses in 1 × SSC at room temperature (RT), a stringent wash 50% formamide–2 × SSC at 52°C for 30 min, a 30-min digestion of ess probe with RNAse A (Boehringer Mannheim) [50 µg/ml dissolved 10 mM Tris, 0.5 M NaCl, 1.0 mM ethylene diamine tetraacetate dihyte (EDTA, pH 8.0; Sigma)] at 37°C, followed by three sequential 30n stringent washes in 50% formamide–2 × SSC at 52°C. Finally, secns were rinsed in three changes of 1 × SSC for 10 min each on an orbital ıker at RT and then transferred into PBS.

Post-hybridization treatments of sections hybridized with the oligodeoxycleotide probe consisted of four 30-min stringent washes in 50% formade–2 × SSC at 40°C and sequential rinses in 1 × SSC and PBS solutions.

Sequential Detection of Fos Immunoreactivity, LHRH mRNA or Imnoreactivity, and Galanin mRNA

Pre-treatment. Hybridized sections were treated with 3% H_2O_2 in thanol for 10 min to eliminate endogenous peroxidase (POD) activity, n washed twice for 5 min in PBS and blocked against nonspecific antibodyding with 2% bovine serum albumin (BSA, Fraction V heat shock; :hringer Mannheim) in PBS for 10 min. The primary and secondary anodies were both diluted with 2% BSA and 0.1% sodium azide in PBS.

Detection of Fos Immunoreactivity. For ICC detection of Fos, the sections were incubated in rabbit anti-Fos primary antibody (Oncogen Ab-2, 1:5000; Uniondale, NY) for 48 hr at 4°C, then in biotinylated anti-rabbit secondary antibody (Jackson, 1:800; West Grove, PA) for 1 hr at RT. Next, the antigen–antibody complexes were reacted with the ABC-Elite reagent (Vector; Burlingame, CA) for 1 hr and POD-containing sites were visualized with a developer consisting of 0.014% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma), 0.4% nickel ammonium sulfate hexahydrate (Sigma), 0.01% H_2O_2, and 0.23% sodium acetate trihydrate in 0.05 M Tris buffer solution (pH 8.0). Sections used for multiple labelings were treated with 3% H_2O_2 in methanol for 10 min to destroy POD activity that might interfere with the subsequent ICC detection system. Optionally, Fos immunoreactivity was detected with the alkaline phosphatase (AP)-based ABC detection system (ABC-AP; Vector). In this case, sections were first rinsed in Buffer A (100 mM Tris-HCl, pH 7.5, 100 mM NaCl) three times for 10 min, then in Buffer B (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 min. The developer was freshly prepared by the addition of 45 µl 4-nitroblue tetrazolium chloride solution (NBT; Boehringer Mannheim), 35 µl 5-bromo-4-chloro-3-indolyl-phosphate solution (BCIP, X-phosphate; Boehringer Mannheim), and 2.4 mg levamisole (Sigma) in 10 ml Buffer B. For color development, sections were incubated in this chromogen solution at 4°C in the dark for several hours.

Detection of LHRH Neurons. LHRH neurons were detected using either nonisotopic ISHH or ICCH

To detect digoxigenin-labeled cRNA probes hybridized to LHRH mRNA, sections were incubated with either sheep anti-digoxigenin antibody conjugated to horseradish POD (Antidig-POD, Fab fragments, 1:200; Boehringer Mannheim) or sheep anti-digoxigenin antibody conjugated to AP (Antidig-AP, Fab fragments, 1:1000; Boehringer Mannheim). The color reaction for demonstration of the POD-conjugated antibody was developed in Buffer A containing 0.02% DAB and 0.005% H_2O_2, whereas the BCIP-NBT chromogen described above was used for visualization of the AP reaction.

For visualization of LHRH immunoreactivity, either a rabbit polyclonal antiserum (LR-1, 1:100,000; kindly donated by Dr. R.A. Benoit, Montreal, Quebec, Canada) or a mouse monoclonal antibody (33) (HU4H, 1:1000; generously provided by Dr. H.F. Urbanski, Beaverton, OR) was used for

ICC detection of LHRH by the biotinylated secondary antibody-ABC-POD system. LHRH-immunoreactive (IR) sites were visualized with the brown DAB chromogen.

Detection of ^{35}S-labeled cRNA Probe or Oligodeoxynucleotide Probe Hybridized to Galanin mRNA. After detection of LHRH mRNA or protein, sections were copiously washed in PBS and placed in 50% ethanol containing 300 mM ammonium acetate for 5 min. This solution was replaced with 70% ethanol–300 mM ammonium acetate and the sections were mounted on gelatin-coated glass slides with a fine-bristled paintbrush and air-dried. Some mounted sections were further dehydrated through 100% ethanol, air-dried, then dipped into 1% Parlodion (Sigma) in acetone (39). After a short drying period, sections were coated two more times with Parlodion solution in the same way. Then, both Parlodion-coated and untreated slides were dipped into Kodak photographic emulsion (NTB-3; Kodak, Rochester, NY) and exposed for 3–5 days at 4°C. Autoradiographs were developed with Kodak Dektol developer and fixed with Kodak Fixer. Finally, sections were dehydrated in increasing ethanols, transferred into xylenes, and immediately coverslipped with Permount. BCIP-NBT-stained sections were coverslipped with Crystal/Mount (Fisher Scientific), an aqueous mounting medium.

Quantitation by Cell Counting. Sections from 10 animals sacrificed between 1530 and 1700 hr were analyzed to estimate the degree of overlap between the Fos and galanin mRNA-containing LHRH neuronal subpopulations. A total of 674 LHRH neurons in the OVLT and MPOA regions were included in this study.

Results

We verified by radioimmunoassay that the estrogen and progesterone treatment of ovariectomized rats we used reliably induced LH surge release, which peaked between 1500 and 1700 hr (Figure 2). This steroid treatment also induced Fos expression in a subset of LHRH neurons during the surge.

The methods described above resulted in well-preserved ICC and ISHH signals after triple-labeling procedures. The regions of the OVLT and the MPOA of female rats contained many Fos-IR nuclei, which were visualized with the purple-black BCIP-NBT chromogen (AP-based detection system; Figure 3A) or the dark blue–black nickel-DAB chromogen (POD reaction product; Figures 3B–3E). The subsequent detection of LHRH neurons with the DAB chromogen using either ICC (Figures 3A–3D) or nonisotopic ISHH (Figure 3E) revealed many LHRH neurons in the OVLT–MPOA region that coexpressed Fos immunoreactivity in their nuclei. LHRH neurons without Fos immunoreactivity were also present in the same areas (Figure 3C). LHRH neurons with either irregular profiles (Figure 3B) or a characteristic fusiform shape (Figure 3C) were co-localized with Fos. Although detection of digoxigenin-labeled LHRH probe with the AP-enzyme reaction yielded comparable staining intensity to that provided by LHRH ICC, the BCIP–NBT chromogen gave insufficient contrast with nickel–DAB, and therefore this combination was not optimal for double labeling of Fos- and LHRH-containing structures (data not shown). Both the mouse monoclonal (Figures 3A–3D and 3I–3K) and the rabbit polyclonal anti-LHRH antibodies resulted in excellent immunostaining for LHRH-containing perikarya. With the double ICC approach, LHRH-IR axons were often seen in juxtaposition to LHRH-IR neurons (Figure 3C), as well as Fos-IR, LHRH-negative preoptic neurons (Figure 3D).

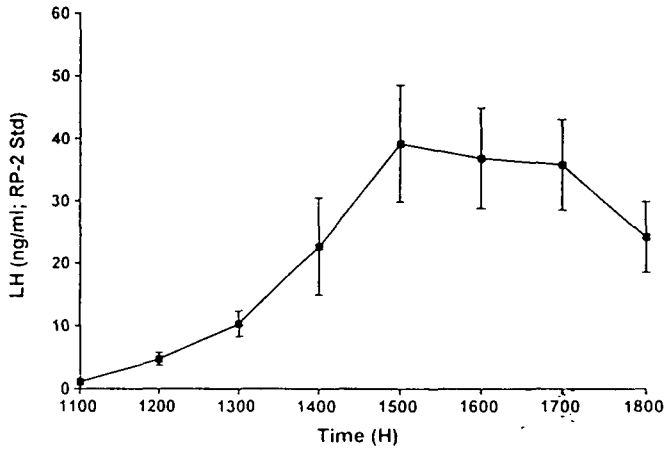


Figure 2. Time course of the estrogen- and progesterone-induced surge of LH release in 1-week ovariectomized rats (see Materials and Methods). Values represent the mean serum LH concentrations (± SEM) of serial blood samples obtained hourly via jugular cannulae from six animals/group.

As observed earlier on slides that were processed for autoradiography without Parlodion coating, the presence of BCIP-NBT immunostaining in tissue sections resulted in nonspecific chemographic artifact (24,39). In the present study, we found that Ni–DAB was also capable of producing high levels of nonspecific autoradiographic background (Figures 3F–3H). Moreover, treatment of unprotected sections with the photographic developer and fixer resulted in severe losses of the Ni–DAB signal from Fos-IR nuclei (Figures 3G and 3H). Although some triple-labeled neurons could still be recognized, the color contrast between nuclear Ni–DAB and cytoplasmic DAB was greatly diminished (Figure 3H). In contrast, coating of slides with several layers of Parlodion, which was introduced earlier against chemographic artifacts on BCIP-NBT-stained material (38), fully prevented decoloration of the Ni–DAB precipitate and also eliminated emulsion fogging. In this way, single-, double-, and triple-labeled LHRH neurons were detected in the regions of the MPOA (Figure 3I) and OVLT (Figure 3J). The oligodeoxynucleotide probe to galanin was also successfully used in triple-labeling studies after Parlodion coating of the sections (Figure 3K).

It is noteworthy that several subpopulations of galanin mRNA-containing, LHRH-immunonegative neurons also expressed Fos immunoreactivity. Areas containing such galaninergic neurons included the sexually dimorphic anteroventral periventricular nucleus (AVPV) and the anteroventral preoptic nucleus (AVPO; Figure 3K). In the latter area, Fos immunoreactivity was generally faint, but a high proportion of Fos-IR neurons expressed mRNA for galanin.

Quantitative analysis of LHRH neurons identified 66.2 ± 2.5% of LHRH neurons that contained IR Fos and 65.2 ± 2.9% of LHRH neurons that coexpressed galanin mRNA in the OVLT and MPOA regions. Only 18.1 ± 1.9% of LHRH neurons did not contain either of these substances. Almost half (49.5 ± 2.8%) of LHRH neurons were triple labeled. This represents 76.8 ± 2.7% of the total of Fos-containing LHRH neurons and 73.6 ± 3.2% of galanin mRNA-coexpressing LHRH neurons in the analyzed areas.

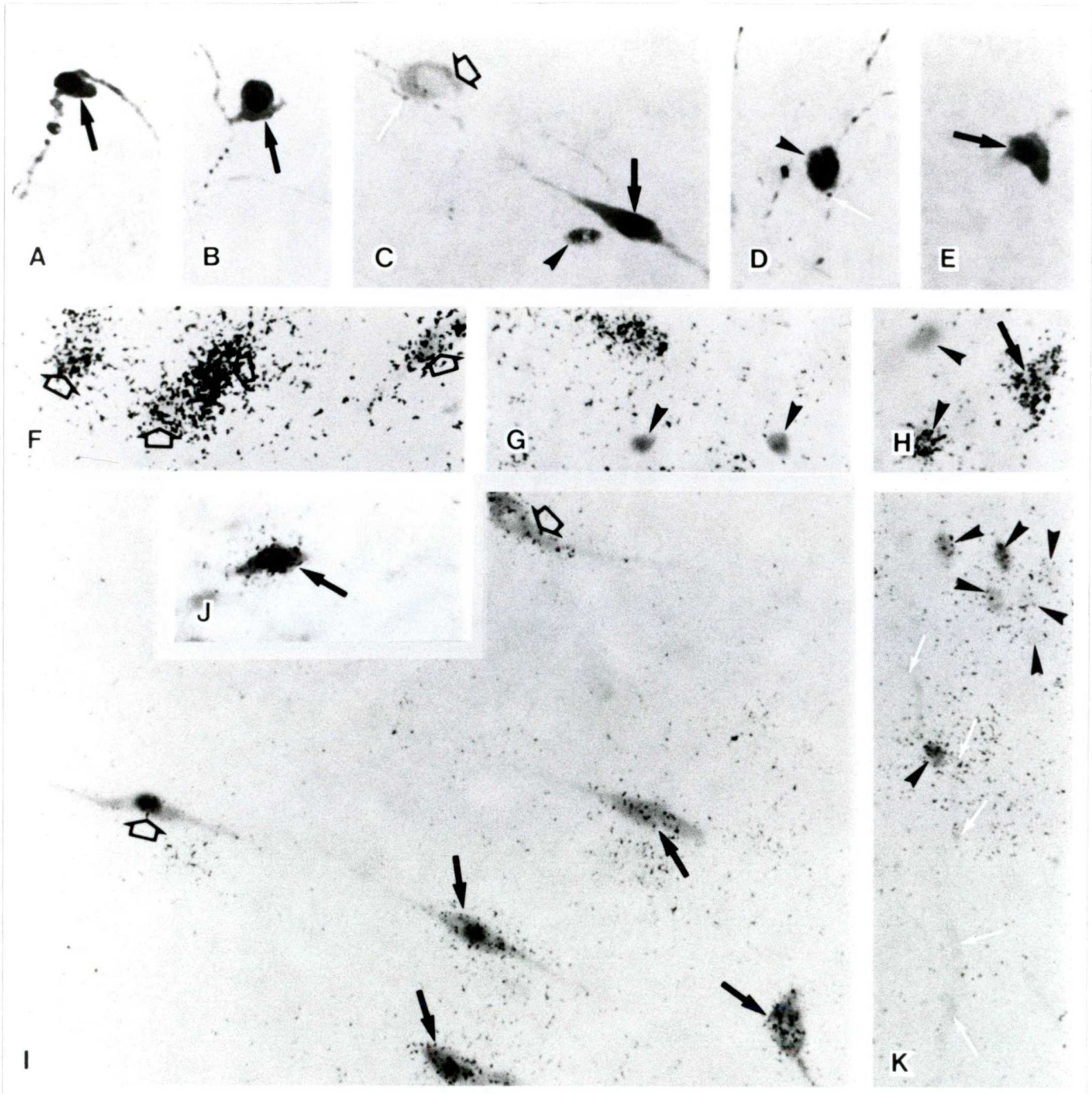


Figure 3. Combined ICCH and ISH for simultaneous detection of Fos immunoreactivity, LHRH immunoreactivity or mRNA, and galanin mRNA within the same tissue sections of the OVLT–POA region during the LH surge of the female rat. (**A–D**) Double-label immunocytochemical detection of FOS- and LHRH-expressing neuronal structures. (**E**) Immunocytochemical detection of Fos combined with non-isotopic ISHH detection of LHRH mRNA by the anti-dig–POD–DAB method. Black arrows in **A–C** and **E** indicate double-stained neurons. Fos immunoreactivity (dark chromogen in the cell nuclei) was visualized by the ABC–AP method and BCIP-NBT chromogen (**A**) or, optionally, the ABC–POD–Ni–DAB approach (**B–E**), whereas LHRH neurons were immunostained with the brown DAB–POD reaction product. Most LHRH neurons were bipolar and exhibited either irregular (arrow in **B**) or typical fusiform morphology (black arrow in **C**). In addition to double-labeled LHRH neurons in the region of the OVLT and MPOA, some immunostained neurons only contained LHRH (open arrow in **C**) or Fos immunoreactivity (arrowheads in **C** and **D**). LHRH-IR axons (white arrows in **C** and **D**) were often juxtaposed to LHRH (black arrows in **C**)- or Fos (arrowhead in **D**)-IR neurons. (**F–H**) Demonstration of chemographic artifacts on Ni–DAB-stained sections processed for galanin autoradiography without previous Parlodion-coating. LHRH mRNA was detected with the anti-dig–POD–DAB method. During the visualization of galanin mRNA (grain clusters shown by open arrows in **F**) in LHRH neurons by the use of the ^{35}S-labeled cRNA probe, high background (scattered grains in **F–H**) also occurred. The original blue–black color of Ni–DAB in Fos-IR nuclei (arrowheads in **G** and **H**) faded and turned brown, thus providing very poor color contrast with cytoplasmic DAB stain in triple-labeled neurons (arrow in **H**). (**I,J**) Parlodion-coated slides had low levels of autoradiographic background and well-preserved Ni–DAB immunostain. In this way, LHRH neurons could be classified to the triple (black arrows in **I** and **J**)-, double (open arrows in **I**)-, and single (not shown)-labeled categories in the MPOA (**I**) and in the vicinity of the OVLT (**J**). (**K**) Although triple labeling could also be performed with ^{35}S-labeled oligodeoxynucleotide probe to galanin, longer autoradiographic exposure time was necessary. Interestingly, a large population of galanin neurons in the ventrolateral part of the MPOA contained faint immunostaining for Fos in their nuclei (arrowheads). White arrows show the course of an LHRH-IR axon approaching a Fos-IR, galanin-expressing preoptic neuron (arrowhead on the left). Original magnifications: **A,C,E,F,H–K** × 550; **B,G** × 460; **D** × 940. Bars: **A,B** = 10 μm; **D** = 5 μm.

Discussion

We describe here a sensitive triple-labeling technique for characterizing the receptor or peptide coexpression in IEG-IR subpopulations of chemically identified neurotransmitter/neuropeptide systems. This triple-labeling technique will be particularly valuable for analysis of the receptor complement of functionally heterogeneous neuronal systems.

In the present application of the triple labeling, we determined that 76.8% of the LHRH neurons that express Fos immunoreactivity during periods of LH hypersecretion also express mRNA encoding galanin. Furthermore, 73.6% of the galanin mRNA-containing LHRH neurons also express Fos. Therefore, a high proportion, but not all, of the LHRH neurons that appear to be activated also express mRNA encoding galanin. It is possible that we underestimated the number of triple-labeled cells by underestimating either the number of LHRH neurons that express Fos or those that express galanin. However, the percentage of Fos-positive LHRH neurons we detected was approximately 15% greater than reported previously in estrogen- and progesterone-treated rats during peak LH release and maximal Fos expression (14). For this reason, we believe that it is unlikely that we are underestimating the number of Fos-positive neurons. The percentage of LHRH neurons we detected which express galanin during the LH surge (65.2%) is also in agreement with values previously reported (19) for female rats. Therefore, we conclude that although a majority of LHRH neurons that appear to be active during LH surge release contain galanin, there is not a complete overlap in these subpopulations.

In the development of our triple-labeling method, we addressed several technical concerns. First, to successfully combine ICC and ISHH, tissues must be fixed sufficiently to retain proteins of interest, but not so heavily fixed that the mRNA is not readily accessible to probes. We found, in agreement with previous studies (31,32), that perfusion-fixation with 4% paraformaldehyde in PBS satisfies these criteria. Penetration of the probe did not pose a problem, even when we used a cRNA probe that was approximately seven or eight times longer than synthetic deoxynucleotidyl probes used in previous studies of perfused tissues (31,32).

Our second concern was that the method must be sensitive enough to detect mRNAs of low abundance. Therefore, we chose to use cRNA probes rather than oligomeric DNA probes as described previously (32) because the former can be labeled to higher specific activities and therefore can be used to detect even low-abundance mRNAs (25). This capability of cRNA probes is particularly advantageous for examining coexpression of mRNAs encoding specific receptors that may not be abundant in individual neurons. For example, in a previous study we used dual-label ISHH with cRNA probes to demonstrate that approximately 75% of LHRH neurons in the region of the OVLT–rMPOA express mRNA encoding the β_3-receptor subunit of the $GABA_A$ receptor (25). By using triple labeling we can now determine whether these neurons comprise the same population that expresses Fos and/or galanin during the LH surge.

A third concern in the development of this method was the storage of tissue sections before hybridization. We chose to store tissue sections in cryoprotectant solution as described previously (17). Our work confirmed that of previous studies demonstrating that long-term storage of brain sections in cryoprotectant solution preserves antigenicity of proteins, tissue morphology (37), and mRNAs (17). In our application, the main advantage of cryoprotectant storage is that we could process floating sections from a relatively large number of treated and control animals simultaneously within the same hybridization. This is an important requirement for studies in which changes in mRNA induced by specific treatments are measured by quantitative autoradiography.

In developing our method, a final concern was that conditions of hybridization and post-hybridization treatments might damage the antigenicity of proteins. However, we found that incubation of sections at temperatures as high as 55°C did not seem to interfere with subsequent ICC detection of Fos and LHRH. Although we did not specifically investigate the reason for the thermostability of antigenic sites, it seems possible that it was attributable to efficient cross-linking of proteins by the fixative.

For any specific application of our method, either the combination of dual-label ICC and isotopic ISHH or the combination of dual-label ISHH and ICC can be used. In the present studies we found the former combination preferable, because our antibodies provided higher staining intensity in labeling LHRH neurons than did the non-isotopic ISHH detection of digoxigenin-labeled probes for LHRH mRNA. We tested several chromogen combinations for double labeling of Fos- and LHRH-containing neurons. The best contrast was obtained when LHRH neurons were visualized with either ICC or non-isotopic ISHH using the brown DAB–peroxidase reaction product. Non-isotopic ISHH detection of LHRH mRNA with the AP–BCIP-NBT method also provided high sensitivity and dark labeling of LHRH neurons; however, the contrast between the chromogen reaction product and silver grains was not sufficient for reliable computer-assisted quantitation. Fos immunoreactivity could be detected at high sensitivity with either BCIP-NBT (ABC–AP technique) or Ni–DAB (ABC–POD method) chromogens. Unfortunately, both endproducts left chemographic artifacts when slides were dipped in Kodak NTB3 nuclear track emulsion without first coating them with Parlodion. In addition, solubilization and decoloration of Ni–DAB-stained structures by the photographic developer and fixer occurred on the sections not protected with Parlodion. Therefore, use of the Parlodion treatment before autoradiography was required in both instances. As an alternative to Parlodion coating, chemography can be eliminated by using Ilford K-5 instead of the Kodak NTB3 nuclear track emulsion to visualize autoradiographic signals in BCIP-NBT-labeled sections (39). This approach, however, does not prevent solubilization of the Ni–DAB chromogen during the photographic development process.

On the basis of the results of these studies, we believe that this method will be important in determining the receptor complement of LHRH and other neurons. The use of isotopic ISHH to detect mRNA encoding the receptor protein rather than ICC to detect the protein itself has several advantages. First, probes for cloned receptors are readily available. Second, even in receptor families in which there is a high percentage of conserved sequences among subtypes, very specific probes can be made to distinguish between mRNAs encoding structurally similar proteins, whereas raising specific antibodies might be difficult. Third, mRNAs usually reside in the perikaryon and proximal dendrites of neurons,

whereas receptor protein is often found in terminal regions of complex neuropil (3). The proximal distribution of mRNAs makes it easier to verify co-localization. A final advantage is that the autoradiographic signal for regulatory peptide or receptor mRNA can be quantitated using a computer-assisted image analysis system.

In conclusion, we developed a sensitive triple-labeling technique to study the receptor or regulatory peptide complement in IEG-IR subpopulations of functionally heterogenous neuronal systems. Using this technique, we established a significant overlap between two subsets of LHRH neurons that differentially express Fos-IR galanin mRNA during the estrogen- and progesterone-induced LH surge. With this sensitive approach, it will now be possible to determine the receptor and/or regulatory peptide complement of subpopulations of LHRH neurons that express Fos protein after various physiological manipulations. We believe that this method will be a valuable tool for identifying the complex afferent neurocircuitry by which estrogen and progesterone regulate LHRH neuronal activity. Furthermore, this technique will be suitable for similar applications in studies of a variety of other functionally heterogeneous neuronal systems.

Acknowledgments

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Effects of Continuous and Repetitive Administration of a Potent Analog of GH-RH(1-30)-NH2 on the GH Release in Rats Treated with Monosodium Glutamate

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Key words: growth hormone-releasing hormone analog, MSG-lesion, hypothalamic GH-RH deficiency, continuous administration, repetitive administration.

Abstract

To assess the efficacy of a potent GH-releasing hormone (GH-RH) analog (D-Ala^2,Nle^{27},Gaba^{30}-GH-RH-(1–30)-amide) in the treatment of GH deficiency, we investigated the effects of chronic administration of this analog (A-495) on growth responses in monosodium glutamate (MSG)-lesioned rats. Basal serum GH concentrations, GH responses to bolus injections of GH-RH, as well as acceleration of body gain and linear growth were compared after long-term continuous and repetitive administration of A-495. The effects of continuous and repetitive administration of the analog on GH responses *in vitro* were also compared using the superfused pituitary cell system method. Treatment with MSG reduced the body weight and linear growth of the animals (−22% and −11%, respectively), the basal serum GH concentration (−66%), and the GH-RH-induced absolute GH responses (−61%) but did not alter the relative GH responses (to basal GH concentrations). Repetitive administration of 10 μg daily doses of A-495 at 24 h intervals for 2 weeks highly increased the GH responsiveness to GH-RH and induced catch-up growth, by which MSG-treated animals achieved the growth rate of normal controls. However, basal serum GH concentrations were only modestly enhanced. Continuous infusion of A-495 at the same daily dose resulted in slight increases in the GH-RH-induced GH rises, moderate acceleration of body gain, and no change in linear growth. Basal serum GH concentrations were not significantly influenced by this treatment. These results demonstrate that exogenous GH-RH pulses administered at lower frequency than the frequency of the physiological GH secretion are able to fully restore the normal growth rate of the GH deficient rats. The effectivity of the treatment is rather dependent on the magnitude of GH rises than the basal GH level. Although continuous administrations of the GH-RH is also have some effect on the body gain, repetitive administration is more effective at the same daily dose. Our results from *in vitro* experiments show that, in addition to the low magnitude of the GH-RH-stimulated GH rises, desensitization of the GH secretory response might also be accounted for the low effectivity of the continuously administered GH-RH. Present results demonstrate the therapeutic usefulness of our new GH-RH analog and are the first to evidence that GH-RH need not be administered as frequently as the appearance of the endogenous GH pulses to restore the normal growth of the GH deficient rats.

It is well established that parenteral administration of MSG or related excitatory amino acids to neonatal rats induces local brain lesions, most prominently in the arcuate nucleus of the hypothalamus (1, 2). Because MSG destroys most of the GH-RH-producing neurons (3, 4). MSG-treated rats have been used as a model to study GH-RH deficiency (5, 6, 7). Similarly to the patients having GH deficiency due to hypothalamic dysfunction (8), significant reductions in the pulse amplitude and frequency with markedly reduced baseline GH concentrations have been observed in this experimental model (9). Since GH deficiency is caused by hypothalamic dysfunction in half or more of the patients (8), GH-RH and its potent analogs have a great importance both in the therapy and the etiological diagnosis of the pituitary dwarfism. In children with GH deficiency both the prolonged repetitive (10, 11) and the continuous administrations of GH-RH (12) or GH-RH (1–29)-amide (13) accelerated linear growth. In contrast to human, in GH-deficient rats repetitive pulses of the GH-RH(1–29)-amide given at 3 h intervals accelerated growth, whereas continuous infusions of this GH-RH fragment at the same daily dose were ineffective (6).

In the present study we used the MSG-lesioned animal model to answer the following questions: (1) How does the sensitivity of the GH secretory response to GH-RH change after MSG-lesion in rats? (2) Is the chronic treatment with a potent GH-RH analog able to restore the normal growth rate of these animals? (3) What is the difference between the effects of continuous and repetitive administration of GH-RH on the hypothalamic GH-RH deficiency syndrome? To answer these questions, a newly

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developed GH-RH analog (14, 15) was used in our *in vivo* and *in vitro* experiments.

Results

1. Results from in vivo *experiments*

1.1. Effects of MSG treatment on the GH-RH immunoreactivity of the hypothalamus

In control rats, numerous immunoreactive cells were detected in the arcuate nucleus and the ventral and dorsolateral hypothalamus. GH-RH immunoreactivity was constantly found in dense bundles of fibers in the median eminence (ME), terminating in contact with portal vessels. In rats neonatally treated with MSG, no GH-RH immunoreactive cell bodies could be observed in the arcuate nucleus, they were only found in the dorsolateral and ventral hypothalamus. The ME showed very few GH-RH immunoreactive fibers. (Fig. 1).

1.2 Effects of pulsatile and continuous administration of the GH-RH analog on growth rates, GH-RH-induced GH responses, and basal serum GH concentrations in MSG-treated rats

As expected, treatment with MSG significantly reduced body weights of the animals by 4 weeks of age, and the reduction became more accentuated at 8 weeks of age (-22%, $P<0.01$ and -34%, $P<0.01$, respectively *vs* normal controls). Repetitive administration of the GH-RH analog by daily injections for two weeks caused 16% increase in body weights of the MSG-treated animals by the end of the treatment (at 6 weeks age), and a further 21% increase was detectable at 8 weeks age ($+16\%$, $P<0.01$ and $+37\%$, $P<0.01$, respectively *vs* MSG-treated controls). By these increases the MSG-treated animals reached the body weight of the normal controls. Continuous infusion of the GH-RH analog for two weeks resulted in a moderate elevation of body weights of the MSG-treated rats ($+11\%$, $P<0.05$ and $+17\%$, $P<0.05$ at 6 and 8 weeks age, respectively *vs* MSG-treated controls), but a distinct backwardness could still be found at 8 weeks age of the animals (-23%, $P<0.01$ *vs* normal controls). (Fig. 2).

Treatment with MSG significantly reduced linear growth of the animals by 4 weeks of age, and the reduction became more accentuated at 8 weeks of age (-11%, $P<0.01$ and -22%, $P<0.01$, respectively *vs* normal controls). Daily injections of the GH-RH analog for two weeks resulted in significant acceleration of the linear growth of the MSG-treated animals ($+7\%$, $P<0.01$ and $+11\%$, $P<0.01$ at 6 and 8 weeks of age, respectively *vs* MSG-treated controls), by which the animals attained BL similar to the normal controls. However, continuous infusion of A-495

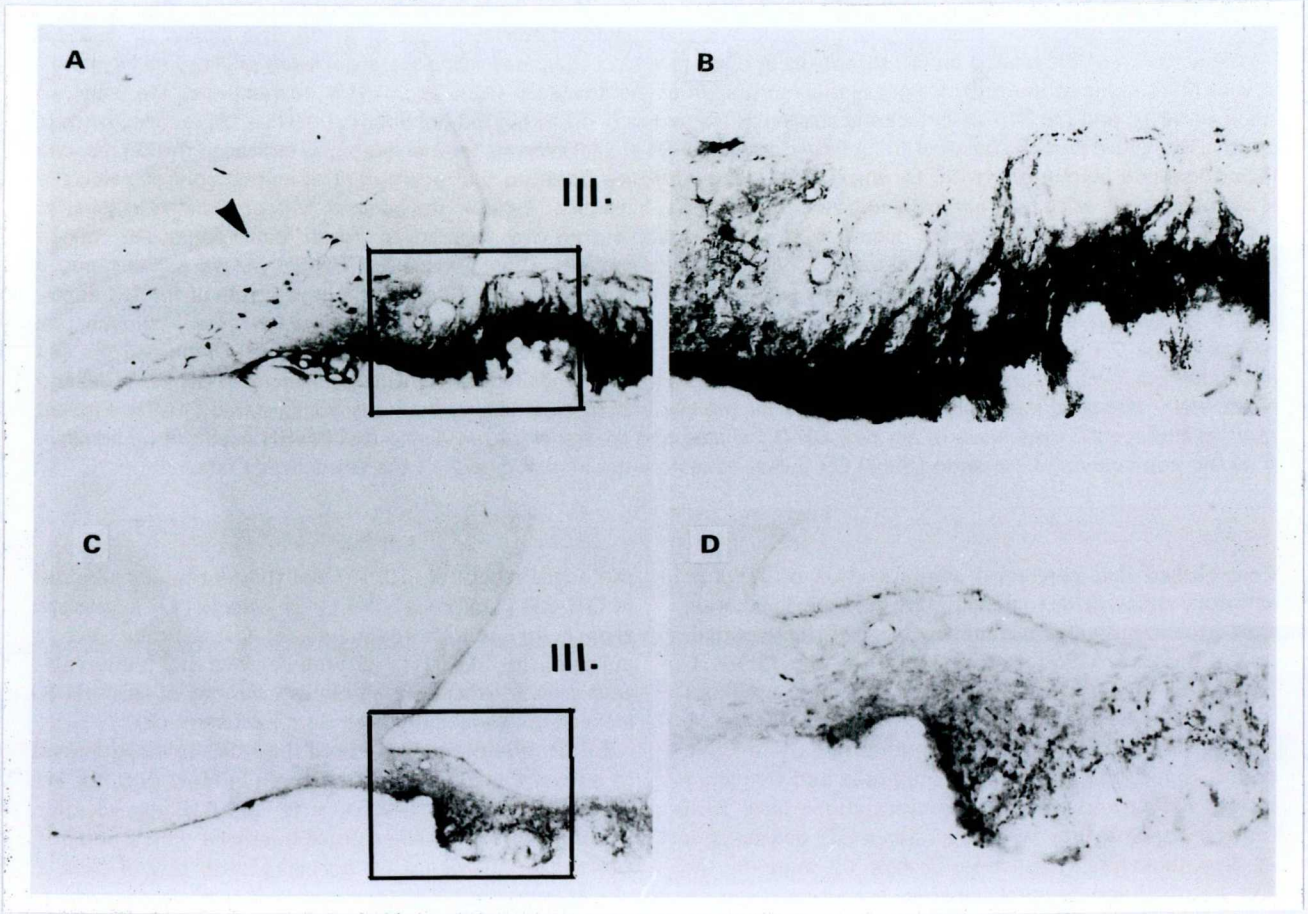


FIG. 1. Immunohistological staining of arcuate nucleus and median eminence of normal control and MSG-treated rats. A In control rats, numerous GH-RH immunoreactive cells are seen in the arcuate nucleus (arrowhead) and dense bundles of fibers in the ME, terminating in contact with portal vessels (×80). C In rats neonatally treated with MSG, no GH-RH immunoreactive cell bodies are observed in the arcuate nucleus and very few GH-RH immunoreactive fibers are present in the median eminence (×80). Micrographs in B and D correspond to the enframed region in A and C, respectively and are shown at higher magnification (×200).

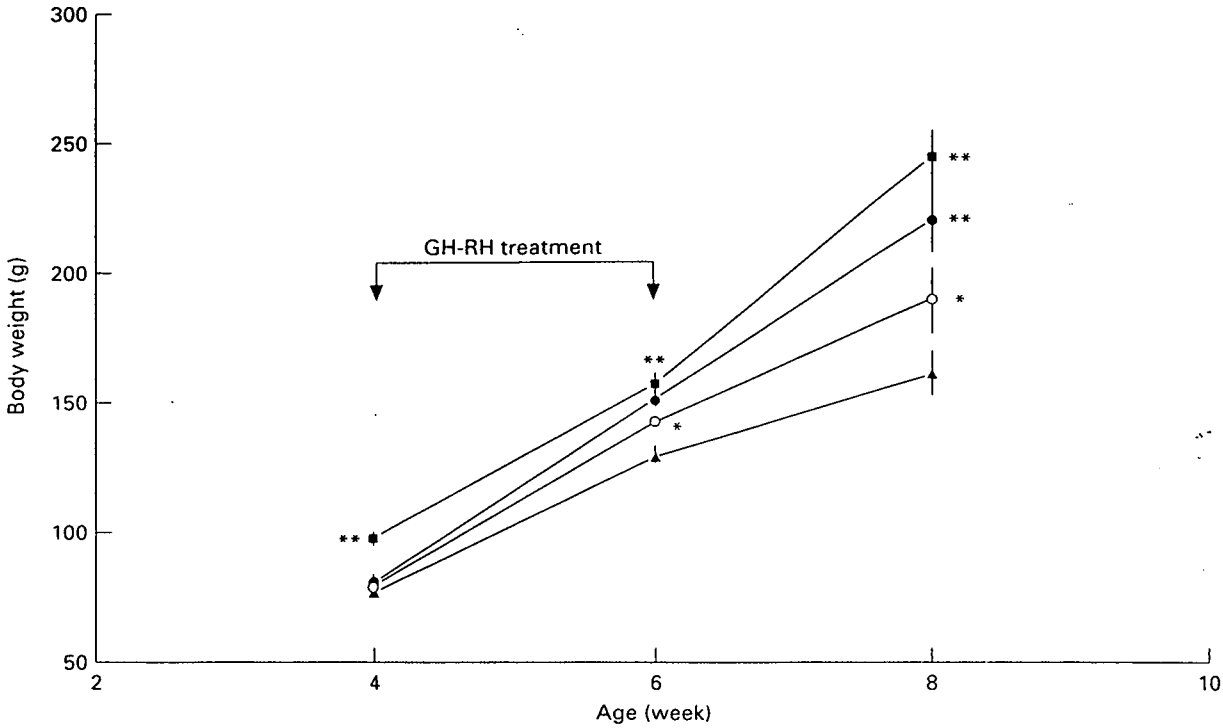


FIG. 2. Body weight of MSG-treated rats received GH-RH by repetitive (n=7) or continuous (n=7) administration of A-495 for two weeks from 4 to 6 weeks of age. MSG control (n=12) and normal control (n=15) rats received solvent. Data points represent mean ±SEM. *P<0.05, **P<0.01 *vs* MSG control rats. ■ Normal control; ▲ MSG control; ● GH-RH pulsatile; ○ GH-RH continuous.

analog for two weeks caused no significant changes in the linear growth. (Fig. 3).

Treatment with MSG markedly reduced the basal GH secretions (−66%, P<0.01 *vs* normal controls) measured at −1 min to the minipump implantation and/or to the first bolus injections of GH-RH on the day 0. Although, chronic repetitive administration of A-495 augmented the mean basal serum GH concentration (+40%, P<0.05 and +63%, P<0.05 at 6 and 8 weeks of age, respectively), it still remained extremely low, compared to normal control values (−59%, P<0.01 *vs* normal controls). Continuous infusion of A-495 did not induce significant changes in the basal serum GH levels. (Fig. 4).

MSG-treatment resulted in attenuation in the magnitude of the GH responses (absolute response) to 10 μg/kg bolus injections of the GH-RH analog (−61%, P<0.01 *vs* normal controls). However, the ratio of the absolute response values to the basal GH concentrations (relative response) was calculated to be equal in the normal and the MSG-treated groups (6–7 fold increase above baseline values). Daily injections of A-495 increased both the absolute and the relative GH responses to bolus injections of the same analog already by the 7th day of treatment (+142%, P<0.01 and +5 fold *vs* MSG treated controls), and no differences were found in the absolute GH secretory responses between the analog-treated MSG-lesioned and the normal control group of animals. Further elevations of the absolute values of GH responses were observed on the 14 day of treatment (+49%, P<0.05 *vs* response on the 7th day) but the degree of elevation was not significantly different from that observed on the 7th day (+133% *vs* +142%, respectively), because GH responses of the MSG-treated control animals also increased between the 7th and the 14th day (+55%, P<0.05).

Continuous infusion of the GH-RH analog evoked slight increases in the absolute GH responses of the MSG-lesioned rats by the 14th day of treatment (+42%, P<0.05 *vs* MSG-lesioned controls), but mean GH responses still remained significantly blunted (−27%, P<0.05 *vs* normal controls). The relative GH responses showed 2 fold decrease between the 7th and 14th day of the continuous GH-RH treatment (*vs* MSG-treated controls). Comparing the relative GH responses of the two different control groups on the 14th day of treatment, 5 fold response over baseline values was found in the normal control rats, while 11 fold elevation could be detected in the MSG-treated rats. (Fig. 5).

2. Results from in vitro *experiments*

Perfusion of 1 ng/ml GH-RH analog for 7.5 h (150 ng/150 ml) resulted in pulsatile GH secretion. The amplitude of pulses was higher at the beginning (3 times of the basal release, P<0.01) than at the end of the experiments. A gradually decreasing GH secretion could be detected during the continuous perfusion, and GH pulses decreased to the initial basal GH level by the end of the perfusion. The last GH response to the exposure of K^+ was higher (168±22%) than the first one (reference response: 100%), showing that the intracellular GH pool was rather increased than depleted. (Fig. 6A). Repetitive 3 min pulses of 30 ng/ml of the GH-RH analog in every 90 min (150 ng/5 ml during the experiment) induced high GH responses. The maximum peak concentrations rose to 9–13 times of the basal GH, P<0.01), and the amount of GH induced by the first to fifth pulses (net integrated

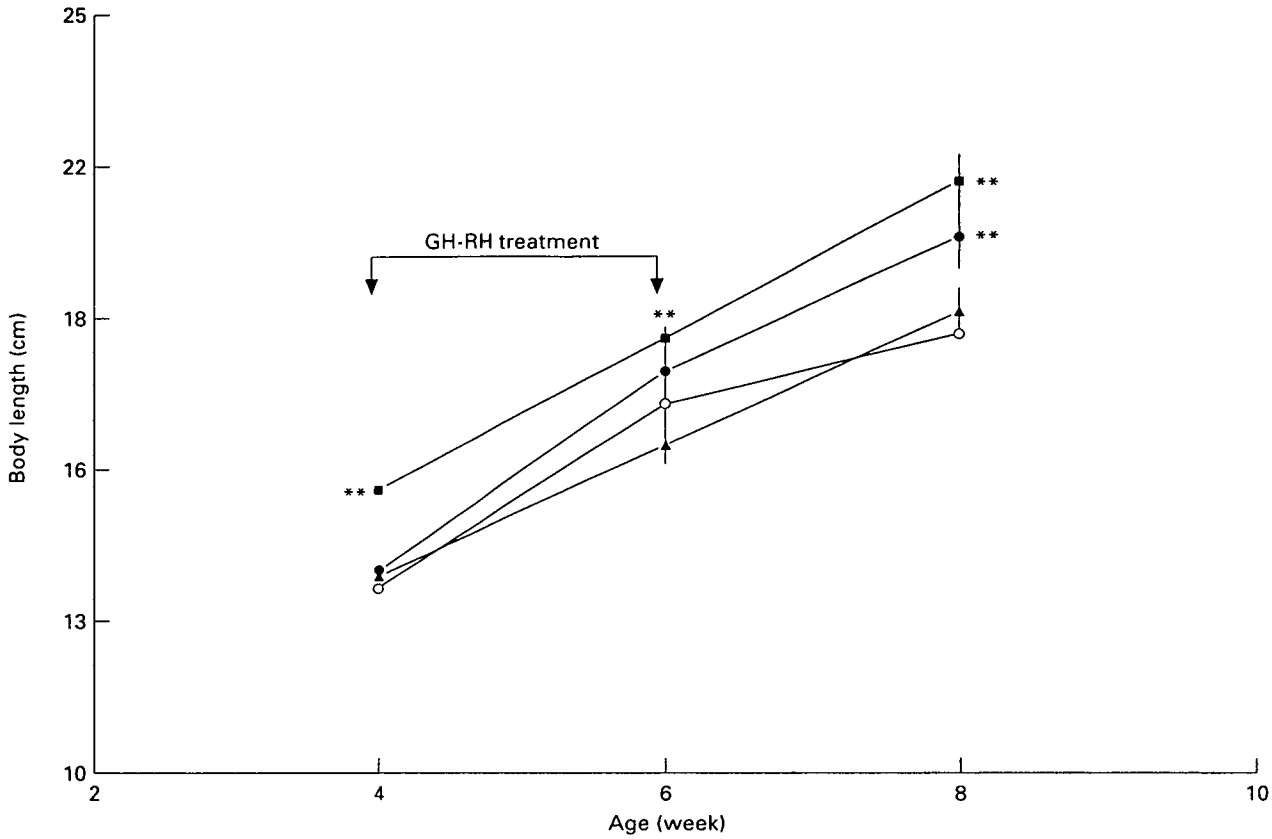
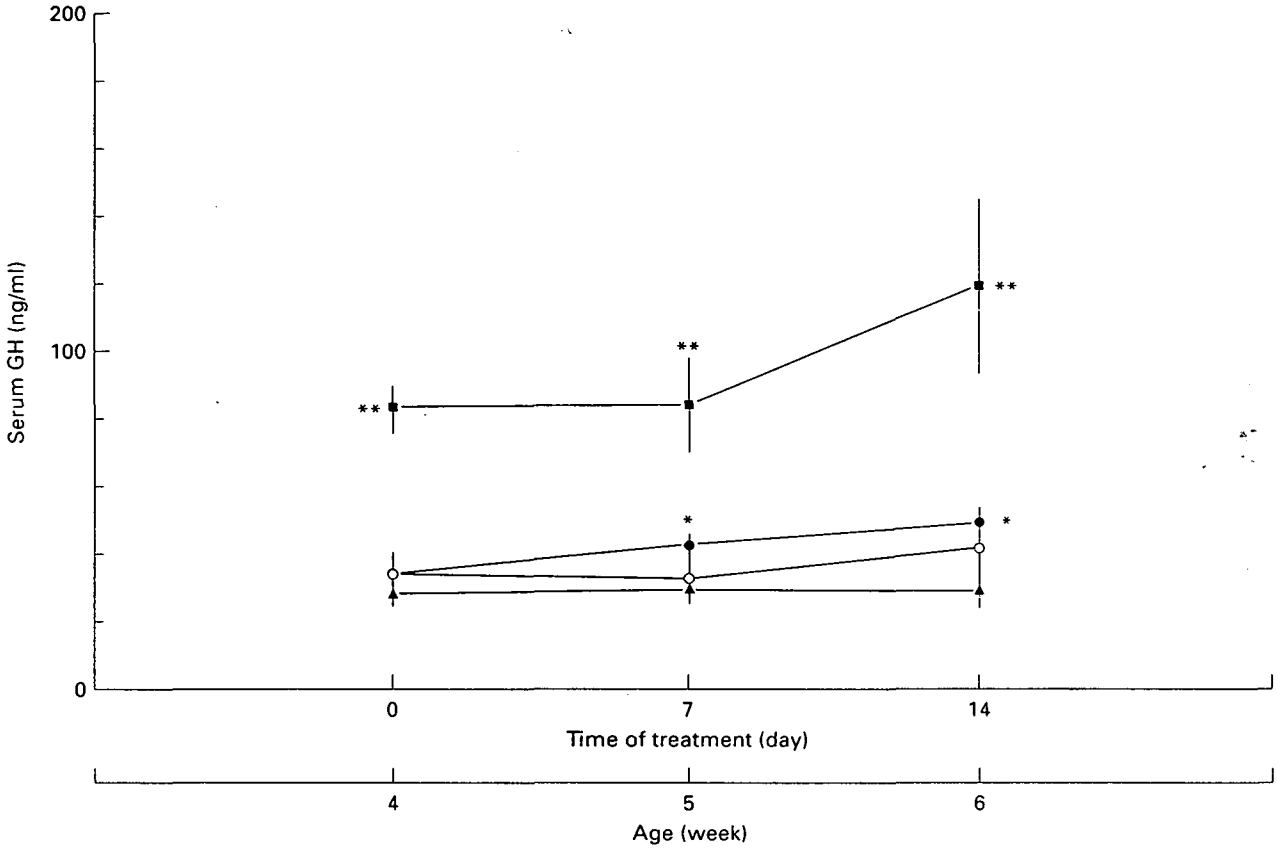


FIG. 3. Linear growth of MSG-treated rats received GH-RH by repetitive (n=7) or continuous (n=7) administration of A-495 for two weeks from 4 to 6 weeks of age. MSG control (n=12) and normal control (n=15) rats received solvent. Data points represent mean ±SEM. **P<0.01 *vs* MSG control rats. ■ Normal control; ▲ MSG control; ● GH-RH pulsatile; ○ GH-RH continuous.

values) were not significantly different from each other (87±15%, 74±13%, 80±11%, 64±10%, and 60±13% of the reference, respectively). In these experiments, the terminal response to the membrane depolarizing K^+ also was higher than the initial one (230±29% to 100%, P<0.01). (Fig. 6B). Comparing the magnitude of GH responses to the membrane depolarizing K^+ applied at the end of the two different patterns of GH-RH administration, significantly higher responses were produced by the cells after the repetitive, than after the continuous administration (230±29% *vs* 168±22%, P<0.01). A significant difference was also found in the total hormone secretion (integrated area under the superfusion curve), i.e. repetitive administration of the GH-RH analog induced higher GH secretion than continuous administration (3.09±0.48 μg and 1.97±0.31 μg, respectively, P<0.01). However, no significant difference could be detected in the remaining hormone content of the cells between the two types of experiments (continuous perfusion: 19.67±2.54 μg; repetitive administration: 18.46±2.38 μg).

Discussion

Injection of MSG in neonatal rats produces selective lesions of the arcuate nuclei, resulting in a 70–90% destruction of neuronal cell bodies (3, 4, 22), including most of the GH-RH-producing neurons (3, 7). This causes disappearance of GH-RH immunoreactive fibers in the ME (4) and markedly diminished GH secretion (5, 23). In keeping with this finding, we have observed marked reductions of GH-RH immunoreactivity in the ME and decreased serum GH concentrations in the MSG-treated animals. Despite the significant reduction in absolute GH responses in MSG treated animals, GH-RH bolus injections given at 4 week age induced comparable 6–7 fold increases of GH over baseline levels in both the MSG-treated and the normal control animals. These findings suggest that despite the highly diminished GH-RH influence, pituitary cells keep the normal responsiveness to GH-RH. The simplest explanation for this finding is, tha although neonatal exposure to MSG results in smaller anterio pituitary gland and lower GH content in the adult, the pituitary GH concentration remains unchanged (5, 9). In addition to the preserved GH concentration, a significant reduction of the pituit ary somatostatin binding (24), may also be accounted for the preserved GH responses to GH-RH after the MSG treatment Supplementation of the endogenous GH-RH by repeated adminis tration of the GH-RH analog markedly increased both the absolute and the relative GH responses. Since the stimulatory effect of repeated administration of GH-RH on the pituitary GH synthesis in normal female rats has been published (6), the mos obvious explanation for this finding is, that the regular exposure of the pituitary to high GH-RH pulses might have augmented the GH and the GH-RH receptor biosynthesis. However, furthe experiments on pituitary GH content and GH-RH recepto determination in MSG-treated rats also have to prove this to be



. 4. Basal serum GH concentrations of MSG-treated rats received GH-RH by repetitive (n = 7) or continuous (n = 7) administration of A-495 for) weeks from 4 to 6 weeks of age. MSG control (n = 12) and normal control (n = 15) rats received solvent. Data points represent serum GH mean SEM measured on days 0, 7, and 14 related to the treatment. *P < 0.05, **P < 0.01 *vs* MSG control rats. ■ Normal control; ▲ MSG control; GH-RH pulsatile; O GH-RH continuous.

case. The high GH peaks, induced at 24 h intervals, resulted a catch-up growth of the GH deficient animals by which they ıched the weight and length of the normal controls. In contrast the previous experiments applying GH-RH every 3 h in MSG-ioned rats (6, 11), we administered the GH-RH at a much ver frequency (every 24 h), and it was enough to achieve the ne effect. Our results are the first to demonstrate that GH-RH ed not be administered as frequently as the appearance of the logenous GH pulses to restore the normal growth rate of the H deficient animals, and that growth responses correlate rather h the amplitude than the frequency of GH pulses. We have nd that those animals which received high GH-RH stimuli at g intervals and responded high GH pulses to the GH-RH atment achieved a catch-up growth, despite the extremely low sal serum GH concentration. These results support the earlier dings, that growth responses appear to be related to the gnitude of the GH-RH stimulated rises in GH levels rather n to basal GH levels (11), and that the deleterious consequence GH-RH deficiency is resulted from the loss of the GH pulses . The effect of GH-RH treatment on the growth rate of rats ted for 2 weeks after the treatment had ceased. The maintained wth of the animals no longer receiving GH-RH injections ght be resulted from an increased GH and IGF biosynthesis due to previous repetitive GH-RH administration. Since we found that GH responsiveness to GH-RH bolus injections was gradually increasing from the 0 to the 14 day of the pulsatile GH-RH treatment, we can rightly suppose that a gradually increasing GH and IGF biosynthesis was resulted from this treatment, which lasted for 2 weeks. Nevertheless, as there are no direct data proving this explanation, further experiments have to confirm it.

Interestingly, in the MSG-treated control animals both the absolute and the relative GH responses to GH-RH bolus injections increased between the 7th and 14th day of treatment, while in the normal control rats no increase could be seen in either GH response. On the basis of these observations, we can suppose that two bolus injections of the GH-RH analog (on the 0th and the 7th day) were enough to increase GH responsiveness of the MSG-lesioned but not of the normal rats. These results also suggest that pituitary cells being out of GH-RH stimulus for a long time are more sensitive to the repeated exogenous GH-RH stimulus than the normal pituitary gland. Chronic continuous administration of the GH-RH analog at the same daily dose was ineffective on the linear growth of the MSG treated rats, and only the body weight was slightly increased by this treatment. Although the absolute GH responses to bolus injections of the GH-RH analog were moderately enhanced by the 14th day of the continuous

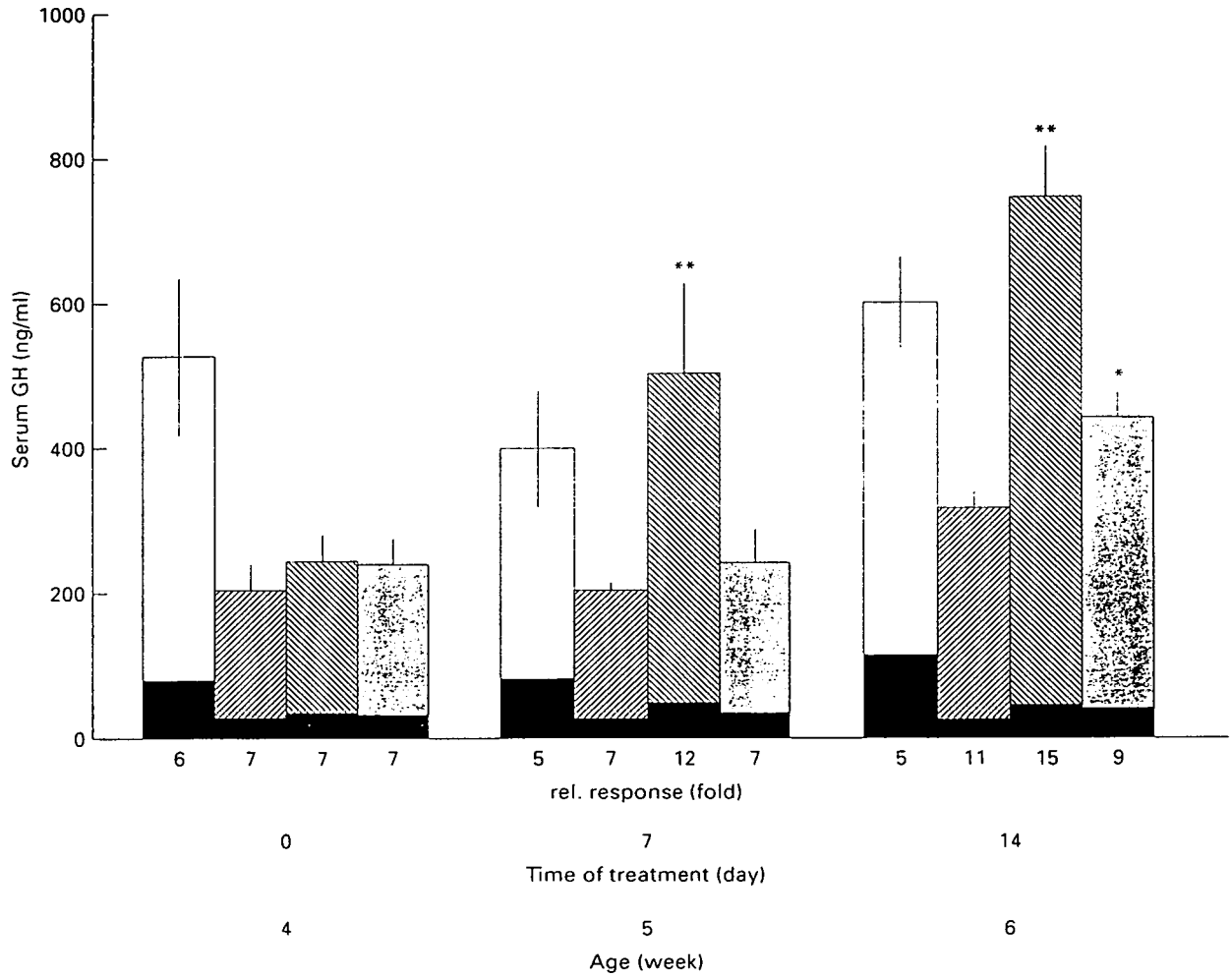
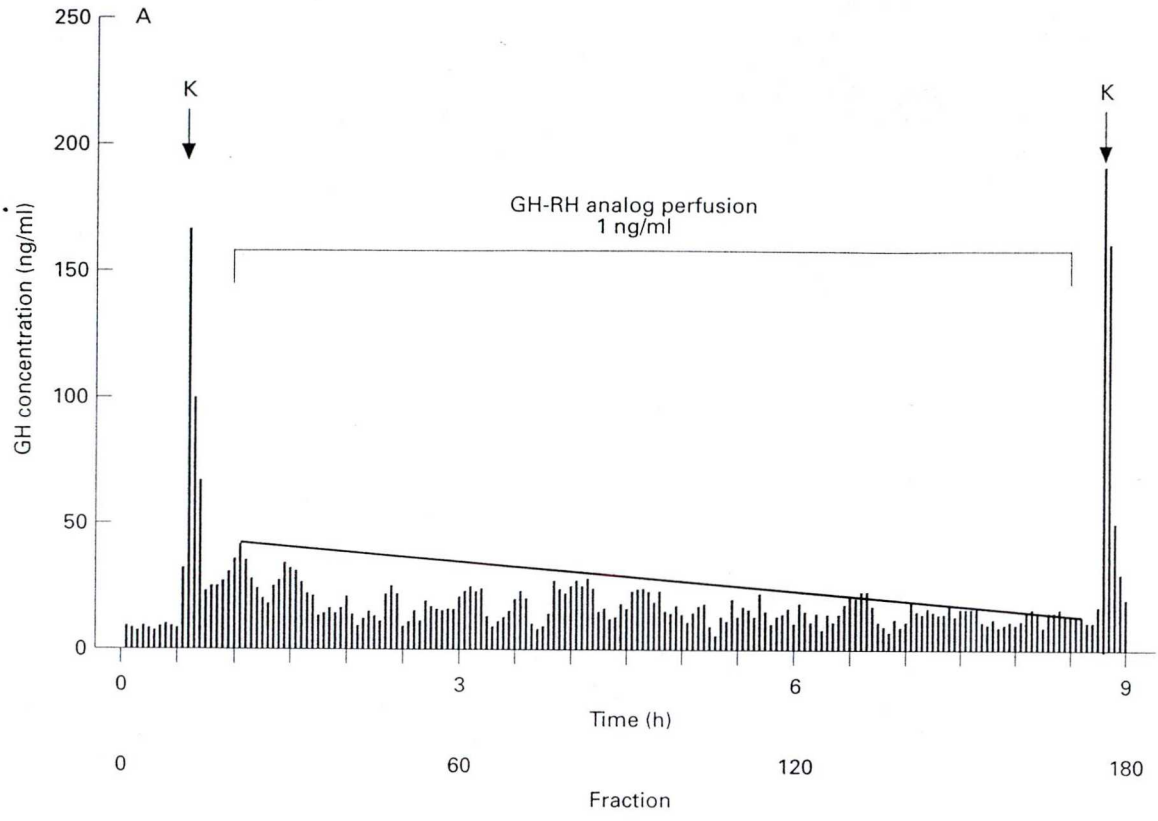


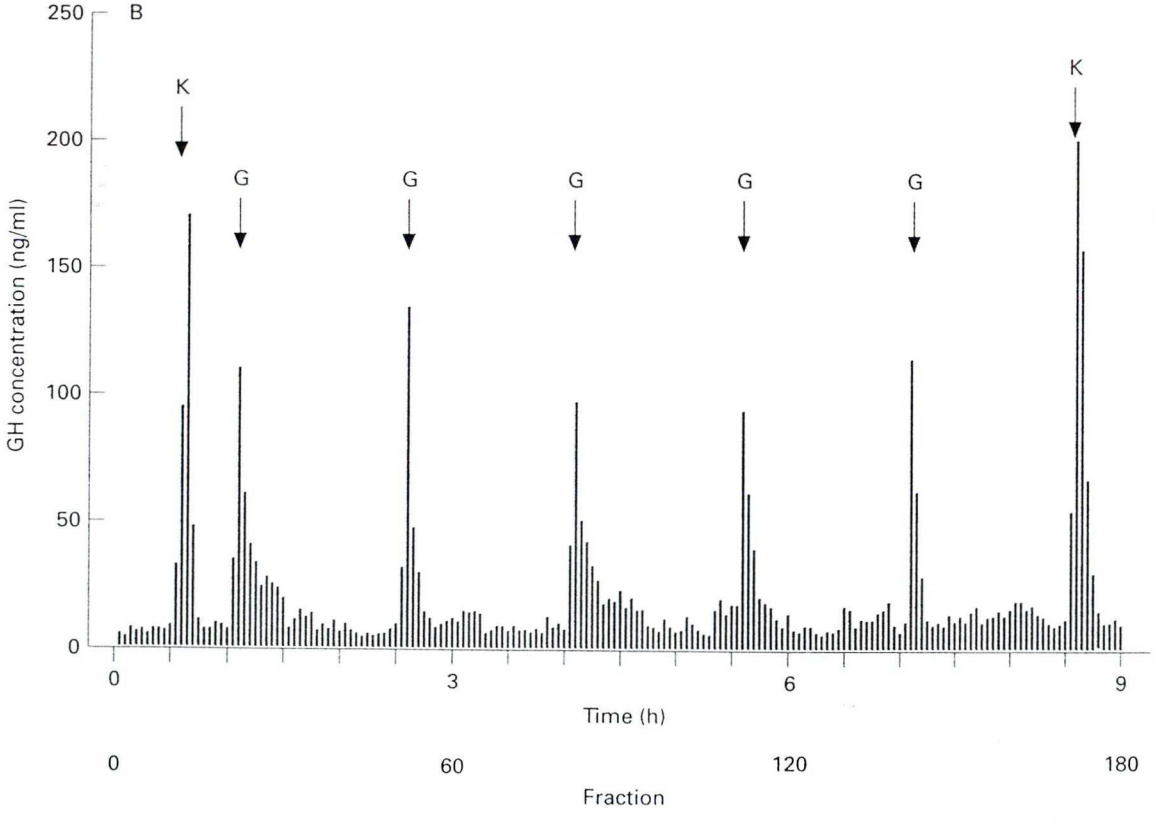
FIG. 5. GH-RH stimulated absolute and relative serum GH responses in MSG-treated rats received GH-RH supplementation by repetitive (n = 7) c continuous (n = 7) administration of A-495 for two weeks from 4 to 6 weeks of age. MSG control (n = 12) and normal control (n = 15) rats receive solvent. Bars represent serum GH mean ±SEM measured at +10 min to 10 μg/kg bolus injections of A-495 on days 0, 7, and 14 related to th GH-RH treatment (absolute responses). The inserted black areas at the bottom of the bars represent basal mean serum GH concentrations measure at −1 min to the bolus injections. Relations of absolute GH responses to basal GH concentrations are expressed as relative responses. *P < 0.0 **P < 0.01 *vs* MSG control rats. □ Normal control; ▨ MSG control; ▧ GH-RH pulsatile; ▢ GH-RH continuous.

treatment, the relative GH responses of these animals were found to be slightly lower than those of the untreated MSG-lesioned control rats. These results show that the moderate increases in GH responses of both latter groups of animals between the 7th and 14th day of treatment must have been caused by the GH-RH bolus injections on the 0th and 7th day, and that continuous administration of the GH-RH at the same daily dose is ineffective to increase either the sensitivity of GH secretion or the linear growth rate of the MSG-lesioned animals. Above data strengthen those of our conclusions as well, that growth responses appear to be related to the magnitude of the GH-RH stimulated rises in GH levels. Similar observations have also been made in children with GH deficiency (11) and in MSG-lesioned rats (6).

On the base of our previous results from normal (25, 26) an present results from MSG-treated rats, we arrived at the conclu sion that continuously administered GH-RH can be effective o the growth rate if the dose is high enough to markedly eleva the serum GH concentration. This dose was found to be betwee 18 and 36 μg/day in our previous experiments in normal ra (25), but it could be even higher depending on the potency c the GH-RH analog used. In our present experiments in MSC treated rats, 10 μg daily doses of our GH-RH analog had onl moderate, but not significant effects on the basal serum GH an linear growth and it was only slightly effective in increasing th body gain. Considering, that this GH-RH analog has more tha 2 times higher potency than GH-RH(1-29)-amide (15) used b

FIG. 6. GH responses to continuous (A) and repetitive (B) administration of GH-RH (A-495) in the superfused pituitary cell system. A A-495 w perfused onto the cells for 7.5 h in a concentration of 1 ng/ml. B 3-min pulses of 30 ng/ml of A-495 (G) were applied every 90 min for 7.5 h. Ba indicate GH concentrations of 3-min fractions collected during the experiment for 9 h. The first and the last GH peak of the panels (K) was evoke by 3-min pulse of 50 mM KCl.


A
K
K
GH-RH analog perfusion
1 ng/ml
GH concentration (ng/ml)
250
200
150
100
50
0
0
3
6
9
Time (h)
0
60
120
180
Fraction


B
K
G
G
G
G
G
K
GH concentration (ng/ml)
250
200
150
100
50
0
0
3
6
9
Time (h)
0
60
120
180
Fraction

Clarke and Robinson (6), it is understandable that 8 μg daily dose of the GH-RH(1-29)-amide proved to be ineffective to accelerate growth in their experiments in MSG treated rats.

Our results from the *in vitro* system correspond with the results from our *in vivo* experiments. These results demonstrate that pituitary cells respond with much lower GH pulses to continuous perfusion than to repetitive administration of the GH-RH. The magnitude of the high GH responses was maintained during the pulsatile application, while GH responses were gradually attenuating during the continuous perfusion of GH-RH. Since no depletion of the intracellular GH content could be detected after the attenuated GH release, we can conclude that desensitization of the GH secretory response might be responsible for this finding. (11, 26) In contrast, no desensitization showed up during the repetitive administration of the GH-RH analog.

Those of our results that the releasable GH pool of the pituitary cells was higher at the end than at the beginning of each *in vitro* experiment indicate that either the continuous or the pulsatile administration of GH-RH stimulates the *in vitro* GH biosynthesis of the GH-deficient pituitaries. The trophic effect of GH-RH on GH m-RNA transcription *in vitro*, using normal but not GH deficient pituitaries, has been published before (27). Since both the releasable hormone pool and the total hormone secretion were found to be higher in the experiments on repetitive than on continuous GH-RH administration, and the remaining hormone contents of the cells were identical, we can conclude that the former administration provides stronger stimulus for the GH biosynthesis than the latter. This conclusion corresponds with the previous *in vivo* results from MSG treated female rats, demonstrating that administration of GH-RH in pulsatile manner significantly increases the pituitary GH content, whereas continuous GH-RH infusion is without effect (6). Considering these and those of our earlier results that pulsatile administration of GH-RH is able to induce both sensitization and desensitization, depending on the frequency of pulses (25), we can rightly suppose that daily exposure of the pituitary GH cells to GH-RH induced sensitization, while the continuous perfusion evoked desensitization of the GH secretory response in our *in vivo* experiments.

We observed some difference between the *in vivo* and *in vitro* effects of repetitive administration of the GH-RH analog on GH responses to GH-RH bolus injections, i.e. repetitive administration of GH-RH increased the sensitivity of GH secretion *in vivo*, but not *in vitro*. This finding can be explained by the different frequency of GH-RH exposure (24 h *vs* 90 min intervals, respectively), that is an autocrine and/or paracrine negative feedback effect of the high GH concentrations might have acted against the increase of GH secretion *in vitro* (28), while this effect could have been eliminated during the 24 h intervals between GH responses *in vivo*.

In conclusion, evidence was provided that the highly attenuated growth rate of the MSG-lesioned rats can be fully restored by chronic treatment with GH-RH. Although continuous administration of the GH-RH also has some effects on the body gain, repetitive administration is more effective at the same daily dose. Present results demonstrate that the growth response appears to be related to the magnitude of the GH-RH-stimulated rises in GH levels rather than basal GH concentrations and are the first to evidence that GH-RH need not be administered as frequently as the appearance of the endogenous GH pulses to restore the normal growth of the GH deficient rats. In addition, above data prove the therapeutic usefulness of our new GH-RH analog.

Materials and methods

1. In vivo *experiments in MSG-treated rats*

Neonatal female rats (Wistar R-Amsterdam) received subcutaneous (SC) injection of MSG in doses of 4 mg/g body weight (BW) or hyperosmotic saline (0.01 ml 10% sodium chloride/g BW; controls), on days 1, 3, 5, 7, 9 of life. At 28 days of age, the pups (6–9 per group) were weaned and grouphoused according to treatment. The rats were maintained under controlled conditions of lighting (lights on from 06:00–20:00 h) and temperature (22 ± 1 °C), with free access to standard rat chow pellets and tap water. BW and body length (BL; head-tail distance) of the animals were recorded weekly from 4 to 8 weeks of age. The effectivity of the MSG treatment was controlled immunohistologically by using the peroxidase-anti-peroxidase (PAP) method of Sternberger (16). 36 h before scarifying, the animals were treated with single dose of colchicine (80 μg/100 g BW, dissolved in 5 μl of saline) injected into the central part of the lateral cerebral ventricle, under Nembutal (40 mg/kg BW) anaesthesia. Re-anesthetized animals were perfused through the ascending aorta first with phosphate-buffered saline (PBS) and than with 4% paraformaldehyde solution, used at variable pH (6.6 and 11) according to the concept of Berod *et al.* (17). Satisfactory visualization of GH-RH antigen required the addition of glutaraldehyde (0.02%) to the alkaline component of the fixative. Finally the animals were perfused with the high pH fixative solution without glutaraldehyde. The same glutaraldehyde free solution was used for overnight postfixation. Thereafter, the brains were removed from their skull and 30 μm thick coronal section were cut on a Lancer vibratome. The rat hypothalamic GH-RH antiserum (R567) was raised in rabbit and used at 1:5,000 working dilution. The details of the production and characterization of this antiserum have been published elsewhere (18). All antibodies were diluted in PBS containing 1% normal goat serum and 0.1% sodium azide. The sections were incubated in the primary antibody for 36–48 h and than for 1–2 h in both the bridging (goat, anti-rabbit IgG, 1:200. Jackson Immunoresearch Laboratories, Inc.) and PAP-complex (Jackson Immunoresearch Laboratories, Inc.). The antigen-antibody sequences were visualized by according to the method of Streit and Reubi (19).

1.1. Chronic repetitive administration of the GH-RH analog in MSG-treated rats

The GH-RH analog D-Ala^{2},Nle^{27},Gaba^{30}-GH-RH-(1-30)-amide (A-495) was synthesized by solid-phase methods in our laboratory as it was published earlier (14). Daily injections of 10 μg doses of A-495 or 0.9% saline (control) were applied intramuscularly (IM) to 7 MSG-treated rats for 14 days. Six MSG-treated (control) and 9 normal (control) animals were injected with the solvent of A-495 (0.9% saline). On the day 0, 7 and 14, GH responses to bolus injections of GH-RH were tested by injecting 10 μg/kg test-doses of A-495 intravenously (i.v.). GH concentrations were measured by RIA from sera obtained from the jugular vein under ketamine anesthesia (50 mg/kg) at −1 and +10 min to the injections. Blood was replaced by saline, centrifuged, and sera were stored at −20 °C until assayed by RIA.

1.2. Chronic continuous administration of the GH-RH analog in MSG-treated rats

At 4 weeks of age osmotic minipumps (Alzet, CA, USA) releasing 0.42 μg/h (10 μg/day) of A-495 were implanted into the peritoneal cavity of 7 MSG-treated animals, under ketamine anesthesia (50 mg/kg). Six MSG-treated (control) and 6 normal (control) animals received minipump releasing the solvent of the GH-RH analog (0.9% saline). The delivery capability of the pump lasted for 14 days. On days 0, 7, and 14, GH responses to bolus injections of GH-RH were tested by injecting 10 μg/kg test-doses of A-495i.v. At −1 and +10 min to the injections blood was drained from the jugular vein and was replaced by saline under ketamine anesthesia (50 mg/kg). Blood was centrifuged, and sera were stored at −20 °C until assayed for GH concentration by RIA. At the end of the experiments the pump was explanted from the animals, it was cut in half crosswise using a razor blade, and reservoir was checked whether it was collapsed or not. Since all pumps appeared to be collapsed into

riangular or figure eight form, they were considered to be empty and to nction appropriately.

In vitro *experiments on pituitary cells from MSG-treated rats*

uperfused rat anterior pituitary cell system (20, 21) was used for the *in tro* experiments. Pituitaries from 4 MSG-treated female rats were moved, cut into small pieces, incubated with collagenase (Type I, 0.5%, Vorthington, USA) for 50 min in a metabolic shaker, dispersed, gently ixed with 1 ml of swollen Sephadex G-10 which had been equilibrated ith oxygenated tissue culture medium (Medium 199, Sigma), and transfer to the superfusion chamber. To assure stable baseline values the cells ere perfused with the enzyme-free Medium 199 overnight before col-cting fractions. GH release during and after the continuous perfusion f 1 ng/ml of A-495 for 7.5 h (150 ng/150 ml), and GH responses to 5 petitive 3 min pulses of 30 ng/ml (150 ng/5 ml) of this peptide given at 0 min intervals were measured. At the beginning and the end of each xperiment, the membrane-depolarizing K^+ (50 mM KCl) was adminis-ered to obtain standard GH releases and control the amount of releasable H in the cells. The first GH response to K^+ was used as reference, hich the additional responses were related to. 1 ml fractions of the perfusion medium were collected every 3 min, and GH concentrations ere determined by RIA. After finishing the superfusion, the remaining ormone content of the cells were extracted with 0.1 N HCl and deter-ined by RIA. These experiments were repeated 3–4 times, and data om them were expressed as mean ±SEM.

Hormone determination

H concentrations of the sera and the samples from the *in vitro* periments were measured by RIA, using materials (antigen for iodina-on, a-rGH S-5 antiserum, and rGH RP-2 reference hormone) supplied y the National Hormone and Pituitary Program. Inter- and intra-assay ariation was 10% or less.

Statistical analysis

esults expressed as means ±SEM were evaluated by analysis of variance; hen the P value was less than 0.05, the analysis was completed using uncan's multiple range test. The superfusion data were analyzed by a ecial computer program (21). With the help of this program we alyzed the peaks, calculated the amount of GH secreted above the aseline during and/or after the stimulation. GH responses during the periments were related to the first response to K^+ (reference response) d were expressed as % of the reference response.

cknowledgements

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ccepted 22 June 1995

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VI.

COMBINATION OF IMMUNOCYTOCHEMICAL (ICC) AND *IN SITU* HYBRIDIZATION (ISH) TECHNIQUES TO STUDY THE MOLECULAR REGULATION OF INDIVIDUAL NEURONS IN THE RAT BRAIN.

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Immunocytochemical (ICC) and *in situ* hybridization (ISH) multiple labeling techniques are powerful tools for studying the coexpression of neuronal compounds in the brain (references, 1, 2, 3). In this paper, we describe novel double- and triple-labeling approaches that combine ICC and ISH in order to investigate the molecular mechanisms of neuronal regulations.

In our hands, the same histological paradigm was used successfully for various ICC and ISH detections. Steps of the tissue processing included perfusion-fixation of rats with 4% paraformaldehyde (pH 7.6), and then, sucrose-infiltration of the brains. Small tissue blocks containing the area of interest were snap-frozen on dry-ice, and 20-μm thick floated sections were prepared on a freezing microtome. The sections were stored in a cryoprotectant solution at -20 ^{o}C until hybridized with one or two probes. Following stringent post-hybridization treatments, antigens of interest were visualized by means of ICC, and then, the hybridization signals were developed. Non-isotopic probes used digoxigenin as a marker for detection. Digoxigenin was reacted with anti-digoxigenin antibody conjugated to alkaline phosphatase, and the signal was revealed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) chromogen. Isotopic probes were labeled with ^{35}S and visualized by autoradiography. The combined methods described below were developed for various applications.

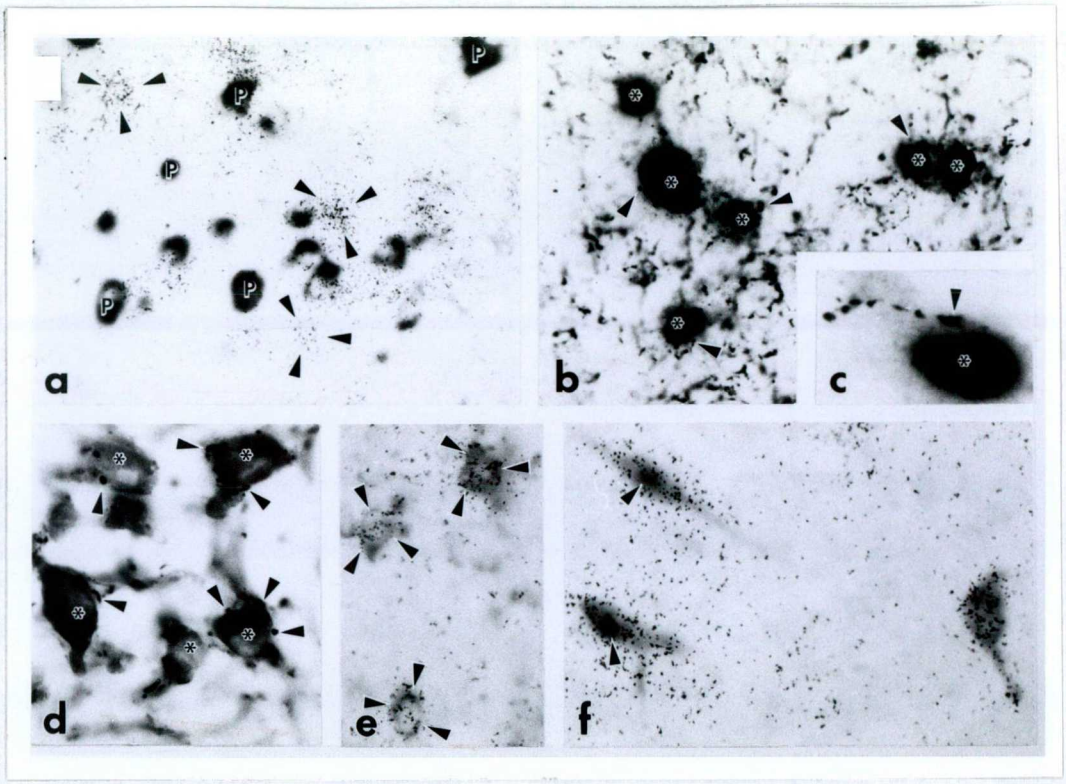
1./ We assayed several hybridization temperatures (with other hybridization parameters unchanged) to develop a method for the simultaneous application of a ^{35}S-labeled oligodeoxynucleotide probe and a digoxigenin-labeled cRNA probe within the same dual-label ISH. Double-labelings were successful following ISH at 50 ^{o}C, and none of the signal components seemed to be compromised. In turn, we found that lowering the temperature to a constant 37 ^{o}C considerably impaired the signal intensity of the cRNA hybridization. Dual label ISH in **figure a** exhibits proopiomelanocortin mRNA-containing neurons of the hypothalamic arcuate nucleus (AN) which were identified by non-isotopic ISH using a cRNA probe. Preprogalanin mRNA-expressing neurons of the AN, in turn, were demonstrated autoradiographically following the use of an oligodeoxynucleotide probe labeled with ^{35}S.

2./ We combined ICC and non-isotopic ISH to investigate the putative regulation of thyrotropin releasing hormone (TRH) neurons by galanin-containing afferents in the hypothalamic paraventricular nucleus (PVN). Sections were hybridized with a digoxigenin-labeled cRNA probe to pro-TRH mRNA, thereafter, galaninergic axons of the hypothalamus were visualized with ICC using silver-gold-intensified diaminobenzidine (DAB) chromogen (reference 1). Finally, the ISH signal was developed. **Figures b** and **c** demonstrate the intimate relationship between galaninergic axon varicosities and neuronal perikarya containing pro-TRH mRNA.

3./ Dual-label ICC, as well as combined ICC and ISH were used to study the molecular regulation of tuberoinfundibular dopaminergic (TIDA) neurons by TRH. Dual-label ICC (reference 1) revealed the apposition of TRH-immunoreactive (IR) axons to tyrosine-hydroxylase (TH)-IR neurons of the TIDA system (**fig. d**). In other experiments, TH-IR neurons were tested for the presence of TRH receptor mRNA, by means of combined ICC and isotopic ISH. Autoradiographic signal in **figure e** delineates TH-IR neurons which co-express the mRNA encoding for TRH receptor.

4./ We developed a triple-labeling method which combines dual-label ICC and isotopic ISH, in order to study the co-expression of preprogalanin mRNA in the subpopulation of luteinizing hormone-releasing hormone (LHRH)-IR neurons that exhibit immunoreactive c-Fos in the cell nuclei, following steroid activation (reference 2). We were able to demonstrate the presence of preprogalanin mRNA in 76.8 % of LHRH-IR neurons that co-expressed immunoreactivity for the immediate-early gene product, c-Fos (**fig. f**).

In conclusion, our newly developed double- and triple-labeling techniques provide further methodological tools for studying the molecular regulation of individual neurons.



LEGENDS

Fig. a: Simultaneous use of a digoxigenin-labeled cRNA probe to proopiomelanocortin (POMC) mRNA and a ^{35}S-labeled oligodeoxynucleotide probe to preprogalanin mRNA in dual-label *in situ* hybridization (ISH). Neurons exhibiting non-isotopic ISH signal for POMC mRNA (P), as well as preprogalanin mRNA-containing neurons (clusters of grains, indicated by arrowheads) are identified in the arcuate nucleus (AN).

Figs. b and **c:** Combined use of immunocytochemistry (ICC) and ISH for the demonstration of neuronal contacts between silver-stained galanin-immunoreactive (IR) axons (arrowheads) and neuronal perikarya expressing pro-thyrotropin releasing hormone (TRH) mRNA (asterisks) in the paraventricular nucleus of the hypothalamus.

Fig. d: Dual-label ICC demonstration of contacts between TRH-IR axons (arrowheads) and tyrosine hydroxylase (TH)-IR neurons (asterisks) in the AN. Silver-gold-intensified diaminobenzidine (DAB) and DAB chromogens were used for the staining of TRH- and TH-IR structures, respectively (reference 1).

Fig. e: Application of ICC and isotopic ISH for the simultaneous demonstration of TH immunoreactivity and TRH receptor mRNA-expression in the AN. Arrowheads indicate TH-IR neurons exhibiting also the hybridization signal for TRH receptor mRNA (grain clusters).

Fig. f: Conjoint application of dual-label ICC and isotopic ISH for simultaneous detection of c-Fos immunoreactivity (dark nickel-DAB staining in cell nuclei, arrowheads) and preprogalanin mRNA-expression (autoradiographic grains over the cytoplasm) in luteinizing hormone-releasing hormone-IR (cytoplasmic DAB staining) neurons.

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VII.

Synaptic regulation of tuberoinfundibular dopaminergic (TIDA) neurons by thyrotropin-releasing hormone (TRH) in the rat

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ABSTRACT

Prolactin secretion is regulated by the hypothalamus via releasing and release-inhibiting factors secreted into the hypophyseal portal circulation. In episodes of stimulated prolactin secretion, a transient blockade of the inhibitory tuberoinfundibular dopaminergic (TIDA) neuronal system potentiates the action of the putative prolactin-releasing factor, thyrotropin-releasing hormone (TRH), upon the lactotrophs. To partially elucidate the central mechanism underlying the reciprocal regulation of dopamine and TRH secretion into the portal blood, we investigated the possibility of an intrahypothalamic communication between the neurosecretory TRH and the TIDA neuronal systems of the rat.

The light microscopic examination of double-immunostained sections revealed axo-somatic and axo-dendritic contacts between TRH-immunoreactive (IR) fibers and tyrosine hydroxylase (TH)-IR, dopaminergic nurons in the arcuate nucleus of both the male and the female rat. By means of preembedding dual-label immuno-electron microscopy, we identified synapses of TRH-containing axons established with tyrosine hydroxylase (TH)-immunoreactive (IR) neurons. Furthermore, by combining the immunocytochemical localization of TH and simultaneous *in situ* hybridization (ISH) detection of TRH receptor mRNA, we demonstrated the co-expression of TRH receptor in a population of TIDA neurons.

Our data indicate an intrahypothalamic link between TRH-possessing fibers and the TIDA system, and contribute to the understanding of the complex central mechanisms which regulate PRL secretion via the hypophysiotropic dopamine- and TRH-secreting neuronal pathways.

INTRODUCTION

The secretion of prolactin (PRL) by the anterior pituitary gland is controlled by a complex interplay between hypothalamic releasing and release-inhibiting factors which reach the lactotrophs via the hypophysial portal circulation[1]. The tuberoinfundibular dopaminergic (TIDA) neuronal system[2] with perikarya in the arcuate nucleus (AN) and axon terminals in the median eminence (ME) represents the final common inhibitory pathway in this regulation, whereas the chemical identity of PRL-releasing factor(s) is still controversial[1]. Thyrotropin-releasing hormone (TRH), which is one of the PRL-releasing factor candidates in the rat, stimulates the secretion of PRL both *in* vivo[3,5,9] and *in vitro*[21,34]. The removal of the tonic dopaminergic inhibition of lactotrophs highly increases the PRL-releasing potency of TRH, suggesting that central mechanisms regulating the secretion of PRL might involve inhibition of dopamine and simultaneous stimulation of TRH release into the portal blood of the hypophysis. The sensitization of lactotrophs by transient dopamine antagonism, accompanied by an increased TRH secretion represents a physiological mechanism in the suckling-induced[6,8,29] as well as the estrogen-stimulated[10] surges of PRL. To elucidate partially the central mechanism underlying the reciprocal regulation of dopamine and TRH secretion into the hypophysial portal blood, we examined the possibility that a neuronal communication exists between the hypothalamic TRH and TIDA neuronal systems of the rat. Our studies were performed on sections from the hypothalamic arcuate nucleus (AN) which contains the cell bodies and dendrites of the TIDA system[2] and also receives abundant innervation by TRH-immunoreactive axons[4]. We used two strategies to provide evidence for the synaptic regulation of TIDA neurons by TRH-containing axons:(i) We performed dual-label immunocytochemical studies at the light- and electron microscopic levels, in order to reveal neuronal communication between TRH-immunoreactive (IR) axon terminals and TIDA neurons of the hypothalamic AN. In addition, (ii) we used combined immunocytochemistry and *in situ* hybridization (ISH) to demonstrate the co-expression of TH immunoreactivity and TRH receptor mRNA in TIDA neurons. A preliminary report of these data has been published recently in a short communication form[17].

MATERIALS AND METHODS

Preembedding dual-label immunocytochemical studies

Tissue preparation

Adult male Wistar rats (N=5; 200-250 g. b.w.) were anesthetized with Nembutal (40 mg/kg, i.p.) and perfused transcardially with 400 mls fixative containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffered saline (PBS, pH 7.4). The hypothalami were dissected and postfixed in 4% paraformaldehyde for 2 h. Thereafter, 25-μm thick coronal sections containing the AN were cut on a Vibratome (Technical Products International, St. Louis, Mo., USA). The sections were incubated in 1% sodium borohydride ($NaBH_4$) for 20 min to inactivate excess aldehydes, permeabilized with 0.2% Triton X-100 for 20 min, and blocked against non-specific antibody-binding with 2% normal sheep serum (NSS) in PBS for 30 min.

Female adult Wistar rats (N=10; 200-250 g. b.w.) were ovariectomized in Nembutal anesthesia. Five animals recieved subcutaneous silastic capsule implants with estradiol (3cm; 150μg/ml in sesame oil) 12 days after the ovariectomy, and were perfusion-fixed two days later. The other 5 animals were perfused on the same day without prior estrogen replacement.

Dual-label immunocytochemistry

We used light- and electron microscopic dual-label immunocytochemistry[25] to simultaneously visualize TRH- and dopamine-containing structures of the AN. By the application of this technique, we have recently published data on the galaninergic[16] and adrenergic[15] innervation of TIDA neurons. TRH-immunoreactive (IR) axons were immunostained first with the peroxidase-antiperoxidase complex (PAP)-3,3' diaminobenzidine (DAB) technique of Sternberger et al.[33], and then, the DAB-containing sites were silver-gold intensified[24]. The sections were incubated for 36 h in a primary antiserum (1/4000) generated in rabbit against pyroglutamyl-histidyl-proline (TRH) conjugated to bovine serum albumin. The preparation and specificity-testing of this antibody was described elsewhere[27]. Then, the secondary antiserum (sheep, anti-rabbit IgG, 1/500; Arnel) and the rabbit PAP complex (1/500; Arnel) were sequentially administered to the sections for 1 h each. All antisera were diluted with 2% NSS in PBS. The peroxidase-containing sites were initially visualized by the brown DAB chromogen[32], and then the DAB was silver-gold-intensified to yield a final black reaction end-product, as reported by Liposits et al.[24]. Subsequently, TIDA neurons were detected by means of immunocytochemistry using an antiserum to the rate-limiting enzyme of dopamine synthesis, tyrosine hydroxylase (TH). The primary antibody was raised in rabbit against TH purified from bovine adrenal medulla, and used at a 1:1000 working dilution[19]. The antigen-antibody complexes were visualized by the PAP-DAB method. Double-immunostained sections were either mounted on glass slides, coverslipped and examined with a light microscope, or further processed for electron microscopy.

Electron microscopy

Immunostained sections from male rats were also processed for electron microscopic studies. They were osmicated, dehydrated and flat-embedded into Epon resin (LX-112; Ladd Research Industries, Burlington, VT), and then, semithin and ultrathin sections were prepared on a Reichert ultratome. The ultrathin sections were mounted on Formvar-coated single-slot grids, contrasted with uranyl acetate and lead citrate and examined with a Tesla BS 500 transmission electron microscope.

Combined immunocytochemical and *in situ* hybridization techniques

We have recently published a detailed protocol for combined use of ICC and ISH within the same sections of perfusion-fixed hypothalami[18].

Preparation of anti-sense RNA probe

A 486-bp fragment of the rat TRH receptor cDNA was inserted into the EcoRI-XbaI site of a pGEM-4Z vector[35]. The plasmid was linearized with XbaI and ^{35}S-labeled anti-sense RNA probe was transcribed with SP6 RNA polymerase, as described earlier[28]. For preparation of sense control, the plasmid was digested with EcoR1 and transcribed with T7 RNA polymerase.

Tissue preparation procedure

Male Wistar rats (N=5; 200-250 g. b.w.) were fixed by transcardiac perfusion with 4% paraformaldehyde in 0.1M PBS (pH 7.4). Small tissue blocks containing the medial basal hypothalami were dissected from the brains, infiltrated with gradually increasing concentrations of sucrose through 30%, and snap-frozen on dry-ice. Then, 20-μm thick coronal sections of the AN were prepared on a cryostat (Reichert-Jung). The sections were stored permanently in cryoprotectant solution[26] at -20 °C.

Preincubation, hybridization and washes

Immediately before hybridization, the sections were removed from the cryoprotectant solution and the following sterile incubation steps were performed: Permeabilization for 30 min with proteinase K (Serva; 0.1μg/ml, dissolved in 2XSSC), five min fixation in 4% paraformaldehyde/0.1M PBS; 2 min rinse in 2X standard saline citrate solution (2XSSC; 1XSSC=0.15M NaCl/0.015M sodium citrate, pH 7.0); 20 min acetylation in 0.25% acetic anhydride/0.9% NaCl/0.1M triethanolamine (pH 8.0); 5 min rinse in 2XSSC. For hybridization, the sections were transferred into a microcentrifuge tube containing the hybridization solution [50% formamide, 4XSSC, 10% dextran sulfate (500,000 MW; Sigma), 1XDenhardt's solution, 500μg/ml heparin sodium salt (Sigma), 0.5 mg/ml yeast tRNA (Boehringer Mannheim), 0.4 mg/ml sheared single-stranded salmon sperm DNA (Boehringer Mannheim), 0.1% SDS, 0.1M DTT, and 20,000 cpm/μl of the hybridization probe.]. The tube was sealed with Parafilm and placed in an incubator for hybridization overnight at 50 °C. The hybridized sections were rinsed in 1XSSC for 10 min, followed by a 1 h stringent wash step in 50% formamide/2XSSC, at 50 °C.

Then, the excess probe was digested with RNAse A (Boehringer Mannheim; 100μg/ml in 2XSSC buffer) for 1 h at 37 °C, followed by two 1h stringent treatment steps in 50% formamide/2XSSC, at 50 °C. Finally, the sections were transferred into 0.1M PBS and washed copiously.

Immunocytochemical detection of TH

Hybridized sections were immersed in a 9:1 mixture of methanol and 30% H_2O_2 for 10 min, thereafter, the immunocytochemical detection of TH was carried out as described earlier.

Detection of the hybridization signal

Immunostained sections were gradually desalted in 1X, 0.5X and 0.1X SSC solutions, transferred into 50% ethanol-300mM ammonium acetate for partial dehydration, and mounted from this solution onto gelatin-coated glass slides. After air-drying, they were further dehydrated in increasing concentrations of ethanol through 100%. Finally, the slides were air-dried, dipped into photographic emulsion (NTB-3; Kodak, Rochester, NY) and exposed for 5 weeks at 4 °C. Autoradiograms were developed for 3 min with D19 developer (Kodak) and fixed for 5 min with Kodak fixer. Finally, the sections were dehydrated with ethanol, cleared with xylenes and coverslipped with Permount.

RESULTS

Preembedding dual-label immunocytochemical studies

Light microscopy

In the male rats, the hypothalamic AN was rich in TRH-IR axon varicosities as well as TH-IR neurons. The black color of silver-gold-intensified DAB allowed an easy distinction of TRH-IR structures from TH-IR elements, characterized by the brown DAB deposition (Fig. a). TRH- and TH-immunoreactive structures were observed in both the dorso-medial and the ventro-lateral parts of the AN. Analysis of dual-labeled sections at higher power demonstrated juxtaposition of TRH-IR axon varicosities to TH-IR perikarya and dendrites (Fig. b). Axo-somatic and axo-dendritic juxtapositions occurred most frequently in the dorso-medial subdivision of the AN, where virtually all TH-IR neurons were contacted by TRH-IR axon varicosities. Juxtapositions were obvious in semithin sections as well (Figs. c, d).

The double-immunostained medial basal hypothalami of ovariectomized and ovariectomized plus estradiol-substituted female rats showed similar innervation pattern of TH-IR neurons by TRH-IR axons in the female, to the male rat. Furthermore, the use of dual-label ICC did not reveale major estrogen-dependent differences in the staining of arcuate nuclei. Because sex and estrogen levels did not seem to have a visible effect on the innervation pattern, only male arcuate nuclei were further processed for electron microscopic studies.

Electron microscopy

At the ultrastructural level, TH-IR neurons were recognized by the presence of DAB deposits which resulted in a medium degree of electron density, whereas TRH-IR axons contained silver-gold particles exhibiting high degree of electron density. Electron microscopic analysis of ultrathin sections from male rat medial basal hypothalami revealed synaptic specializations between TRH-IR terminals and TH-IR neuronal profiles (Fig. e).

Combined immunocytochemical and *in situ* hybridization studies

As we observed earlier, hybridization treatments did not interfere with the subsequent immunocytochemical procedure[18]; therefore, dopaminergic neurons remained detectable in hybridized sections of the AN. Autoradiographic exposure of sections hybridized with the antisense cRNA probe for 5 weeks resulted in the appearance of grain clusters mainly in the dorso-medial part of the AN. In this subdivision of the nucleus, the autoradiographic signal for TRH receptor mRNA often appeared above the cell bodies of TH-IR neurons (Fig. f). In turn, after 5 weeks of exposure, autoradiographic signal was only occasionally evident in the ventro-lateral area of the AN and cases of colocalization with TH immunoreactivity were scarce (Fig. g). Hybridization of control sections with the sense probe did not result in the appearence of autoradiographic signal over background levels.

DISCUSSION

In these studies, we presented evidence for the neuronal regulation of the TIDA system[2] by TRH-containing axons. Using preembedding dual-label immunocytochemistry[25], we identified synapses of TRH-IR axons with TH-IR perikarya and dendrites of TIDA neurons, consistent with a previous observation of TRH-IR synapses on ^{3}H-dopamine-labeled neurons in the AN[31]. In addition, using a novel approach of combined immunocytochemistry-*in situ* hybridization (ISH)[18], we co-localized the mRNA encoding TRH receptor with TH-IR neurons of the AN. Altogether, these findings suggest that dopaminergic neurons of the TIDA system are synaptically regulated by TRH.

PRL secretion by the anterior pituitary gland is controlled by the hypothalamus via releasing and release-inhibiting factors[1]. Dopamine, the major PRL release-inhibiting factor is synthesized and released into the hypophysial portal vessels by the TIDA system[2], neurons of which, in turn, are regulated by specific synaptic afferents from various neurotransmitter/neuromodulatory systems[14,15,16,22]. Our present observations that TRH, a putative PRL-releasing factor[1] is present in synaptic afferents to TIDA neurons, and that TIDA neurons express the mRNA encoding for TRH receptor, merit special interest from the viewpoint of the central regulation of PRL secretion. Although the role of TRH as a major PRL-releasing factor is controversial[23,30], TRH has long been known to be a potent secretagogue of PRL[3,5,9,21,34]. A great deal of evidence suggests a complex interplay between hypothalamic dopamine and TRH in the stimulatory regulation of PRL secretion, under various physiological and pharmacological stimuli. In ovariectomized and estrogen-primed rats, a decrease in dopamine is followed by an increase in TRH in the hypophysial stalk blood during the PRL surge. Likewise, a transiently decreased dopaminergic tone at the anterior pituitary increases the potency of TRH to release PRL in the lactating rat[6,8,29]. This dual mechanism involving reciprocal regulation of dopamine and TRH releases has also been proposed as a cause of the steroid-stimulated PRL surge[10], as well as PRL hypersecretion during pregnancy[12] and pseudopregnancy[11].

Our data increase the knowledge of the hypothalamic connectivity of neuronal systems which regulate PRL secretion via TIDA neurons; nevertheless, at present, the precise role of this interaction is not understood. In the domestic fowl, the dopamine turnover in the medial basal hypothalamus is increased following intracerebroventricular administration of TRH[13]. This observation suggests a stimulatory action of TRH upon dopamine neurons of the AN in this species. Hypothetically, if TRH axons also exert a stimulatory action upon TIDA neurons in the rat, this might prevent prolonged inhibition of TIDA neurons by the ascending catecholaminergic[15], serotonergic[22] or other afferent neuronal pathways during episodes of PRL hypersecretion. On the contrary, if the function of TRH-containing synaptic terminals is inhibitory upon dopaminergic neurons, TRH might have a central, in addition to the widely known pituitary site of action, whereby it increases PRL secretion.

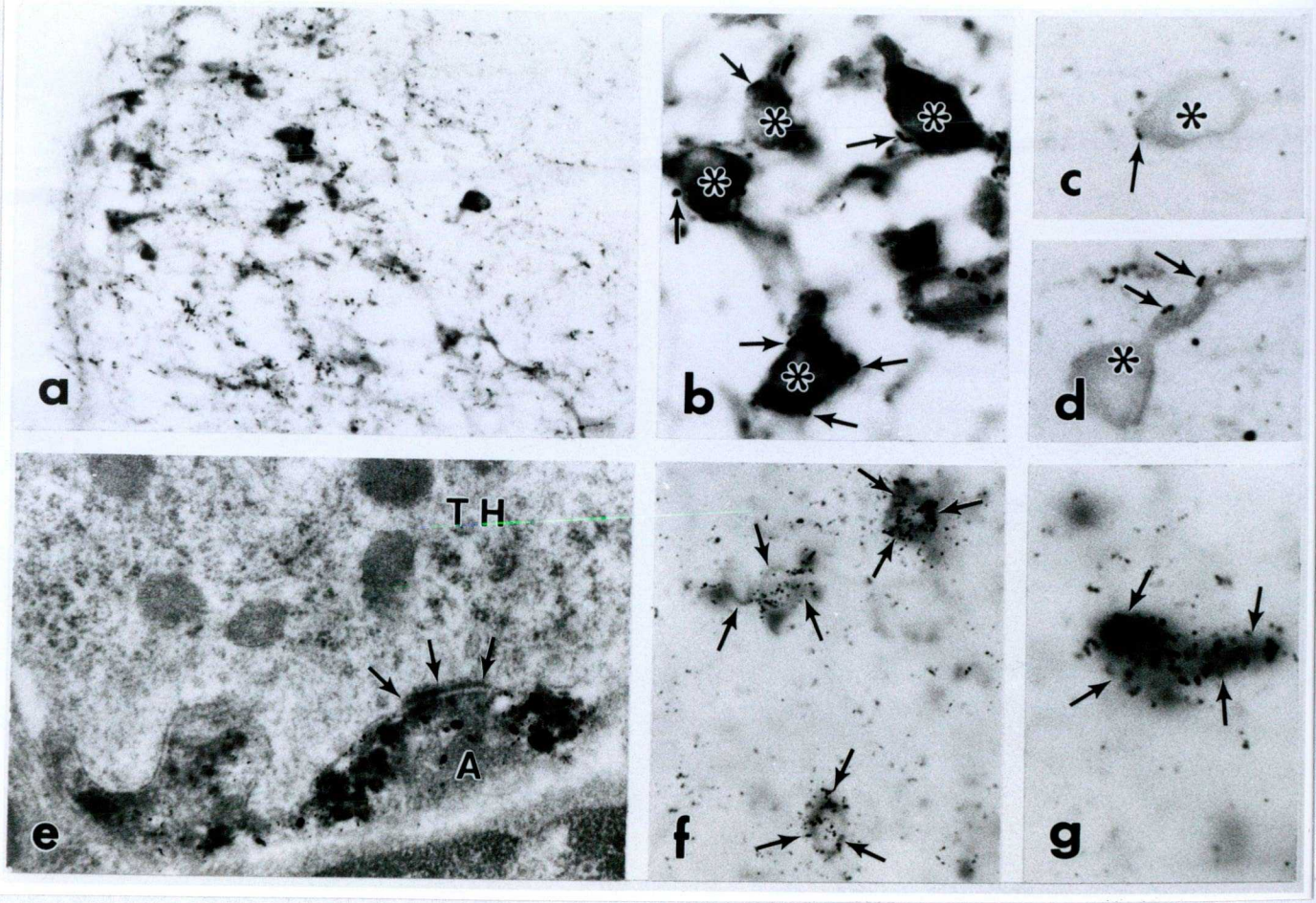
Three different animal models, the male, the ovariectomized female, and the ovariectomized and estrogen-primed female rat were tested in light microscopic dual-label ICC studies to reveal crude differences in the innervation pattern and density of TIDA neurons by TRH-IR axons. It seems likely that sensitivity of immunocytochemical approach is too low to reveal subtle quantitative differences depending on the sex or hormonal status of the animal.

The studies of the dorso-medial arcuate nuclei in all cases demonstrated that virtually all TH-IR neurons were contacted by TRH-IR axons and the innervation in the ventro-lateral AN was more scarce. The combined ICC-ISH studies of male rats showed that TRH receptor mRNA was also expressed preferentially in the dorso-medial subdivision of the AN, and only occasionally in its ventro-lateral subdivision.

The precise origin of TRH-IR axons regulating TH-IR neurons of the AN is still to be determined. Earlier studies revealed a 65% depletion of the TRH content in the AN following bilateral lesions of the paraventricular nucleus[4], indicating that TRH neurons of the PVN are involved in the innervation of TIDA neurons. It still remains to be clarified whether TRH neurons of the PVN that innervate the TIDA system represent a separate group from the hypophysiotropic population of TRH neurons[20]. If the opposite is true and the same paraventricular TRH neuron population fulfills both functions, fibers synapsing with TH-IR neurons of the AN might be either collaterals or "en passant" contacts of axons with a final destination in the external zone of the ME.

The expression of TRH receptor mRNA in cell bodies of TH-IR neurons indicates that TRH-IR synaptic terminals influence TIDA neurons via these receptors. Although, the synaptic pathway reported in our studies might only represent one type of potential interactions between TRH and the TIDA system. Other ways of neuronal communication might include volume transmission and/or regulation of TIDA axon terminals by TRH-secreting axons at the level of the ME. In fact, the latter hypothesis seems to be corroborated by our present study, in which we observed similar distribution patterns of TRH- and TH-IR axons in the ME, and also dual-labeled axo-axonic contacts were frequently observed in this area.

In conclusion, we described a synaptic connection between TRH-IR axons and TH-IR neurons in the hypothalamic AN and identified the mRNA encoding for TRH receptor in TH-IR neurons of the TIDA system. These results support the hypothesis that TRH-containing neurons directly regulate TIDA neurons. Thus, TRH has at least two sites of action to regulate PRL release: (i) It stimulates the secretion of PRL via a direct action on lactotrophs, and (ii) it influences TIDA functions at the level of the hypothalamus via a synaptic mechanism.



LEGEND

Synaptic regulation of tyrosine hydroxylase (TH)-immunoreactive (IR), dopaminergic neurons by thyrotropin-releasing hormone (TRH) in the hypothalamic arcuate nucleus (AN) of the male rat

Fig. a: Dual-label immunocytochemical detection of TRH-IR axons (black axon varicosities, immunostained with silver-gold-intensified diaminobenzidine [DAB]) and TH-IR neurons, stained with DAB chromogen (gray perikarya and dendrites). **Fig. b:** Juxtapositions of TRH-IR axons (arrows) to perikarya and dendrites of TH-IR neurons (asterisks). **Fig. c:** Semithin section exhibiting a neuronal contact between a TRH-IR axon (arrow) and a TH-IR cell body (asterisk). **Fig. d:** Juxtapositions of TRH-IR axons (arrows) to the dendrite of a TH-IR neuron (asterisk). **Fig. e:** Asymmetric synaptic specialization between the apposed surfaces of a TRH-IR axon (A) and a TH-IR perikaryon (TH). Arrows point to the postsynaptic density. **Figs. f and g:** Co-localization of TRH receptor mRNA (autoradiographic grains) with TH in immunostained perikarya of the dorso-medial (Fig. f) and ventro-lateral (Fig. g) aspects of the AN. Arrows indicate the contours of dual-labeled neurons. (Bars in a-d, f and g =10μm; Bar in e=250nm.)

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VIII.

Expression of estrogen receptor-β mRNA in oxytocin and vasopressin neurons of the rat supraoptic and paraventricular nuclei

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ABSTRACT

The regulatory actions of estradiol on magnocellular oxytocin (OT) and vasopressin (VP) neurons of the paraventricular (PVN) and supraoptic (SON) nuclei are well documented. To date it is still debated whether the effect of estrogens is exerted directly or mediated by estrogen-sensitive interneurons. Previous immunocytochemical (ICC) and *in situ* hybridization (ISH) studies detected either low levels or absence of the classical estrogen receptor (ER-α) in the PVN and the SON of the rat. The present experiments using a combined ICC and ISH method were undertaken to examine the expression of the recently cloned beta form of ER (ER-β) in OT- and VP-immunoreactive (IR) neuronal systems of the rat hypothalamus. The results demonstrate that the highest cellular levels of ER-β mRNA in OT-IR neurons can be visualized in the caudal portion of the PVN and in an area ventro-medial to the central core of VP-IR cells. These neurons were previously shown to project caudally to the brainstem and the spinal cord to regulate autonomic functions. In addition, the whole rostro-caudal extent of the PVN and the SON contained OT-IR neurons that co-expressed variable levels of ER-β mRNA. Similarly, the presence of ER-β mRNA was evidenced in a large population of VP-IR paraventricular and supraoptic neurons. In the SON, somewhat stronger hybridization signal was detectable in VP-IR neurons as compared with OT-IR neurons.

Together, these findings provide strong support for the concept that the functions of OT- and VP-IR neurons in the PVN and the SON are regulated directly by estrogen and that the genomic effects of estrogens in these neurons are mediated by ER-β.

Introduction

The magnocellular neurosecretory system consists of vasopressin (VP)- and oxytocin (OT)-synthesizing neurons which send axon projections to the neurohypophysis from the hypothalamic supraoptic and paraventricular nuclei (SON; PVN) (1). While the main function attributed to VP is the central control of water and electrolyte balance (2), OT is primarily involved in the regulation of reproductive functions, including parturition (3), sexual (4; 5) and maternal (6) behavior, and the ejection of milk (7). The neurohormone output from OT-ergic and VP-ergic neurons is controlled partly by neuronal mechanisms (7). Among the humoral agents influencing OT and VP neurons, the actions of ovarian steroids have been studied extensively. The observations that the expression of OT mRNA in magnocellular neurons increases with the onset of puberty and decreases following castration (8), and that the cellular levels of OT mRNA exhibit variations during the estrus cycle (9) indicate that estrogen regulates the expression of OT. Furthermore, increased VP-, in addition to OT mRNA levels were detected in the SON of pregnant and lactating rats (9). While the effects of estrogen on magnocellular functions are well documented, the site of estrogen action, direct or interneuron-mediated, remains a question of controversy. Although ER immunoreactivity was colocalized with the majority of OT neurons in the guinea-pig (10), ligand binding (11, 12), ICC (13) and ISH (14) studies failed to demonstrate estrogen-receptivity of most magnocellular neurons in the rat. New evidence by Herbison indicates that ER-IR GABA-ergic neurons located in the perinuclear zone of the SON might mediate, at least in part, the effect of estrogen on OT neurons in this species (13).

Recently, a new estrogen receptor, termed ER-β has been cloned from the rat prostate gland (15). *In situ* hybridization studies with ER-β-specific cRNA probes have established a wide distribution of ER-β mRNA in the hypothalamus (16) and brain (17) of the rat. An interesting finding of these experiments was the high level of ER-β mRNA in the PVN and

the SON. The aim of the present studies was to analyze the expression of ER-β mRNA in OT- and VP-IR neurons of the hypothalamic PVN and SON, by means of a combined ICC and ISH method (18).

Materials and methods

Preparation of hybridization probes

The preparation of hybridization probes was described earlier (16). Briefly, a 285 and a 558 base-pair fragments (bases 52-610 and 1809-2094, respectively) of the rat ER-β cDNA (15) were selected as templates for transcription of cRNA probes and subcloned into the EcoRI site of the pBluescript plasmid vector (Stratagene, La Jolla, CA). The vectors were linearized with BamHI and antisense RNA probes were transcribed from the T7 promoter in the presence of ^{35}S-UTP. To increase detection sensitivity of the hybridization procedure, the two probes were preferentially combined in a hybridization cocktail, instead of using a single probe. A series of preliminary experiments detailed elsewhere verified the specificity of these hybridization probes (16).

ISH detection of ER-β mRNA by autoradiography

For single-labeling ISH studies of ER-β mRNA expression, fresh-frozen sections including the hypothalamic PVN and SON of the ovariectomized female rat were used as described earlier (16). For single-cell analysis of the hybridization signal, the autoradiograms were visualized on Kodak NTB-3 photograhic emulsion.

Combined use of ICC and ISH in the same sections

The methodology of combined ICC and ISH was modified from a previous protocol (18).

Animals

Five female Wistar rats (200-220 g) were maintained in a controlled environment (lights on 5h, off 19h, 22 °C), with food and water available ad libitum. The animals were anesthetized with Avertin (1ml/100g bw), and gonadectomized bilaterally. On postovariectomy day 12, the

animals were reanesthetized and sacrificed by transcardiac perfusion using a short flush with 0.1 M phosphate buffered saline (PBS, pH 7.6), followed by 400 ml of a 4% paraformaldehyde solution in PBS. Small tissue blocks containing the hypothalamic SON and PVN were dissected from the brains, infiltrated with gradually increasing concentrations of sucrose through 30% (for 24 h total) and snap-frozen on dry-ice. Then, 20-μm thick coronal sections were prepared on a freezing microtome (Reichert-Jung), collected and stored permanently in cryoprotectant solution at -20 °C (19) until use.

Preincubation, hybridization and washes

Immediately before hybridization, the sections were removed from the cryoprotectant solution and rinsed thoroughly in 2X standard saline citrate solution (2XSSC; 1XSSC=0.15M NaCl/0.015M sodium citrate, pH 7.0). Subsequently, they were pretreated with proteinase K (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany; 1μg/ml, dissolved in 2XSSC at room temperature) for 30 min, postfixed with 4% paraformaldehyde for 5 min to stop the digestion, rinsed shortly in 2XSSC, and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0)/0.9% NaCl for 20 min. Finally, the sections were rinsed in 2XSSC and transferred into hybridization solution.

During the hybridization, the sections were incubated in microcentrifuge tubes containing the hybridization cocktail [50% formamide, 4XSSC, 10% dextran sulfate (500,000 MW; Sigma Chemical Company, St. Louis, MI), 1XDenhardt's solution, 500μg/ml heparin sodium salt (Sigma Chemical Company), 0.5 mg/ml yeast tRNA (Boehringer Mannheim GmbH, Mannheim, Germany), 0.4 mg/ml sheared single-stranded salmon sperm DNA (Boehringer Mannheim GmbH), 200 mM dithiothreitol, and 30 000 cpm/μl of each hybridization probe.]. The tubes were sealed with Parafilm to exclude atmospheric air and placed in an incubator for hybridization overnight at 52 °C.

In the morning, the hybridized sections were rinsed in 1XSSC for 10 min, followed by a 1-hour stringency-wash in 50% formamide/2XSSC at 50 °C and a 1-hour digestion of excess

hybridization probes with RNAse A (Boehringer Mannheim GmbH; 100μg/ml in 2XSSC buffer) at 37 °C. Finally, two sequential stringent treatment steps (1 hour each) were performed in 50% formamide/2XSSC solutions at 50 °C and the sections were rinsed in 0.1M PBS with 0.2% Triton X-100.

Immunocytochemical detection of oxytocin and vasopressin

Before the immunocytochemical detection of either OT or VP was carried out, the sections were immersed in 0.5% H_2O_2 in PBS for 10 min and blocked with 2% bovine serum albumine (BSA) against non-specific antibody-binding for 20 min. The primary monoclonal antibodies for neurophysin-OT (PS-38; 1/1000, kindly provided by Dr. Sharon Key) were diluted with 2% bovine serum albumin (BSA) in PBS and applied to the sections for 12 h at 4^{o}C. The tissue-bound antibodies were reacted with biotinylated antimouse IgG (1/800; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), then with streptavidin-peroxidase complex (1/2000; Jackson ImmunoResearch Laboratories, Inc.) for 1h each. The antiserum against VP (Incstar Corp., Stillwater, MN) was raised in a rabbit and applied to the sections at a 1/4000 working dilution for 12 h. The detection protocol included sequential incubation of sections in the antirabbit IgG (1/500, Arnel Products Co., Inc., New York, NY.) and then, in the peroxidase-antiperoxidase complex (1/2000, Dako Corporation, Carpinteria, CA) solutions for 1h each. Finally, OT- and VP-IR neurons were visualized in a developer containing 0.01% diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Chemical Company) and 0.003% H_2O_2 in 0.1 M Tris-HCl buffer (pH 7.6).

Detection of the hybridization signal

The immunostained sections were rinsed in PBS for 5 min, transferred briefly into 0.3% Elvanol with 300 mM ammonium acetate, mounted onto precleaned double gelatin-coated microscopic slides and air-dried. They were then dehydrated in a graded series of ethanol through 100%, dried and then dipped into photographic emulsion (LM-1; Amersham

International plc, Amersham, UK) and exposed for 3-6 weeks at 4 °C. The slide autoradiograms were developed with Kodak D19 developer for 3 min, rinsed briefly with distilled water, then fixed with Kodak fixer for 5 min. Finally, the sections were dehydrated in ethanols, cleared in xylenes and coverslipped with Permount.

Mapping of double-labeled cells

The immunocytochemical distribution of OT- and VP-IR neurons did not seem to respect the borders of the classical anatomical subnuclei of the PVN. Moreover, a high percent of OT-IR neurons occurred in the classical parvocellular subnuclei of the PVN (20). Therefore, the topographical analysis of double- and single-labeled neurons in the PVN followed a simplified terminology adapted from a previous immunocytochemical study of OT- and VP-IR neurons, which subdivided the PVN into a rostral (rPVN) a middle (mPVN) and a caudal (cPVN) portion (21).

In the caudal portion of the PVN where the highest cellular levels of ER-β mRNA were detected, a semiquantitative estimate of the numbers of ER-β-positive OT-IR neurons was made. From sixteen sections representing the cPVN of four animals, a total of 543 OT-IR neurons were analyzed for co-expression of ER-β mRNA.

Results

Single-labeling studies

Following 3-6 weeks of autoradiographic exposure, a clear hybridization signal was detectable in all three rostro-caudal subdivisions of the PVN and the whole rostro-caudal extent of the SON. In order, the strongest labeling of neurons was observed in the cPVN (Fig. 1a), then in the mPVN (Fig. 1b) and finally, in the rPVN (not shown) and the SON (Fig. 1c). In the SON, the signal had a tendency to be slightly higher in the ventral than in the dorsal part of the nucleus.

Colocalization studies with combined use of ICC and ISH

The method of combined ICC and ISH was used for the simultaneous visualization of OT- or VP-immunoreactivity and ER-β mRNA (Fig. 2).

Paraventricular nucleus

Corroborating the results of single-labeling experiments, the peak ISH signal intensity was observed in the cPVN. The majority of the high ER-β mRNA-expressing neurons in the cPVN were immunoreactive for OT (Figs. 2a, b). Semiquantitative analysis of OT neurons further revealed that 93± 6.8 % of them also expressed ER-β hybridization signal. In addition, the few VP-IR neurons that intermingled with OT neurons in the cPVN often coexpressed detectable, albeit relatively lower levels of ER-β mRNA. In the mPVN, the strongest hybridization signal was detected in cells situated ventro-medial to the central core of VP neurons, and dual-labeling studies with the OT antiserum have established that most of them were OT-ergic. Variable levels of ER-β mRNA were revealed in different populations of OT- as well as VP-IR (Fig. 2c) neurons. Subsets of OT- and VP-IR neurons in the mPVN did not contain detectable hybridization signal. Similarly, the population of OT-IR neurons in

the rPVN consisted of a mixture of ER-β mRNA-expressing and ER-β mRNA negative neurons, reflecting either lack of ER-β-coexpression in some OT-ergic neurons or a limitation of the method.

Supraoptic nucleus

In the SON, ER-β mRNA was coexpressed both in VP- (Figs. 2d, e) and OT-IR (Fig. 2f) neurons. Somewhat higher cellular levels of the receptor mRNA were seen in VP as compared with OT neurons. Dual-labeled cells appeared along the rostro-caudal extent of the nucleus. A longer exposure time was needed to visualize the hybridization signal in the SON, compared with the PVN.

Discussion

The results of the studies described herein have demonstrated that ER-β mRNA is expressed in OT- and VP-IR neurons of the hypothalamic PVN and SON.

Substantial data support an important role for estradiol in the regulation of magnocellular OT and VP neurons. The concentration of OT in the pituitary (9) and in the hypophyseal portal blood (22) as well as OT mRNA levels in the SON (9) change throughout the estrus cycle and estrogen treatment of ovariectomized rats increases plasma OT levels (23). In addition, VP mRNA in the SON is elevated in the lactating and the pregnant rat (9) and lactation also increases the percent ratio of OT neurons that coexpress VP mRNA in this nucleus (24). The effects of estradiol on the magnocellular systems are well documented, although whether they are direct or interneuron-mediated, have long been a question of controversy. In the guinea pig, most OT neurons of the PVN and the SON contain immunoreactive ER (10), supporting the hypothesis of a direct effect of estrogen. In contrast, few magnocellular neurons were found to contain ER immunoreactivity in sheep (25) and monkeys (26). In rats, only a restricted population of paraventricular neurons concentrate tritiated estradiol (11; 12). Similarly, ISH studies of the rat hypothalamus have revealed the absence of ER-α mRNA in the SON and only low levels of its expression in the anterior magnocellular subnucleus of the PVN (17). Therefore, the concept that the actions of estrogen on OT and VP neurons are mediated by estrogen-sensitive interneurons has been raised. Accordingly, an ER-IR GABA interneuron population located in the perinuclear zone of the SON has been implicated in the circuitry whereby estrogens might regulate OT neurons of the SON (13).

Recently, a second form of ER termed ER-β has been cloned from rat prostate (15). Using ISH with complementary probes to the ER-β mRNA, the anatomical distribution of ER-β mRNA-expressing cells has been mapped in the hypothalamus (16) and brain (17) of the rat.

A surprising observation of these studies was the wide distribution of ER-β mRNA in the PVN and the SON. We now show that a large percent of OT- and VP-IR neurons in these nuclei coexpresses ER-β mRNA. The highest hybridization signal was revealed in the cPVN, where 93±6.8 % of OT-IR neurons expressed detectable levels of ER-β mRNA. Interestingly, this topographical distribution of double-labeled OT neurons highly resembles the map of neurophysin-containing neurons which exhibit nuclear uptake of tritiated estradiol (12), and project caudally to the medulla and/or spinal cord to influence autonomic functions (20). Nevertheless, the area populated by ER-β-positive OT neurons is larger and includes the whole rostro-caudal extent of the PVN as well as the SON, comprising regions that project to the posterior lobe of the pituitary gland. Most VP-ergic neurons expressing ER-β mRNA were localized in the middle portion of the PVN (mPVN), and ventrally in the SON. To establish the precise role of ER-β in functionally distinct subpopulations of OT and VP neurons and to identify parvicellular neuropeptide systems which express ER-β mRNA in the subnuclei of the PVN, further studies will be required. The genes regulated by ER-β in OT and VP neurons are presently unclear. However, identification of ER-β mRNA in OT neurons, most of which do not express ER-α, might explain the presence of functional estrogen-response elements on the promoter of the OT gene, itself (27). Among the neuropeptides that have been colocalized in magnocellular VP neurons (28), galanin might represent a putative target for regulation by ER-β because of the presence of estrogen-response elements on the preprogalanin gene (29).

The method of combined ICC and ISH has been used successfully earlier for the colocalization of preprogalanin mRNA with c-Fos- and LHRH immunoreactivities in the preoptic area of the female rat (18). In the present application of the combination technique several efforts have been made to maximize the sensitivity of the ISH detection component. This seemed to be especially important in view of the relatively low cellular abundance of

ER-β mRNA. Modifications we have introduced to the protocol included a proteinase K digestion step prior to hybridization in order to facilitate access of probes to the target mRNA and combination of two antisense hybridization probes in the hybridization cocktail, instead of using a single cRNA probe. These changes resulted in a largely increased sensitivity of the hybridization.

In summary, the present studies have localized ER-β mRNA in paraventricular and supraoptic OT-IR and VP-IR neurons of the rat. These new data suggest that estrogen directly regulates gene(s) in OT-IR and VP-IR neurons.

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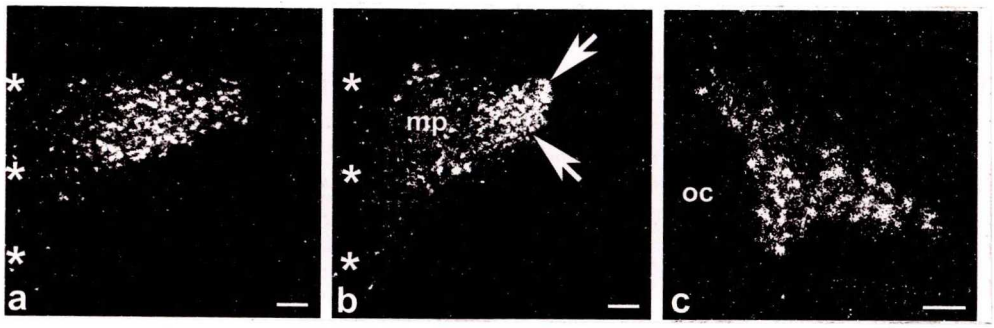

FIG. 1. Detection of ER-β mRNA in representative sections of paraventricular (PVN) and supraoptic (SON) nuclei by ISH. The expression of ER-β mRNA in the PVN is highest in the caudal part (a) and attenuated in the middle part of the nucleus (b). The receptor signal is the strongest in the lateral, magnocellular subdivision (arrows), while the medial, parvocellular subdivision (mp) is weakly labeled. In the SON (c) the hybridization signal is slightly higher in the ventral than in the dorsal part of the nucleus. *, third ventricle; **oc**, optic chiasm. Scale bars: 150μm (in a, b) and 100μm (in c).

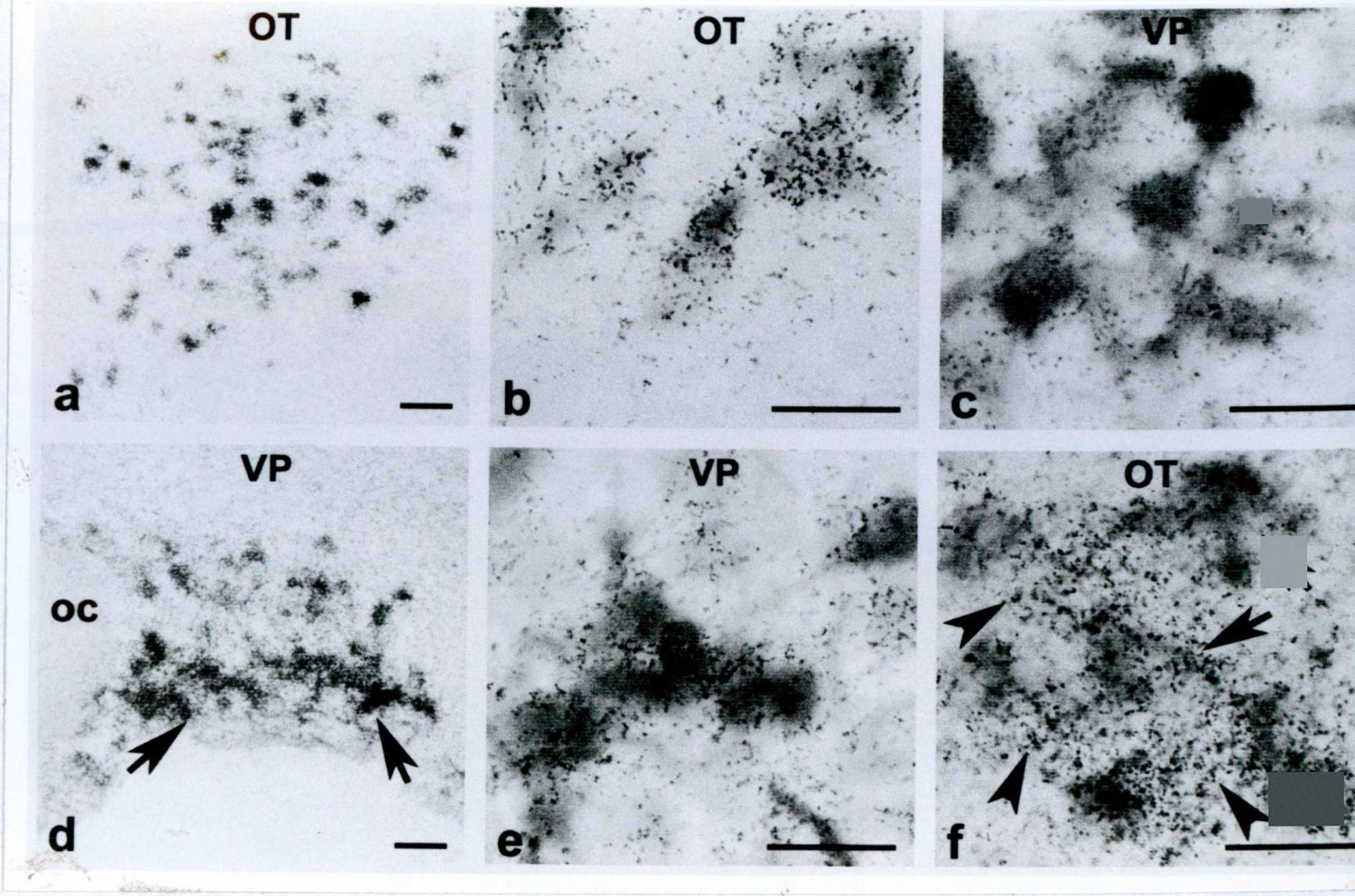

FIG. 2. Expression of mRNA encoding the β type of ER in OT- and VP-immunoreactive (IR) perikarya located in the hypothalamic paraventricular (PVN) (a,b,c) and supraoptic (SON) (d,e,f) nuclei of OVX rats. **oc**, optic chiasm; **OT**, oxytocin; **VP**, vasopressin. (a) Most of the OT-IR neurons in the caudal part of PVN exhibit strong hybridization signal for ER-β mRNA. In this part of the nucleus the OT-IR neurons are rather scattered than forming a solid mass. (b) OT-IR/ER-β mRNA and (c) VP-IR/'ER-β mRNA double labeled cells at higher magnification in the PVN. (d) ER-β mRNA expression in VP-IR neurons of SON. The autoradiographic silver grains are mainly clustered over VP-IR neurons located in the ventral part of the nucleus (arrows). (e) VP-IR/ER-β mRNA and (f) OT-IR/ER-β mRNA double labeled cells shown at a higher power in the SON. In (f) arrows point to double labeled cells, arrowheads mark immunonegative cells that express the ER-β mRNA. Scale bars: 60μm (in a, d) and 25μm (in b, c, e, f)