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An «in vitro» model for combined functional and morphological investigations on the rat pituitary gland

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Summary

We have developed an alternative model for combined «in vitro» functional and morphological studies on the rat pituitary gland. To test its use for biochemical investigations we studied some aspects of the secretion in pituitary thyrotrophs under basal conditions and after stimulation with TRH. After stabilisation of basal TSH release, the stimulation with TRH significantly increased the TSH secretion. To test the usefulness of our model for morphological investigations the tissue was processed for conventional and immunocytochemical light and electron microscopy at the end of the incubation. Different types of pituitary cell resulted to be well preserved. Our model allows combined biochemical and morphological studies on the pituitary gland and thus reduces the number of animal groups, eliminates the extrapolation of results from different groups and replaces the «in vivo» by an «in vitro» experiment.

Key words: **pituitary – «in vitro model» – Thyrotropin-stimulating hormone – morphology – immunocytochemistry – electron microscopy – secretion.**

Ein «in vitro» Modell für kombinierte funktionelle und morphologische Untersuchungen der Hypophyse der Ratte

Wir haben ein «in vitro» Modell für kombinierte funktionelle morphologische Untersuchungen der Rattenhypophyse entwickelt. Um die Anwendbarkeit dieses Modelles in biochemischen Studien zu testen, haben wir einige Aspekte der Sekretion der hypophysären thyreotropen Zellen unter basalen Bedingungen und nach TRH-Stimulation untersucht. Die Stimulation mit TRH hat eine signifikante Erhöhung der TSH-Sekretion induziert. Um abzuklären, ob unser Modell auch für morphologische Untersuchungen brauchbar ist, haben wir das Gewebe am Ende der Inkubation für konventionelle und immunzytochemische licht- und elektronenmikroskopische Studien weiter bearbeitet. Die verschiedenen Typen der hypophysären Zellen haben einen befriedigenden Erhaltungszustand aufgewiesen. Die erhaltenen Resultate erweisen, dass sich unser Modell sowohl für biochemische als auch für morphologische Studien ein und derselben Hypophyse eignet; dazu wird dank Anwendung dieses Modelles die Anzahl der notwendigen Tiergruppen reduziert, die Extrapolation der Resultate zwischen den verschiedenen Gruppen eliminiert und «in vivo» werden durch «in vitro» Untersuchungen ersetzt.

Schlüsselwörter: **Hypophyse – «in vitro Modell» – Thyreotropin – Morphologie – Immunohistochemie – Elektronenmikroskopie – Sekretion.**

Introduction

The combination of biochemical with morphological investigations can substantially contribute to reduce the number of laboratory animals used for the study of the pituitary gland. Several *«in vitro»* systems are available to study the pituitary secretion under basal conditions and after stimulation with different hypothalamic releasing factors (Arimura and Shally, 1975; Mitsuma et al, 1990; Vale and Grant, 1975). However, the structure of the incubated pituitary gland was seldom investigated. Farquhar et al. (1975), reported that in hemipituitary glands «the cellular fine structure was reasonably well preserved up to 1 hour of incubation», but «with longer incubation times there was progressive deterioration in the condition of the tissue, in that the center of the pieces showed increasing nuclear pycnosis, autophagy, and cell death». Perez and Hymer (1990) described a tissue slicing method for the study of the function and position of somatotrophs in the rat pituitary gland and reported that, 1 hour after gyrotory incubation, by light microscopy the tissue showed good cell integrity, and by ultrastructural study the characteristics of the adenohypophyseal cells were maintained. They also examined the basal GH secretion from pituitary slices placed in gyrotory incubation for three consecutive 20-min periods and stated that «in preliminary experiments GHRH had no effect on GH release from tissue placed in gyrotory incubation».

The morphological evaluation of the *«in vitro»* treated pituitary tissue is, among others, essential to confirm the biochemical results, because only data obtained from well preserved tissue are reliable. Moreover, the use of the same gland for both biochemical and morphological studies a) allows morphofunctional correlations b) reduces the number of animals per experiment, and c) avoids to extrapolate results from different groups. Finally, the use of hemipituitary glands instead of dispersed cells allows the study of different cell types in their natural anatomical environment.

Boujon et al. (1987) developed an *«in vitro»* model for morphofunctional investigations of the mediobasal hypothalamus. We have modified this model and applied it to investigate some aspects of various types of pituitary cells.

Animals, Materials and Methods

Animals

The study was undertaken on 8 male albino rats (360 ± 32.1 g body weight). The animals were housed under standard environmental conditions at 22° C with controlled lighting (on at 0600, off at 1800). Standard rat diet and tap water were available ad libitum. The rats were decapitated between 0800–1030 under halothane anesthesia. The skull was opened and the pituitary gland rapidly removed and bisected sagittally under a stereomicroscope (5×), without removing the neurohypophysis. Pilot experiments with entire or quartered pituitary glands gave unsatisfactory results.

Incubation

The *«in vitro»* system is similar to that previously described by Boujon et al. (1987), with some modifications. Briefly, a gas bottle containing 95% O_2 and 5% CO_2 connected through vinyl tubes to two massflowmeters (model 5850TR; Brooks Instrument BV, Veenendaal, The Netherlands) and coupled with two massflow controllers and reading systems (model 5876; Brooks Instruments BV) conveyed 4 ml/min gas mixture through vinyl tubes into two humidification bottles. From each bottle, a silicon capillary tube conveyed the gas to an incubation chamber. After bubbling through the medium the gas exited the incubation chambers through two needle segments mounted on the stopper. We used an incubation and a reserve chamber, each connected to the massflow controller and to the reading system. The two chambers were supported by an acrylic rack mounted in a shaker water-bath at 37° C and 140 oscillations per min. Each chamber contained 0.4 ml of Locke's medium. The basal medium had the following composition: 2 mM Hepes, 154 mM NaCl, 5,6 mM KCl, 1 mM $MgCl_2$, 6 mM $NaHCO_3$, 10 mM glucose, 1.25 mM $CaCl_2$, and 2×10^{-2} mM bacitracin (Sigma, St. Louis, MO, USA).

Four pituitary glands were incubated in basal medium alone and four stimulated with 3 ng/ml TRH (Bachem, Bubendorf, Switzerland).

The two pituitary halves of each rat were incubated together in the same chamber, according to the following schema: two 10-min washings and one 20-min preincubation in basal medium, and two 20-min incubations either in basal medium alone or with added TRH.

At the end of each incubation period the medium was collected in tubes and frozen at -80° C. The tubes were previously washed in Tween 20 diluted 1:20 in distilled water and then rinsed several times in distilled water.

Biochemical studies

The TSH released into the medium during the two incubation periods was quantified by double-antibody radioimmunoassay (RIA), using a commercial kit (Repromed, Hamburg, Germany). The TSH standard used was NIADDK-r-TSH-RP-2. Briefly, 200 µl of medium diluted 1:2 with PBS-BSA-buffer were incubated for 24 h at 4° C with rabbit anti-rat TSH diluted 1:8000 in PBS-BSA. Then [^{125}I]-rat TSH (total activity 10 000 cpm/100 µl) was added and incubated for 24 h at 4° C. Incubation for 15 min at room temperature with goat anti-rabbit antiserum diluted 1:50 in phosphate buffer followed. The separation of bound from free TSH was achieved by centrifugation for 15 min at 2000 g. All samples were assayed in duplicate. The counting time was 1 min.

Tissue processing

At the end of the incubation, the two pituitary halves were fixed in 2% glutaraldehyde/1,5% paraformaldehyde in phosphate buffer (pH 7.4). After rinsing in S-collidine buffer, a 0.3 mm frontal slice was cut from each pituitary half at the level of its maximal height (Pitton and al., 1987). Subsequently, one slice from each hemipituitary was postfixed in 2% osmium tetroxide (pH 7.4) for 2 h; the other was not postfixed and used for immunocytochemistry. All specimens were embedded in Spurr's low viscosity medium, and semithin sections were cut. The sections from postfixed slices were stained by toluidine blue. Thin sections of both kinds of specimens were then cut for electron microscopy.

Immunocytochemistry

The immunocytochemical labelling of GH, PRL, ACTH, TSH, FSH, and LH was performed on semithin sections as described by Bestetti et al. (1987). Briefly, after etching in Mayor and acetic acid (1:7), (Mayor et al., 1961) inhibition of the endogenous phosphatase, the sections were incubated with rabbit anti-human GH (Dako Corporation, Santa Barbara, CA, USA), anti-human PRL (Dako Corporation), anti-human ACTH (Dako Corporation), anti-human TSH (Dako Corporation), anti-bovine FSH (UCB-Bioproducts, Brussels, Belgium), and anti-human LH (Dako Corporation) antisera, all diluted 1:200 in PBS, and goat anti-rabbit IgG labelled with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, USA), diluted 1:100 in acetate buffer. The sections were finally washed in PBS-Tween 20 (1:200). To assay the immunoreaction specificity, we either omitted the primary antisera or preabsorbed it with specific purified rat hormones (4 µg/100 µl; UCB-Bioproducts) according to the protocol of Lloyd and Childs (1988).

Electron microscopy

Thin sections from postfixed specimens were counterstained with uranyl acetate and lead citrate.
To demonstrate TSH, thin sections were cut from not postfixed specimens and stained for immunocytochemistry as described by Bestetti et al. (1987), using rabbit anti-human TSH (Dako Corporation) diluted 1:200, and goat anti-rabbit IgG conjugated to 15 nm colloidal gold particles (Jansen Life Science Products, Beerse, Belgium) diluted 1:40 in PBS-Tween 20 (1:200). No etching was performed.

Statistics

Group mean and standard error (SEM) were calculated. The significance of differences between groups was tested by the Kruskall-Wallis *H*-test.

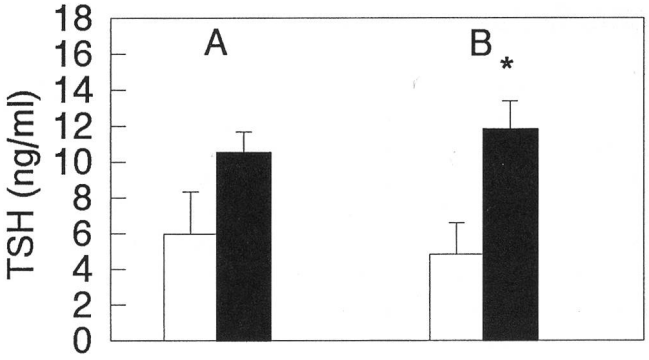

*Figure 1: TSH release during incubation 1 (A) and 2 (B) in basal medium (empty) and after stimunation with TRH at a dose of 3 ng/ml medium (solid bars). * = $p < 0.05$*

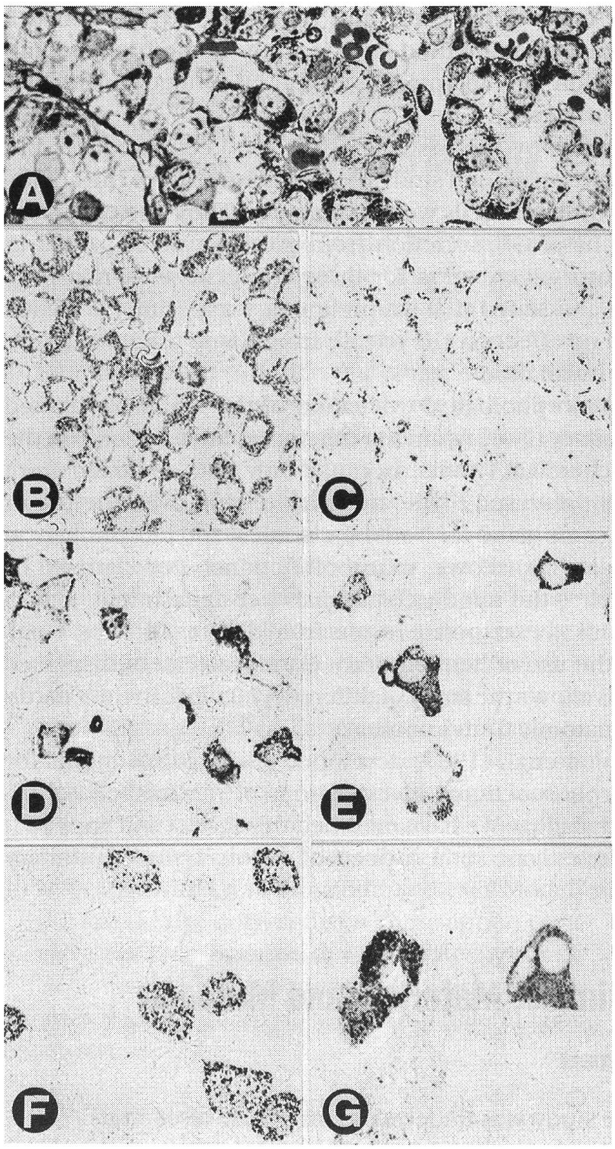

Figure 2: Conventional (A) and immunocytochemical (B–G) light microscopy of pituitary tissue after incubation. Toluidine blue staining (A). Anti-GH (B), -PRL (C), -ACTH (D), -TSH (E), -FSH (F), and -LH (G)-alkaline phosphatase method. 530 X.

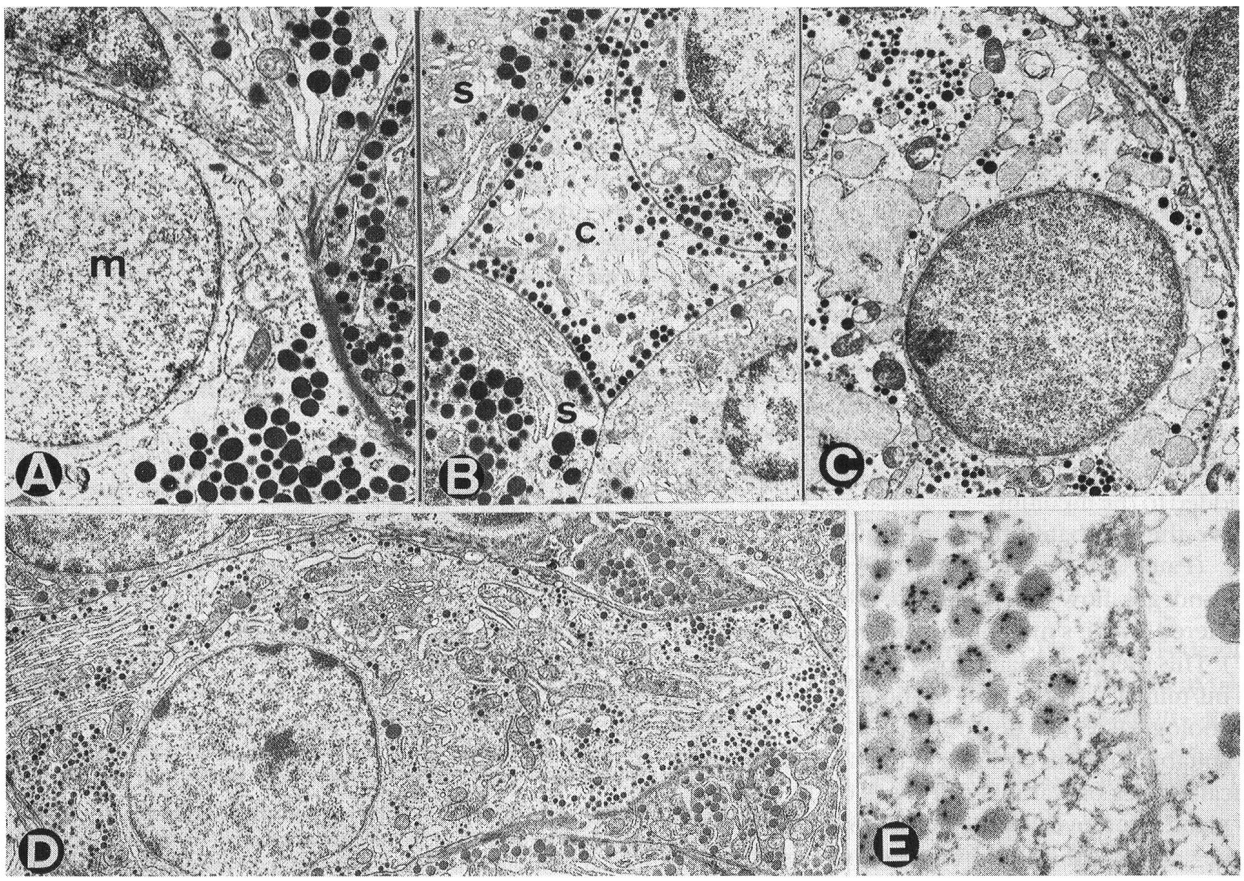

Figure 3: Conventional (A-D) and immunocytochemical (E) electron microscopy of pituitary tissue after incubation. Mammotroph (A, m 6,600 X), somatotroph (B, s) and corticotroph (B, c, 6,600 X), gonadotroph (C, 6,600 X), and thyrotroph (D, 4,500 X). Thyrotroph with secretory granules labeled by anti-TSH (colloidal gold method, E, 30,000 X).

Results

Biochemistry

The amounts of TSH released during the two incubation periods in basal conditions were similar (fig. 1). The addition of 3 ng/ml TRH into the medium resulted in a markedly increased TSH release during the second incubation period (fig. 1).

Morphology

By conventional light microscopy of semithin sections, the incubated tissue was well preserved at the periphery and in the center of the pituitary halves. (fig. 2 A). There was no sign of cell degeneration or necrosis. The nuclei were normal, the nucleoli clearly visible, and the cytoplasm was granulated.

By light microscopical immunocytochemistry, somatotrophs, lactotrophs, corticotrophs, thyrotrophs, and gonadotrophs could be labelled with their corresponding anti-hormones (fig. 2 B–G).

By conventional electron microscopy, the incubated tissue had a normal structure. The various pituitary cells (fig. 3 A–D) presented their typical features (Rappay, 1984). The nuclear and cellular membranes as well as all intracytoplasmatic organelles were well preserved.

By immunocytochemical electron microscopy the secretory granules of the thyrotrophs could be labelled for TSH (fig. 3 E).

Discussion

The functional data indicate that in our model the basal TSH secretion was stable after 40 min and remained constant during the following 40 min. The dose of 3 ng/ml TRH, used for thyrotroph stimulation, is within the range of the physiological TRH concentration in the pituitary portal blood (Ching and Utiger, 1983; De Greef and Visser, 1981; Fink et al., 1982; Guillaume et al., 1986; Sheward et al., 1983). Because of high individual variations the difference between secretion in basal condition and under TRH stimulation was not significant during the first incubation period. Our results thus show that the isolated pituitary gland can respond to a physiological stimulus.

To ensure good tissue preservation till the end of the *«in vitro»* treatment, we used pituitary halves. In fact, preli-

minary experiments showed that with entire pituitary glands nuclear pycnosis and cytoplasm shrinkage occurred in the central tissue portions, probably because the diffusion of medium and oxygen is confined to the gland periphery. Quartered glands were also useless because gas bubbles caused the specimens to float on the fluid surface inducing hypoxia and necrosis of the superficial cell layers. The removal of neurohypophysis before incubation was omitted to avoid the consequent mechanical damage.

Farquhar et al. (1975), reported that the pituitary dissection may cause cell death because of the discharged intracellular proteases on the surface of the cut. These proteases could again catabolise the hormones released into the medium. Therefore bacitracin, a protease inhibitor, was added to the medium.

To preserve the incubated tissue pH must be close to neutrality (Paul, 1970). Medium composition, temperature, and gas flow can influence pH. We used a Hepes-buffered Locke's medium according to Boujon et al. (1987). This medium, maintained at 37° C with a gas flow of 4 ml/min/0.4 ml, has a pH of about 7.

Our morphological results demonstrate that the present model is suitable for conventional light and electron microscopical studies on various cell types of the isolated pituitary gland. Moreover, by means of immunocytochemistry we could label immunoreactive cells and granules. Thus, the tissue could also be used for densitometrical evaluations of the hormone content in single pituitary cells.

Since the results of both functional and morphological investigations were satisfactory, we propose our *«in vitro»* model to be used for combined studies which would allow to reduce the number of animals needed for an experiment, to avoid the extrapolation of results from different groups and to replace an in «in vivo» by an *«in vitro»* experiment.

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Un modèle «in vitro» pour la combinaison d'investigations fonctionnelles et morphologiques sur l'hypophyse du rat.

Nous avons développé un modèle «in vitro» permettant la combinaison d'études fonctionnelles et morphologiques sur l'hypophyse du rat. Pour tester sa validité lors d'investigations biochimiques, nous avons étudié la sécrétion des cellules thyréotropes hypophysaires en conditions basales et après stimulation par la TRH. Après stabilisation de la sécrétion basale de TSH, une stimulation par la TRH engendre une augmentation significative de la sécrétion de TSH. Pour tester la validité de ce modèle lors d'investigations morphologiques, le tissu incubé fut utilisé pour des études en microscopie photonique et électronique conventionnelles et immunocytochimiques. Les cellules hypophysaires de différents types étaient bien conservées. Notre modèle permet donc une combinaison d'études biochimiques et morphologiques sur du tissu hypophysaire et, par conséquent, de réduire le nombre de groupes d'animaux utilisés, d'éviter l'extrapolation de résultats entre différents groupes et de remplacer des expériences «in vivo» par des expériences «in vitro».

Un modello «in vitro» per studi morfologici e funzionali sull'ipofisi di ratto.

Si descrive un modello alternativo per lo studio morfologico e funzionale dell'ipofisi di ratto. Per sperimentare il suo uso per studi biochimici sono stati valutati alcuni aspetti della secrezione delle cellule tireotrope in condizioni basali e dopo stimolazione con TRH. Il tessuto incubato é stato poi utilizzato per studi morfologici convenzionali ed immunoistochimici al microscopio ottico ed elettronico. L'uso di questo modello per effettuare contemporaneamente studi morfologici e biochimici su un'unica ipofisi consente di ridurre il numero di animali utilizzati per l'esperimento, evita l'estrapolazione dei risultati tra differenti gruppi e sostituisce un esperimento «in vivo» con un esperimento «in vitro».

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