Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 82, Suppl. II: 15-22, 1987.
Intern. Symp. on Immunomodulators

CELL BIOLOGY OF THYMIC HORMONE SECRETION

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It is currently known that the thymus gland is the source of peptidic hormones that play a role in T cell differentiation (see Bach, 1983), Some of these hormones had their aminoacid sequences determined, as for example thymosin α-1 (Goldstein et al., 1977), thymopoietin (Schlesinger & Goldstein, 1975) and thymulin (Bach et al., 1977). Interestingly, although thymulin and thymopoietin are exclusively produced in thymus, a recent series of papers published by Horecker's group does suggest that thymosin α-1, and/or its precursor may be also produced in extra-thymic sites (Haritos et al., 1984).

In the present review, we shall foccuse our attention in a series of recent fingings that have thrown some more light on the intracellular pathways for thymic hormone secretion. In addition, we intend to summarize the separate lines of evidence concerning feedback mechanisms and neuroendocrine modulation on the production of these T cell differentiating factors.

THYMIC HORMONES ARE PRODUCED BY THYMIC EPITHELIAL CELLS

Since the beginning of the 80's some papers addressed the question of what cell type in the thymus was the source of thymic hormones. Immunohistochemical studies (using polyclonal immunesera) applied for the detection of both thymosin α-1, thymulin and thymopoietin revealed that these substances were located within thymic epithelial cells (TEC) both *in vivo* and *in vitro* (Dalakas et al., 1981; Monier et al., 1980; Jambom et al., 1981, Haynes et al., 1983). At least for thymulin, these results were further confirmed with the use of anti-thymulin monoclonal antibodies in both mouse and human thymuses (Fig. 1), and even in a continous rat TEC line (Savino et al., 1982; Berrih et al., 1984; Savino et al., 1986).

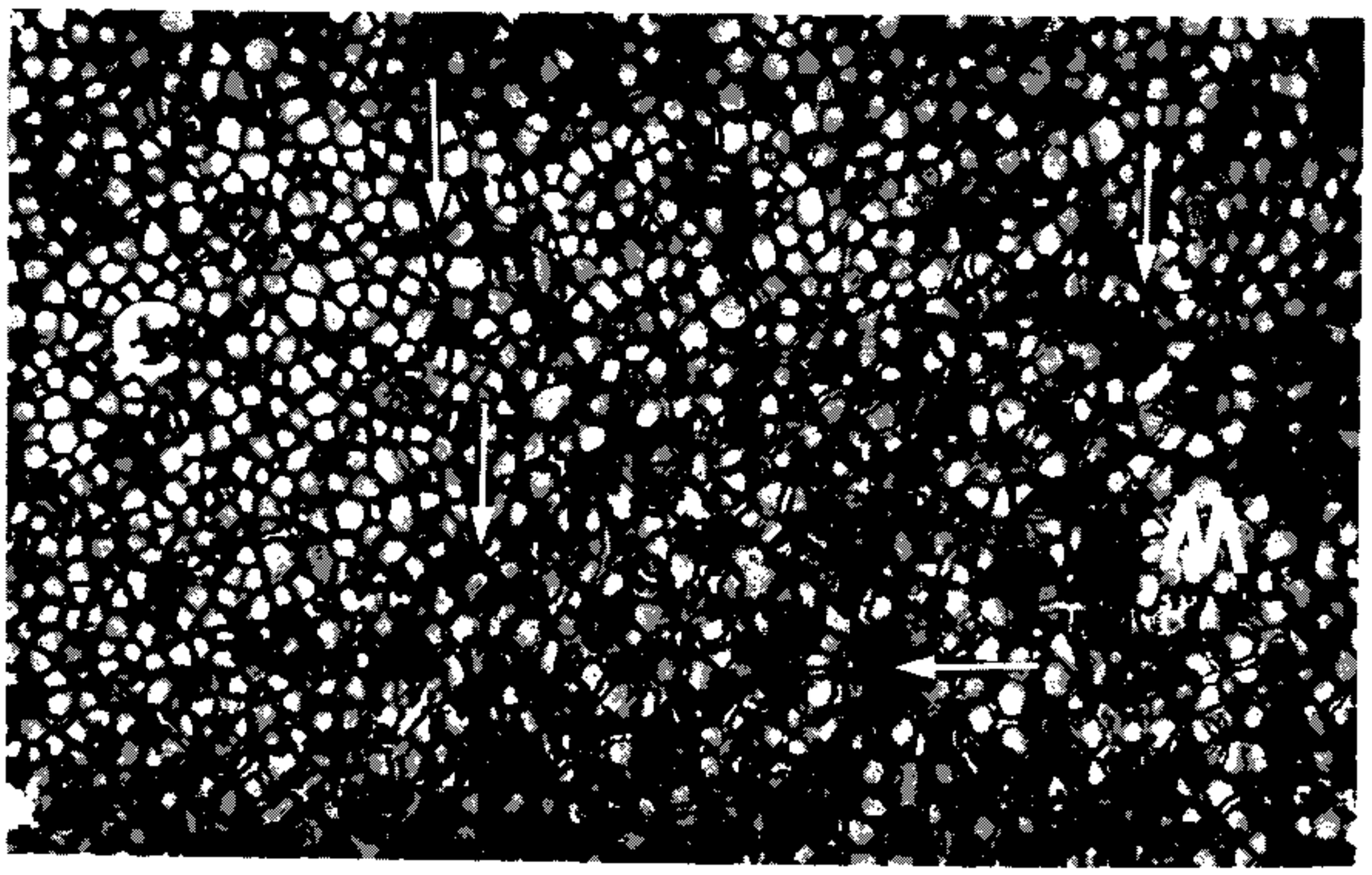

Fig. 1: Immunohistochemical demonstration of thymulin within epithelial cells of a mouse thymus. The frozen section was labeled with an anti-thymulin MAb followed by peroxidase-coupled second antibody. Note that thymulin containg cells (arrows) are more frequent in the medulla (M) than the cortex (C) of the thymic lobule. X 400.

In addition to these findings, we also evidenced (again by the use of immnohistochemistry) that these three well-defined thymic hormones can be simultaneously produced by the same TEC (Savino & Dardenne, 1984).

It should be pointed out that the above question should be further analysed using *in situ* hybridization. Appropriate molecular probes for each thymic hormone will permit – hopefully in a near future – to know if messenger RNAs for distinct thymic hormones can also be simultaneously detected in a given TEC.

INTRACELLULAR PATHWAYS FOR THYMULIN SECRETION

The very first data concerning the intracellular location and/or storage of thymic hormones came out with immunoelectronmicroscopic findings on thymulin. It was clearly demonstrated, firstly with polyclonal antisera (Schmitt et al., 1982) and then with monoclonal reagents (Auger et al., 1982, 1984), that thymulin immunoreactivity was concentrated in cytoplasmic vacuoles. These studies in fact represented the first evidence suggesting a classical pathway for secretion of polypeptides.

This hypothesis was specifically investigated much more recently by the use of drugs that can block the movement of polypeptide-containing vesicles at specific sites within the cell. We applied colchicine, cytochalasin B and monensin on primary cultures of human thymic epithelial cells. Colchicine (largely known to inhibit microtubule polymerization) blocks the coordinated movement of secretory vesicles towards the cell membrane whereas cytochalasin B – a microfilament inhibitor – rather acts on the final step of fusion of the vesicle to cell membrane thus blocking exocytosis. Differently, monensin is known to specifically inhibit the movement of Golgi-derived vesicles (Tartakoff, 1983).

These three agents were proved to inhibit the release of thymulin into the culture supernatants. These effects were all dose-dependent and we noticed that simultaneous addition of colchicine and cytochalasin B was more efficient than each substance alone used in the same dose (Savino & Dardenne, 1986). The immunofluorescence analysis of these treated cells revealed an intra-cytoplasmic accumulation of thymulin in all blocking situations, demonstrating that the hormone synthesis did occur but its release was inhibited.

This bulk of results, together with the fact that we recently identified high molecular weight polypeptides reactive with anti-thymulin monoclonal antibodies, strongly suggest that a thymulin precursor is synthetized at the granular endoplasmic reticulum, moves to the Golgi complex where maturation is likely to take place. From the Golgi, secretory vacuoles are released and move towards the cell membrane eventually fusioning with it, characterizing an exocytotic release of the mature hormone.

A remaining point that merits discussion concerns the coupling of zinc to the molecule of thymulin. As reviewed elsewhere in this volume (Dardenne & Bach, 1987), thymulin is only biologically active when the nonapeptide is coupled to zinc.

The first indications that the incorporation of zinc into the peptide occurs within TEC came from electronmicropobe analysis of ultrathin thymic sections, that revealed zinc-storage sites within TEC cytoplasmic vacuoles (Dardenne et al., 1982). In the same vein, we demonstrated that TEC express large amounts of metallothionein (Savino et al., 1984), a small protein extremely avid to the metal zinc, and typically found in zinc-storing cells in the body. Moreover, we now know that the anti-thymulin monoclonal antibodies we produced specifically recognize a zinc-dependent epitope in the molecule of thymulin (Dardenne et al., 1985). Considering that these antibodies only label secretory vesicles but not the granular endoplasmic reticulum or the Golgi complex, we postulate that zinc incorporation to thymulin molecule is likely to occur within these secretory vesicles, thus representing a late event in the intracellular maturation of the hormone.

A matter that is still open to speculation concerns the existence or not of a polarization for thymulin release. If such polarization exists, one may hypothesize that sites of TEC membrane in close relation to thymocytes might represent priviledged regions for exocytosis. Alternatively, thymulin releasing sites might be rather topographically related to cappilaries. These hypotheses as well as the putative intracelular pathways for thymulin secretion are schematically summarized in Fig. 2.

It should be reminded that virtually no information is to our knowledge avaiable concerning intracellular pathways for the secretion of other thymic hormones. Yet, very recently data by Auger and co-workers (unpublished) suggest that thymosin α-1 can also be detected

within cytoplasmic vesicles. Thus, it should be worthwhile to determine if more that one thymic hormone can be found in the same secretory vacuoles.

FEEDBACK SYSTEMS CONTROLLING THYMULIN SECRETION

The fact the thymulin levels in the blood remain constant for relatively long period of time (Bach & Dardene, 1973) and that physiological levels apparently reflect the intra-thymic status of hormone production (Savino et al., 1983a), led us to imagine that thymulin secretion is under control mechanisms. Actually, this hypothesis was first experimentally suggested after the observation that, when one or several thymuses are grafted into normal mice, a transient increase of the circulating thymulin levels is noted, and then return to normal values (Tubiana & Dardenne, 1979).

In order to further check this hypothesis, we carried out *in vivo* experimental depletion of thymulin in the circulating blood, either by injection of exogenously-produced anti-thymulin monoclonal antibodies, or by induction of endogenous anti-thymulin antibody formation following immunization with synthetic thymulin coupled to BSA (bovine serum albumin). These experimental precedures did result in a strong depletion of peripheral thymulin that followed by an increase in the numbers of thymulin-producing cells, immunohistochemically evidenced with monoclonal antibodies (Savino et al., 1983b). These results immediately drove us to imagine the existence of a classical negative feed-back mechanism for thymulin secretion, that could be triggered, at least in some experimental conditions, when the levels of thymulin itself were altered.

In vitro experiments with cultured human TEC, not only confirmed our previous findings but also extended them showing that when synthetic thymulin was added in excess in the culture supernatant, there was a clearcut decrease in the numbers of thymulin-containing cells.

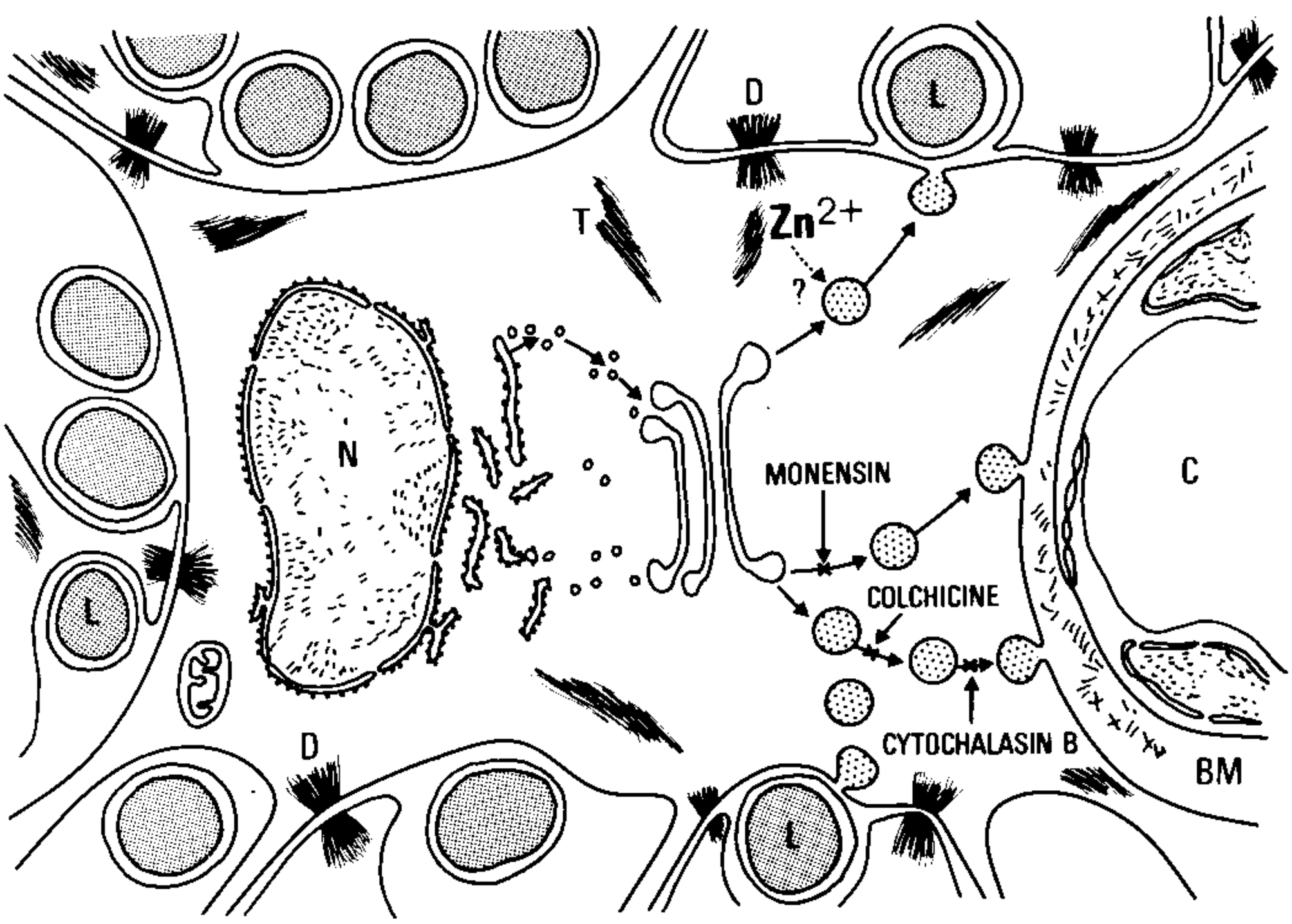

Fig. 2: Hypothetical model to explain the intracellular pathway for thymulin secretion. Nascent molecule (more likely a high molecular weight thymulin precursor) would be synthesized at the granular endoplasmatic reticulum, then moving to the Golgi complex from which secretory vacuoles would be releases. Zn^{2+} would be incorporated into those vacuoles. They would then move towards the cell membrane eventually fusioning with it by exocytosis. L-lymphocytes; N-nucleus; D-desmosome; C-capillary; BM-basement membrane, (reproduced with permission of the Journal of Histochemistry and Cytochemistry).

The above findings raise another interesting question in terms of cell biology, namely, do thymulin-secreting cells also express specific receptors for the hormone? This latter question is still open to experimentation, although very preliminary attempts (umpublished observations) failled in demonstrating receptors fot thymulin on TEC membranes.

NEUROENDOCRINE MODULATION OF THYMULIN SECRETION

The control of thymulin secretion by TEC appears however to be more complex than a simple closed-circuit in which thymulin ultimately controls its own secretion rate. A neuroendocrine axis is probably also involved in the physiological control of thymulin release by thymic epithelial cells. This hypothesis was initially raised based on the findings reported by Pelletier et al. (1976) showing that the dwarf mouse exibited a precocious decrease of seric thymulin. This animal is a mutant essencially characterized by a severe depletion of circulating growth and thyroid hormones. Much more recently, decrease thymulin seric levels were also detected in humans with hypophysary dwarfism, and in hypophysectomized rats. (M. Dardenne, unpublished data).

Target organs for pituitary hormones, can also modulate thymulin secretion, and experimental data are already avaiable concerning thyroid, adrenals and gonads.

Mouse and rats injected with triiodothyronine (T_3) revealed a progressive increase of blood thymulin levels, which (as studied in mice) were paralleled by the high numbers of thymulin-immunoreactive cells (Savino et al., 1984). Conversely, PTU (propriothiouracyl), an inhibitor of thyroid hormone synthesis, promoted an important decrease in the seric and thymic contents of thymulin. Interestingly, Fabris & Mocchegiani (1985) demonstrated that the stimulatory effects of thyroid hormones could still be observed in old animals (known to exibit low levels of thyroxine). These data indicate that thyroid hormones might be acting directly on TEC stimulating them to produce and release thymulin. This latter hypothesis has been recently confirmed by in vitro experiments using both primary cultures of human TEC and a rat TEC line (Fig. 3).

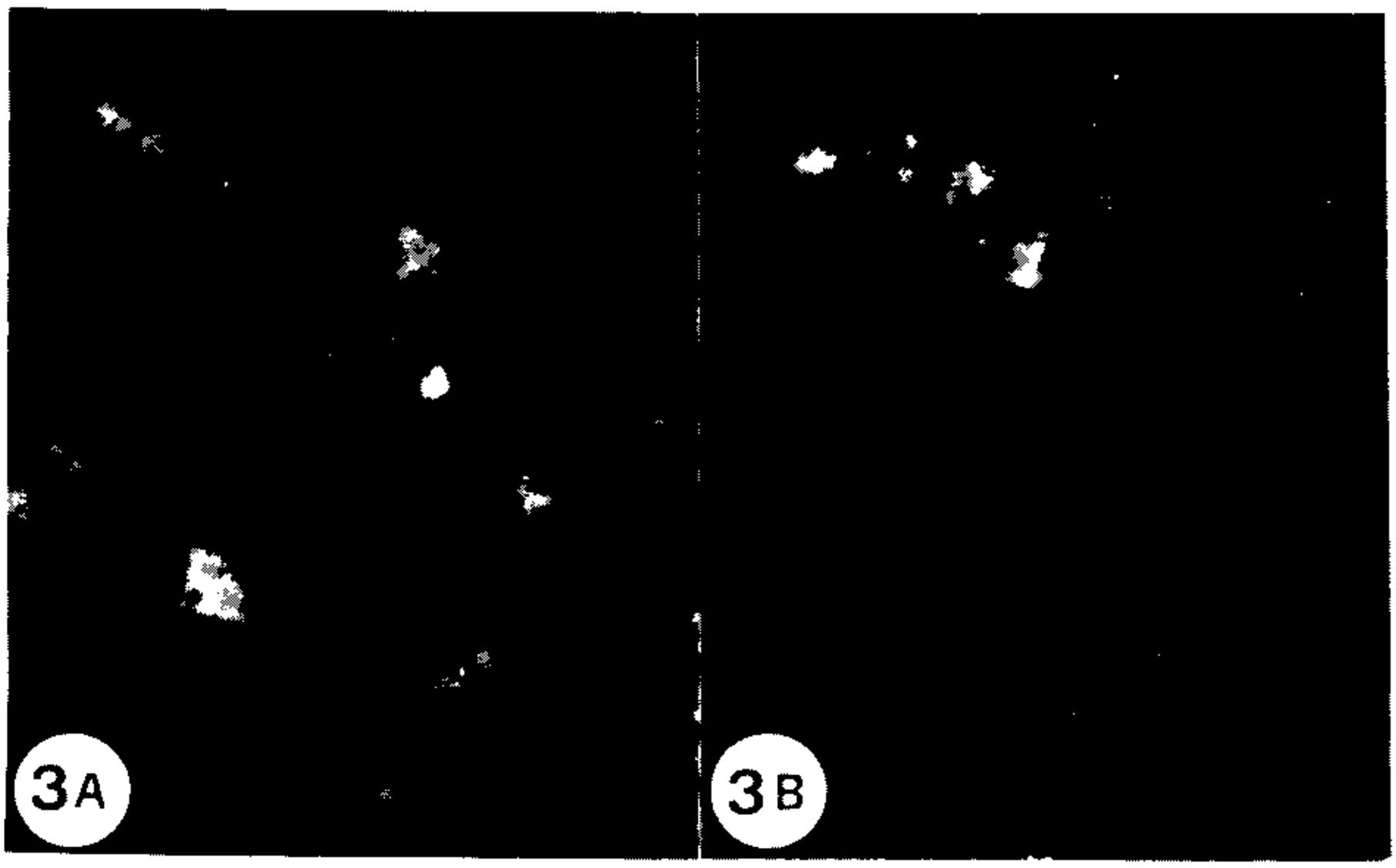

Fig. 3: Immunohistochemical detection of thymulin in vitro using a rat thymic epithelial cell line (experiments performed on day 3 after settling the culture). Panel A depicts a control culture showing a small proportion of thymulin containing cells. This number is greatly augmented after treatment with progesterone. In this picture, the anti-thymulin MAb was revealed with a fluorescein coupled conjugate X 600.

The existence of modulatory activities by steroid hormones on thymulin secretion has also been investigated using both *in vivo* and *in vitro* models. We demonstrated that adrenalectomy (Adx) or castration (Cx) could promote a transient (one-month long) decrease of thymulin seric levels that was interestingly accompanied by a transient augmentation in the thymic content of the hormone (Dardenne et al., 1986a). When Adx and Cx were simultaneously performed the same kind of effect could be observed, but with a much more prolonged kinetics (Fig. 4). Importantly, all these effects could be prevented by appropriate steroid hormone replacement.

In vivo steroid effects on thymulin secretion apparently comprises a paradox in that, for some time, we observe high numbers of thymulin-producing cells concomitant with persistent low seric levels of the hormone. Actually, this was explained by the discovery of low molecular weight thymulin inhibitors that are released after Adx and/or Cx. These substances thus gradually disappear from circulating blood as seric and thymic contents of thymulin get back to normal values.

The influences of steroids on thymic hormone containig cells appear to be via a direct effect on TEC. Data for the existence of specific receptors for sexual hormones had been previously obtained by Grossman and co-workers (Grossman et al., 1979a, b, c), and glucocorticoid receptors have been recently directly demonstrated in cultured TEC (Dardenne et al., 1986b). Definite results were very recently obtained in our laboratory when we demonstrated that both glucocorticoids and sexual steroids were able to stimulate cultured TEC to enhance thymulin secretion and that such effect was blocked by the simultaneous addition of adequate steroid antagonists in the culture supernatants (Savino et al., 1988).

The bulk of findings reviewed above clearly shows that thymic endocrine function is under control of hypophysis and at least some of its target organs. However, hypothalamus influences seem also to be involved. It has been recently demonstrated that a hypothalamic extract was able to restore in old mice thymulin seric level to achieve values as high as those physiologically found in young animals (Folch et al., 1986). It remains however to be clarified if the biological active substance(s) contained in this extract actually corresponds to: a) a releasing factor for a pituitary hormone that would act on its target organ which in turn would release another hormone eventually responsible for TEC stimulation; or b) a hitherto unknown chemical entity that would directly bind to TEC, or act indirectly, but via other biological circuits. It would be important to proceed purification of the extract and carry out *in vitro* studies, in order to check these hypotheses.

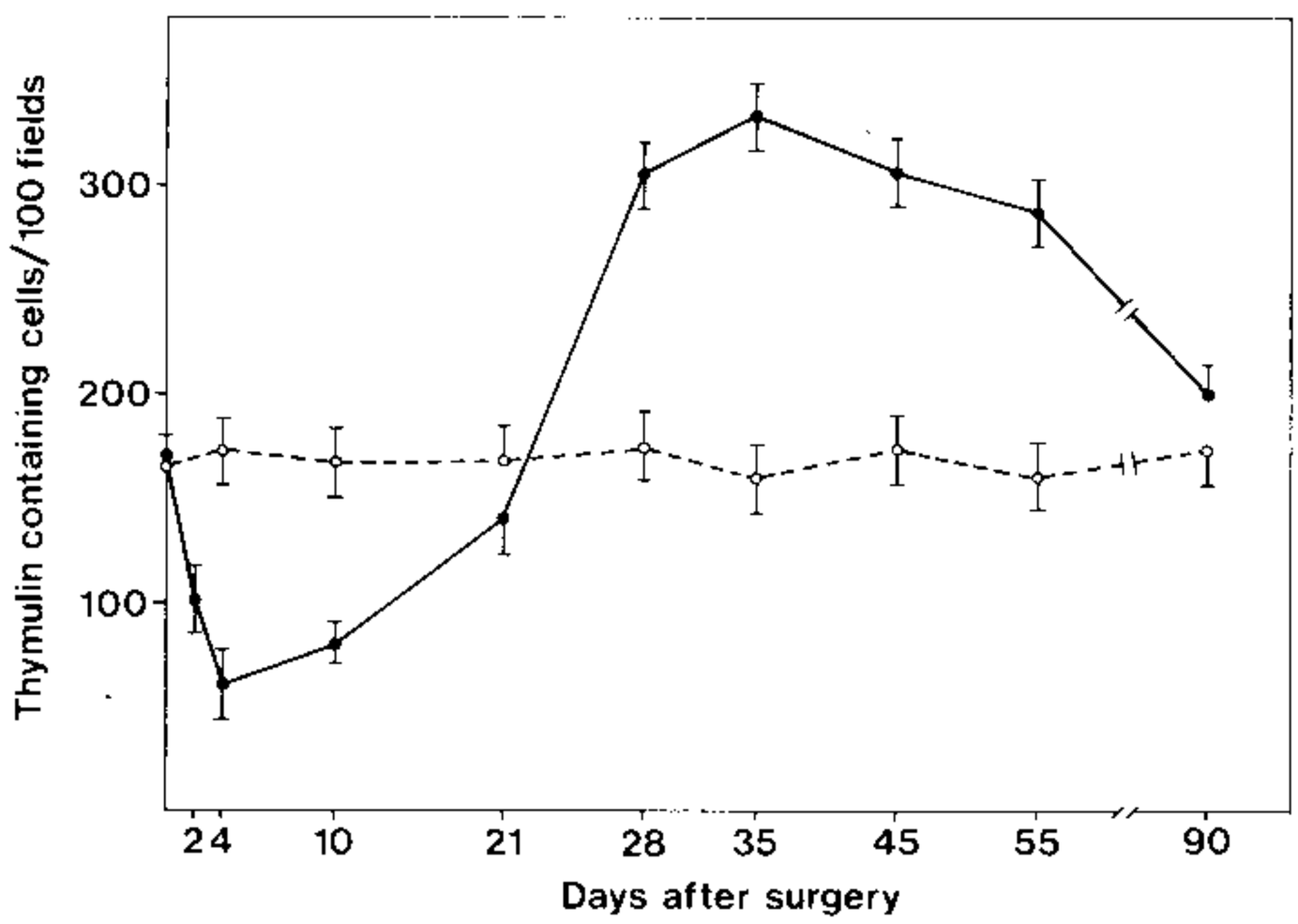

Fig. 4: Thymulin containing cells in thymuses from adrenalectomized/castrated mice. After a brief decrease in the numbers of hormone producing cells there is an increase in cell numbers. This latter effect gradually disappear, to reach normal values 3 months after surgery. (Reprinted with permission of the Journal of Immunology).

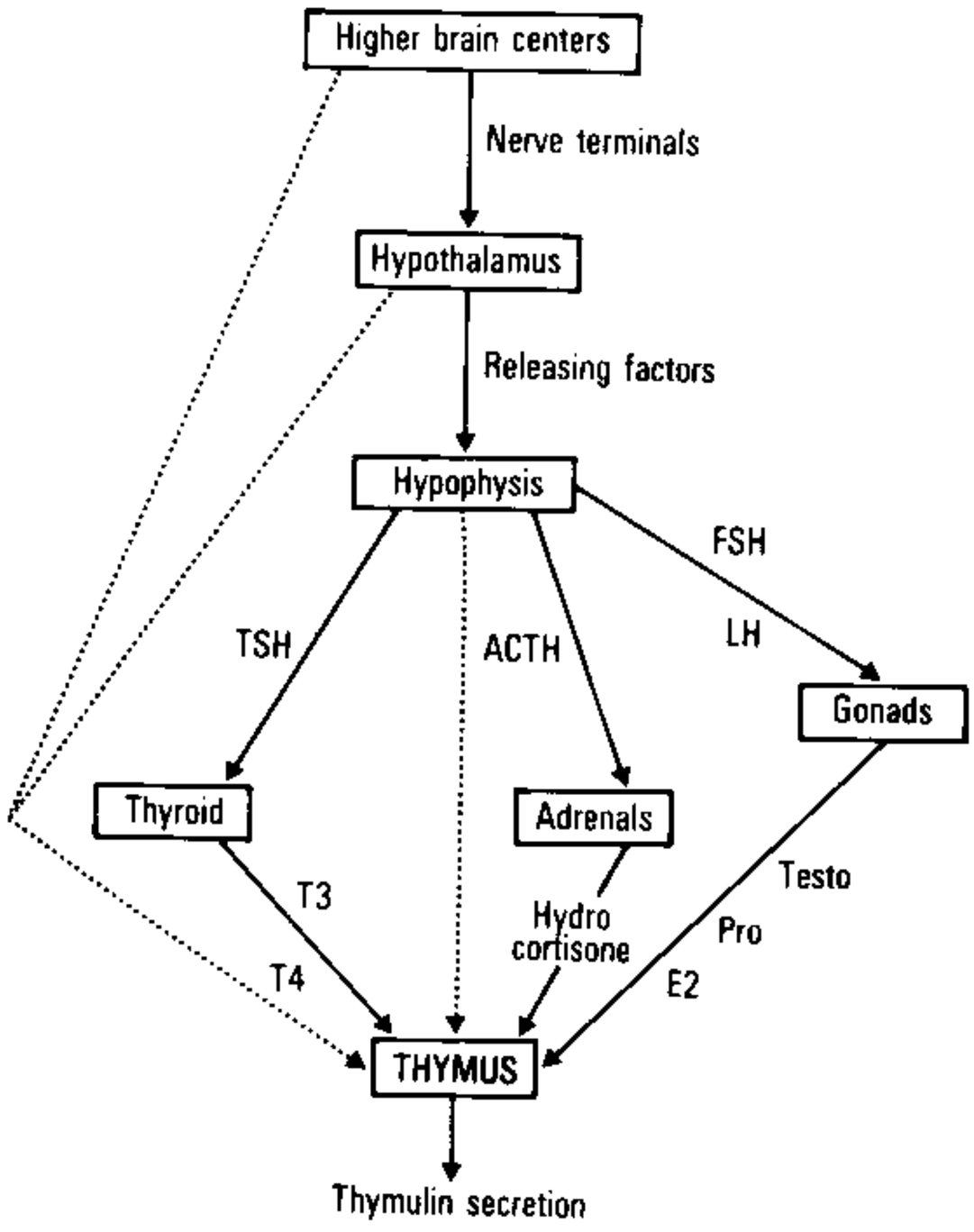

Fig. 5: Schematic representation on the various demonstrated (—) or hypothetical (---) neuroendocrine circuits affecting thymulin secretion.

The multiple neuroendocrine pathways that may be involved in the physiological control of thymulin secretion are schematically depicted in Fig. 5.

CONCLUSIONS AND PERSPECTIVES

The series of results so far appeared in the literature leaves no doubts that thymic epithelial cells are the source of thymic hormones (at least for those strictly produced within the thymus). These molecules appear not to have a common precursor, although the existence of a high molecular weight molecule is likely to be the case, at least in respect to thymulin biosynthesis. However, the gene transcripting the mRNA for such precursor is to be clearly identified as well as its cromosomal location. Still less clear seems to be the biosynthesis of thymopoietin for which is unknown if there is or not a precursor molecule.

Concerning the intracellular pathways of thymulin and/or its precursor, the data so far avaiable indicate that it corresponds to a classical pathway for a peptidic hormone, from synthesis to extracellular release. Again, it should be useful to determine if other thymic hormones follow the same route and if they can be simultaneously stored in common secretory vesicles. We think that this is and important point because it could throw some more light on the question of control mechanism(s) for each thymic hormone.

The putative existence of such mechanism(s) has been actually demonstrated for thymulin. Although it is likely to occur in relation to other thymic hormones, a piece of work has to be carried out before demonstrating that.

A similar reasoning can be apllied concerning the neuroendocrine influences on thymic hormone secretion. The data so far avaiable for thymulin just represent a beginning, and putative roles of other endocrine glands as well as the nervous system should be systematically investigated if we want to built up a detailed model on the biological circuits that are involved in the control of this group of T cell differentiation factors.

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