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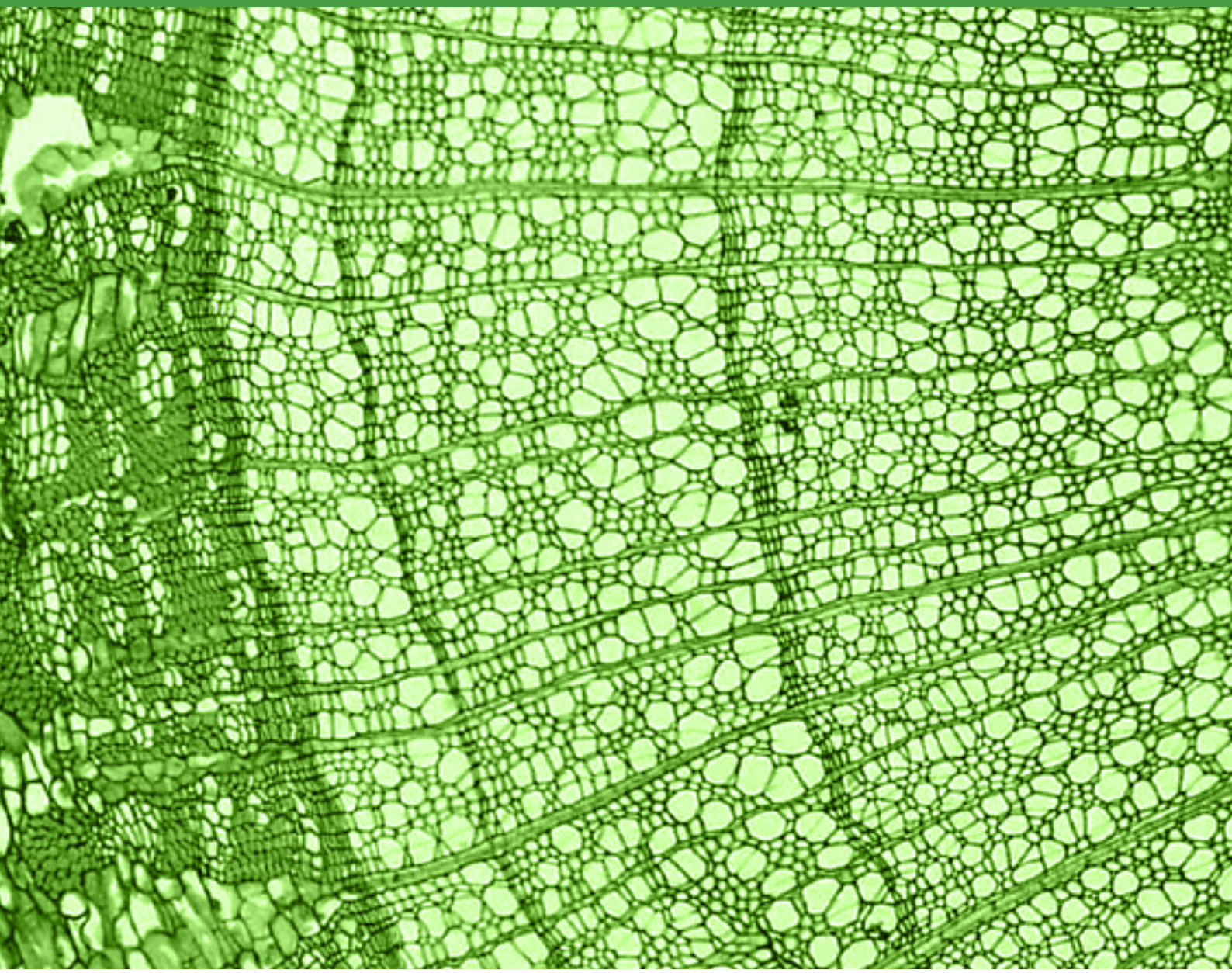

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PHYSIOLOGY, MOLECULAR BIOLOGY AND THERAPEUTIC POTENTIAL OF THE THYMIC PEPTIDE THYMULIN

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Keywords: thymulin; neuroendocrine control; hypophysiotropic activity, artificial gene, gene therapy, anti-inflammatory, ovarian dysgenesis.

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* These two authors contributed equally to this work.

ABSTRACT

Thymulin is a thymic hormone exclusively produced by the thymic epithelial cells. After its discovery and initial characterization in the ‘70s, it was demonstrated that thymulin production and secretion is strongly influenced by the neuroendocrine system. Conversely, a growing core of information, to be reviewed here, points to thymulin as a hypophysiotropic peptide. Additionally, the substantial body of evidence pointing to thymulin and some synthetic analogs as anti-inflammatory and analgesic molecules in the brain and other organs will be also reviewed. In recent years, a synthetic DNA sequence coding for a biologically active analog of thymulin, metFTS, was constructed and cloned in different adenoviral vectors. A number of recent studies suggest that thymulin gene therapy may be a suitable therapeutic strategy to prevent some of the endocrine and reproductive alterations that typically appear in congenitally athymic (nude) mice, used as a suitable model of neuroendocrine and reproductive aging. Summing up, the present article briefly reviews the literature on the physiology of the thymulin-neuroendocrine axis and the anti-inflammatory properties of the molecule and its analogs. The availability of novel biotechnological tools should boost basic studies on the molecular biology of thymulin and should also allow an assessment of the potential of gene therapy to restore circulating thymulin levels in thymodeficient animal models and eventually, in humans.

RELEVANCE OF THE THYMUS IN THE IMMUNE-NEUROENDOCRINE HOMEOSTATIC NETWORK

The immune system is functionally linked to the nervous and endocrine systems thus constituting an integrated homeostatic network (**1).** Within this network the neuroendocrine system monitors and controls the physical and chemical variables of the internal milieu. On its part, the immune system perceives, through antigenic recognition, an internal image of the macromolecular and cellular components of the body and reacts to alterations of this image, effectively participating of the "biological" homeostasis of the organism.

In mammals, the interaction of the thymus gland with the neuroendocrine system seems to be particularly important during perinatal life, when the thymus and the neuroendocrine system influence the maturation of each other. This was initially suggested by early findings showing that in species in which neonatal thymectomy does not produce any evident impairment of the immune capacity, neuroendocrine functions are already highly developed at birth. In mice, the importance of the thymus for a proper maturation of the neuroendocrine system is revealed by the endocrine alterations caused by neonatal thymectomy or congenital absence of the thymus. In effect, congenitally athymic (nude) female mice show significantly reduced levels of circulating and pituitary gonadotropins, a fact that seems to be causally related with a number of reproductive derangements described in these mutants **(2).** Thus, in homozygous (nu/nu) females the times of vaginal opening and first ovulation are delayed, fertility is reduced **(2)** and follicular atresia is increased such that premature ovarian failure results. Similar abnormalities result from neonatal thymectomy of normal female mice. Ovaries of athymic mice respond normally to exogenous gonadotropins, suggesting that the defect is at the level of the hypothalamo-pituitary axis. In homozygous adult nude CD-1 male mice, thyrotropin (TSH), prolactin (PRL), growth hormone (GH) and gonadotropin responses to immobilization and cold stress are reduced as also are serum basal levels of the same hormones as compared to the heterozygous counterparts (**3, 4, 5).** A functional impairment of the hypothalamo-adrenal axis has been reported in nude mice suggesting that humoral thymic factors may play a role in the maturation of this axis.

THYMULIN

Thymulin is a thymic metallopeptide involved in several aspects of intra- and extrathymic T-cell differentiation (**6).** Thymulin which is exclusively produced by the thymic epithelial cells, consists of a biologically inactive nonapeptide component termed FTS (an acronym for serum thymus factor in French), coupled in an equimolecular ratio to the ion zinc, which confers biological activity to the molecule. The metallopeptide active form bears a specific molecular conformation that has been evidenced by nuclear magnetic resonance **(7).**

NEUROENDOCRINE CONTROL OF THYMULIN PRODUCTION

The control of thymulin secretion seems to be dependent on a complex network of events. Initial studies showed that the hormone itself exerts a controlling feedback effect on its own secretion both *in vivo* and *in vitro.* Additionally, thymulin production and secretion is influenced directly or indirectly by the neuroendocrine system**.** For instance, GH can influence thymulin synthesis and secretion. *In vitro,* human GH can stimulate thymulin release from TEC lines **(8)** which are known to possess specific receptors for GH. Animal studies have shown that treatment of aged dogs with bovine GH partially restored their low thymulin serum levels. In old mice, treatment with ovine GH increased their low circulating thymulin levels and enhanced the concanavalin A (Con A)-dependent proliferative response of their thymocytes, as well as interleukin-6 production **(9)**. In old rats, combined treatment with GH and thyroxine (T_4) was also able to restore partially their reduced thymulin levels **(10).** In clinical studies, it was reported that in congenitally GH-deficient children, who consistently exhibited low plasma thymulin levels, GH therapy succeeded in increasing

thymic hormone levels to near normal values**.** Acromegalic middle-aged patients have elevated thymulin serum levels compared to age-matched normal subjects **(8).** It is likely that these effects of GH are mediated, at least in part, by insulin-like growth factor 1 (IGF-1) as suggested by the fact that the GH-induced enhancement of thymulin production could be prevented by previous treatment with antibodies against IGF-1 or IGF-1 receptor **(8).**

There is also evidence for a PRL-thymulin axis. Thus, it is known that TEC possess PRL receptors and that PRL can stimulate thymulin synthesis and secretion both *in vitro* and *in vivo* **(11).** Furthermore, administration of PRL to old mice elevated their reduced circulating levels of thymulin **(11).**

The thyroid axis also influences thymulin secretion. Thus, T_4 has been shown to stimulate thymulin synthesis and secretion in mice (**12).** *In vivo* treatment of mice with triiodothyronine (T_3) enhanced thymulin secretion whereas treatment of the animals with propylthiouracil, an inhibitor of thyroid hormone synthesis, decreased their circulating thymulin levels. In humans, hyperthyroidism brings about an increase in circulating thymulin levels whereas hypothyroid patients show depressed levels of this thymic hormone (1**3).** In *in vitro* studies, it was shown that thyroid hormones stimulate thymulin secretion by a direct action on TEC (14**).** Interestingly, it has been shown that treatment of aged animals with T_4 can reverse their decreased thymulin levels (**14).**

Although there are no studies documenting a direct effect of gonadotropins or adrenocorticotropic hormone (ACTH) on thymulin secretion, gonadectomy or adrenalectomy in mice are known to induce a transient decrease in serum thymulin levels. This effect is potentiated by the simultaneous removal of the adrenals and gonads. In TEC cultures, it was shown that exposure to physiological levels of glucocorticoids or gonadal steroids enhanced thymulin concentration in the cell supernatants (**15).**

HYPOPHYSIOTROPIC ACTIVITY OF THYMULIN

The multilateral influence that the neuroendocrine system exerts on thymulin secretion suggests that this metallopeptide could in turn be part of a feedback loop acting on neuroendocrine structures. This possibility is now supported by a significant body of evidence indicating that thymulin possesses hypophysiotropic activity. Thus, thymulin has been shown to stimulate luteinizing hormone (LH) release from perifused rat pituitaries (**16)** and ACTH from incubated rat pituitary fragments, the latter being an effect mediated by intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) accumulation **(17).** In an *in vitro* study using pituitary cells obtained from female rats in different days of the estrous cycle it was observed that thymulin modulates the stimulatory activity of gonadotropin releasing hormone (GnRH) on LH and follicle stimulating hormone (FSH) release (**18).** Thymulin has been found to stimulate GH, PRL, TSH and gonadotropin release in dispersed rat pituitary cells at doses from 10^{-8} to 10^{-3} M (**19, 20)** whereas others have reported that thymulin doses of 10^{-11} M stimulate LH, inhibit PRL release and have no effect on GH secretion in incubated rat pituitary fragments (**40).** The stimulatory effect of thymulin on hormone release in rat pituitary cells declines with the age of the cell donor which suggests that aging brings about a desensitization of the pituitary gland to thymic signals (**19, 20).**

There is *in vitro* and *in vivo* evidence suggesting that thymulin plays a role in the regulation of female spontaneous puberty, possibly through effects on pituitary gonadotropin release and ovarian steroidogenesis (**16, 18).** Thymulin also modulates gonadotropin-induced testicular steroidogenesis.

ANTIINFLAMMATORY ACTIONS OF THYMULIN AND ITS ANALOGUES

Among the molecules that interact with thymulin are a set of cytokines that play a major role in the inflammatory response. In this regard, several studies have evidenced the potent effect of thymulin in different animal models of pain. Subsequent experiments were designed to examine the effects of intracerebroventricular (i.c.v.) injections a thymulin on cerebral inflammation induced by i.c.v. injection of endotoxin. Pretreatment with thymulin (0.1 to 1 μg) reduced in a dose-dependent manner, the endotoxin-induced hyperalgesia and exerted differential effects on the upregulated levels of cytokines in different areas of the brain, suggesting a neuroprotective role of thymulin in the brain (**21)**.

In a model of neuroinflammation, a peptide analogue of thymulin also resulted in a significant alleviation of endotoxin-induced hyperalgesia and in a reduction of the proinflammatory molecules IL-1ß, IL-6 and TNFα in the hippocampus and the brainstem. Interestingly, injected alone the analog induced an up regulation of IL-10 in the hippocampus and brainstem and produced a marked enhancement of the level of this cytokine in the same regions when endotoxin was also applied (**22)**.

Taken together, all the actions of thymulin and its analogue described above suggest that these molecules have potent analgesic and anti-inflammatory properties.

The beneficial effects of thymulin have also been reported by several groups in various models of organ specific inflammation: In alloxan- and streptozotocin-induced diabetes, pretreatment with thymulin significantly suppressed hyperglycemia and prevented the destruction of pancreatic beta cells **(23)**. A similar protection was observed against myocarditis induced by encephalomyocarditis virus and pulmonary hypertension induced in rats by monocrotaline (**24)**. In this model, thymulin effect was related to the inhibition of expression of the proinflammatory cytokine IL-6 and to the suppression of p38 MAPK pathway.

In a recent report, Lunin *et al*. **(25)** studied the modulation of inflammation by thymulin in New Zealand White (NZW) mice with acute autoimmune encephalomyelitis, induced by myelin basic protein. In this model, it was suggested that thymulin treatment reduced the severity of the desease and the autoimmune response via mechanisms involving the nuclear factor-kappaB (NF-κB) cascade. It was shown that the peptide reduced the level of phosphorylation of the NF-κB signalling protein IKK and the production of HSP72 protein.

The above data demonstrate that, although the mechanisms of action of these molecules are not yet clearly identified, thymulin and its analogues provide promising results in the therapy of inflammatory diseases, including neuroinflammation and neurodegenerative disorders. These molecules are safe, without side effects in a large range of doses. However, the short half life is the main limit to their clinical use. Various methods to prolong this half-life are currently under study. In this context, thymulin gene therapy is emerging as a promising strategy for long-term delivery of thymulin and its analogs (see below). The success of this approach would constitute an important step to improve the efficacy of these molecules and to extend the field of their therapeutic applications.

CONSTRUCTION OF SYNTHETIC GENES FOR THYMULIN

The prospect of implementing thymic hormone gene therapy appears as an interesting avenue of research aimed at restoring endocrine thymic activity when thymus function is compromised. However, none of the genes coding for the known thymic peptides has been cloned, a situation that hinders the implementation of gene or other molecular therapies for thymic hormones. It was suggested that a possible way to overcome this problem could be to construct "artificial genes" coding for those thymic peptides whose amino acid sequences were short and required no posttranslational processing (**26).** This has been recently achieved for thymulin and the corresponding DNA sequence cloned in a recombinant adenoviral (RAd) vector which was subsequently used in a number of experimental gene therapy studies (see

below). Thus a DNA sequence coding for the biologically active FTS analog called metFTS, was constructed and cloned in an adenoviral vector (**27).** The design of the DNA sequence for metFTS was optimized for expression in rat systems by choosing for each amino acid of the native peptide, the codon more frequently used by rat cells**.** A variant of this sequence was used to construct RAd-metFTS, an adenoviral vector that harbors the synthetic gene for metFTS driven by the mouse cytomegalovirus (mCMV) promoter **(Fig. 1).** When intra-muscularly (i.m.) administered to thymectomized (Tx) mice and rats (whose circulating levels of thymulin are nondetectable), RAd-metFTS induced sustained supraphysiological serum levels of biologically active thymulin which remained high for at least 112 days in mice (**27)** and for over 320 days in rats.

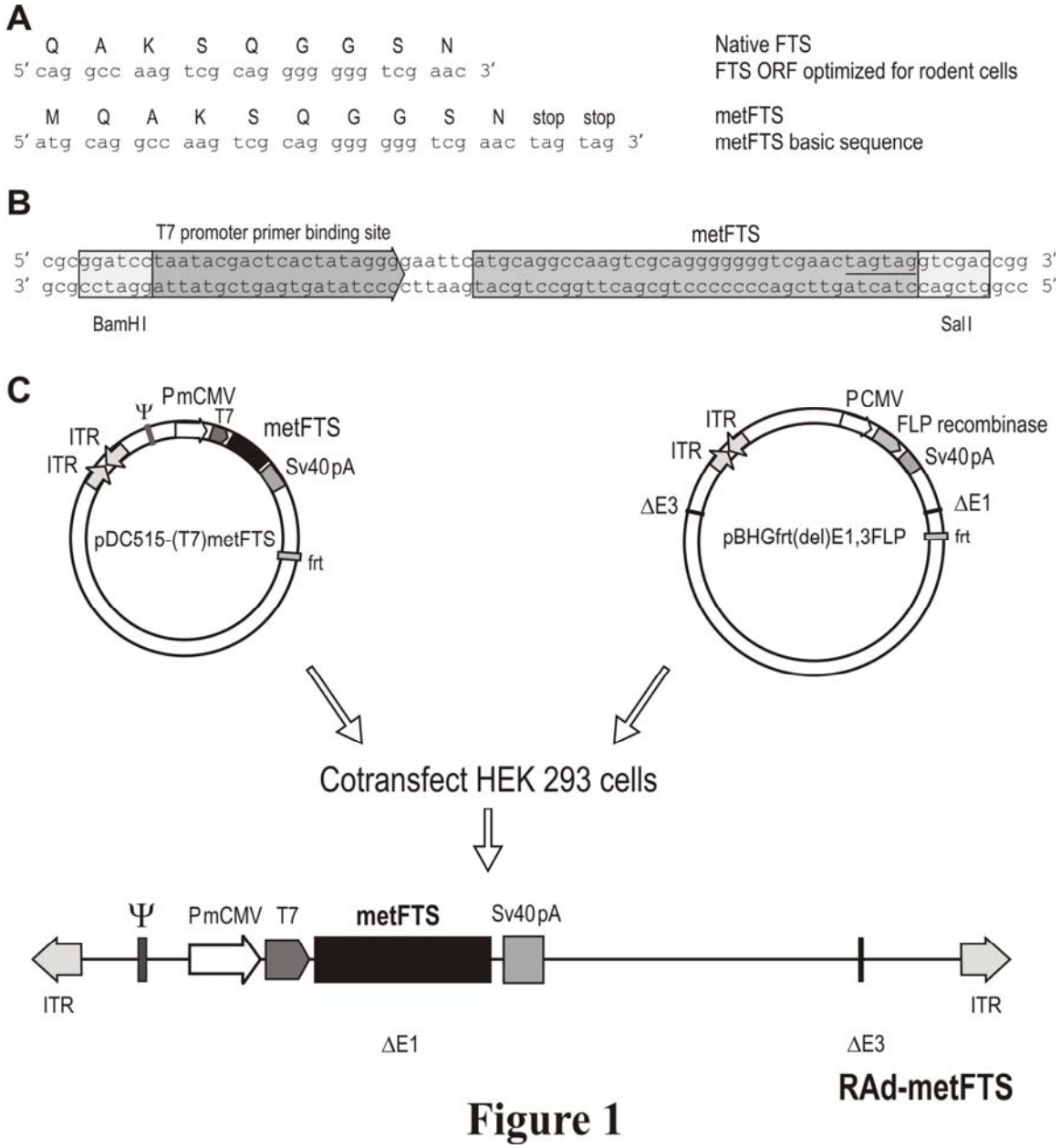

Figure 1

Fig. 1. DNA constructs encoding metFTS and RAd-metFTS construction. A DNA sequence coding for native FTS was designed for optimal expression in rat cells. By adding an ATG starting codon upstream and 2 stop codons downstream this sequence, it was converted into an ORF for the analog metFTS **(A)**. This metFTS ORF was used to generate a construct to be cloned in the shuttle vector pDC515. The construct included the phage T7 promoter primer binding site which was used for sequencing purposes **(B).** The shuttle pDC515-metFTS was generated by inserting the T7-metFTS sequence into the Bam HI Sal I sites of the MCS of the shuttle pDC515. In turn, this construct was

used to generate RAd-metFTS **(C).** PmCMV, mouse cytomegalovirus promoter; frt, recognition element for the yeast FLP recombinase; ITR, inverted terminal repeats; ΔE1 and ΔE3, deletions in the Ad5 genome; SV40, simian virus 40 polyadenylation signal; ψ, packaging signal (From ref. 28).

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GENE THERAPY FOR THYMULIN

A single i.m. injection of RAd-metFTS in newborn nude mice (nude mice have undetectable circulating levels of thymulin) elicited long-term restoration of serum thymulin in these mutants. This treatment was able to prevent the mild reduction in the number of gonadotropin-releasing hormone (GnRH) neurons of the athymic mice **(Fig. 2)** as well as the deficits in serum LH and FSH that typically appear in adult female nudes **(28)**.

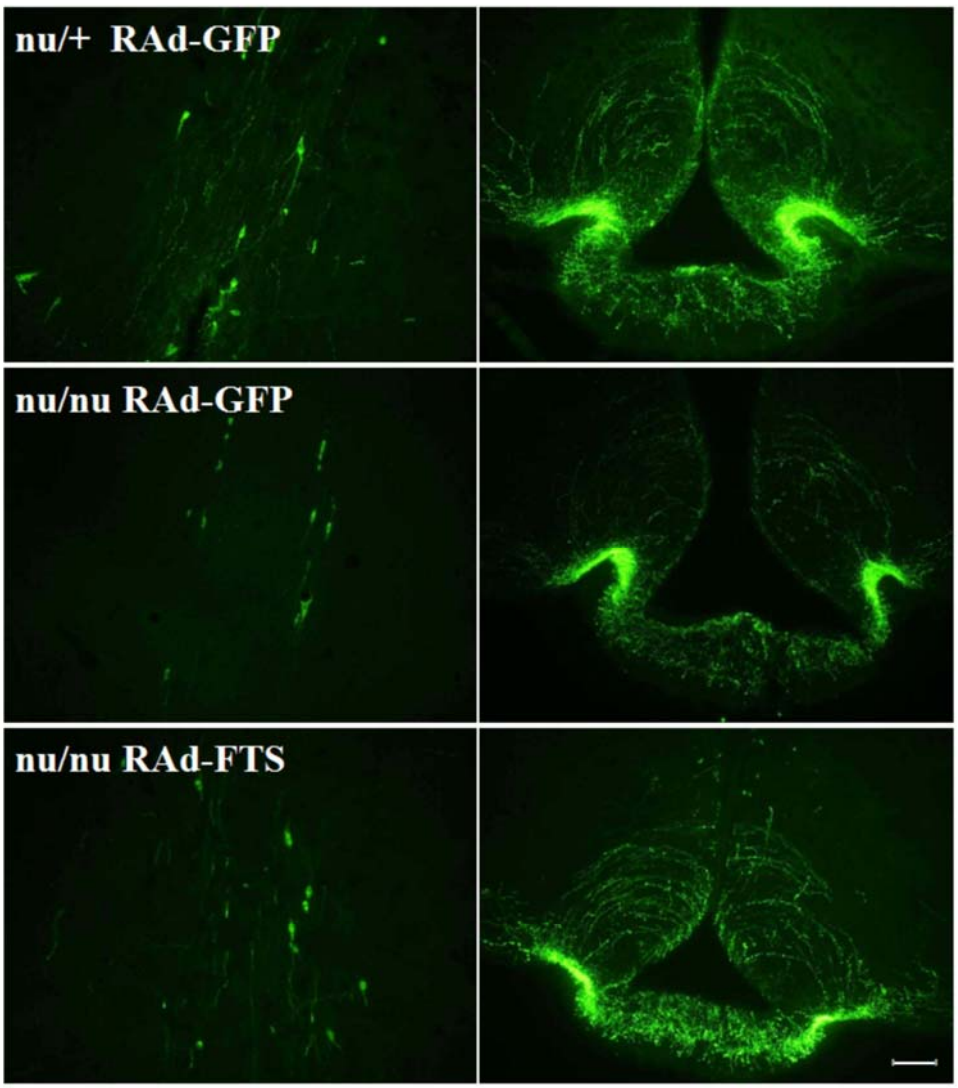

Fig 2. Hypothalamic GnRH neuron populations in control and experimental nude female mice. GnRH perikarya (left panels) and fibers (right panels) in the anterior and mediobasal hypothalamus, respectively, of control hetero and homozygous nude females and in homozygous counterparts submitted to neonatal thymulin gene therapy (From ref.29). Scale bar corresponds to 200 μm.

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Furthermore, neonatal thymulin gene therapy (NTGT) in nude female mice has been found to significantly prevent the ovarian dysgenesis that usually develops in 70-day old female nude mice **(Fig. 3) (29)**.

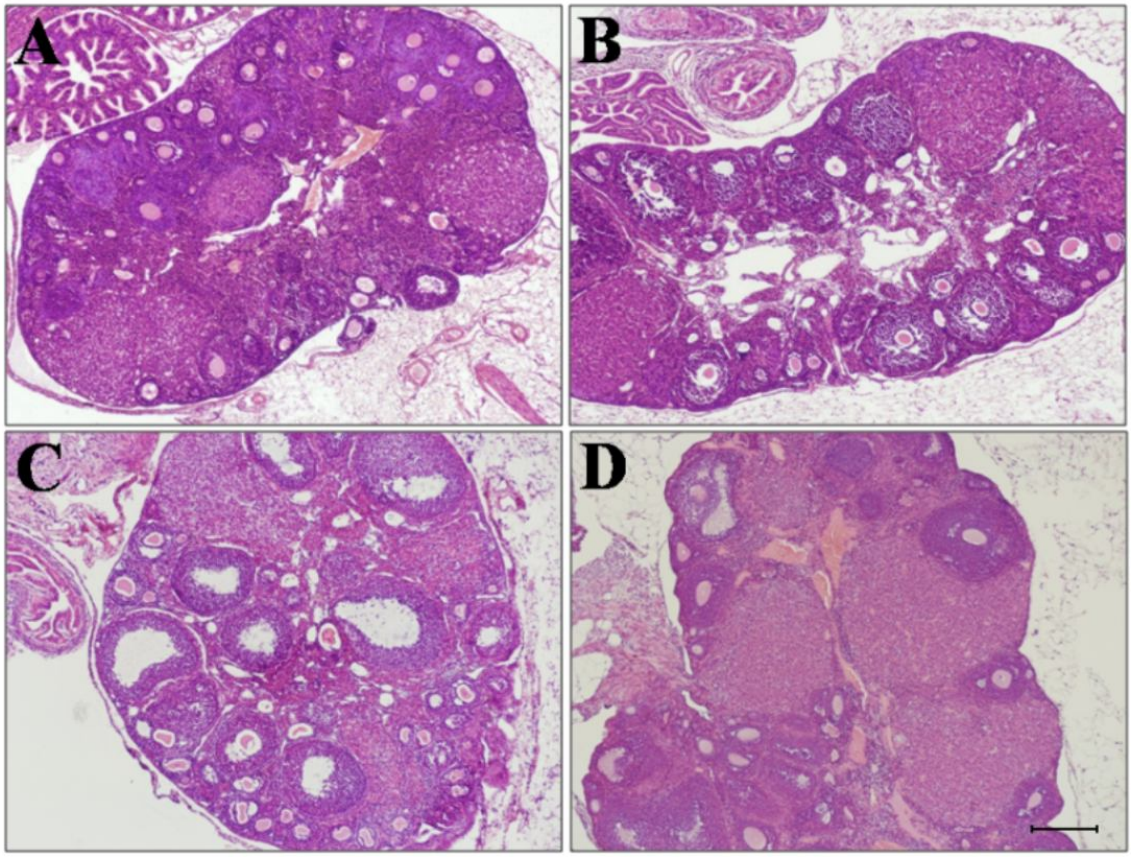

Figure 3

FIG. 3. Effect of thymulin gene therapy on ovarian morphology in nude mice. H&E stained sections of ovaries from nu/+ **(A)**, nu/nu **(C)** mice treated with the control vector (RAd-GFP) and nu/+ **(B)** and nu/nu **(D)** mice submitted to neonatal thymulin gene therapy (From ref. 29). Scale bar corresponds to 200 μm.

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In nude mice, NTGT has also been shown to partially prevent the alterations of some of the endocrine cell populations that occur in these mutants after puberty. Thus, NTGT prevented the reduction in the number of gonadotrophs, thyrotrophs, corticotrophs and somatotrophs in adult nudes **(30-32)**. NTGT had the highest histomorphometric impact on the gonadotrophic population of nudes (**30)**.

CONCLUDING REMARKS

Thymulin is probably the best characterized of all putative thymic hormones and seems to play a physiologic role in thymus-pituitary communication, particularly during perinatal life. Interest in the therapeutic use of thymulin flourished during the ’70 and ’80 when efforts where almost exclusively focused on using thymulin (and other thymic peptides) for the treatment of autoimmune and other immunopathologies as well as cancer (**33**). Subsequent studies, most of them carried out during the last twenty years, established that thymulin is active on the hypophysis and the brain. This awareness and the recent availability of a synthetic gene for metFTS have opened new avenues for the exploration and eventual exploitation of the therapeutic potential of this metallopeptide.

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