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Physiology and Therapeutic Potential of the Thymic Peptide Thymulin

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Abstract: Thymulin is a thymic hormone exclusively produced by the epithelial cells of the thymus. After its discovery and initial characterization in the '70s, it was demonstrated that the production and secretion of thymulin are strongly influenced by the neuro-endocrine system. Conversely, a growing body of evidence, to be reviewed here, suggests that thymulin is a hypophysiotropic peptide. Additionally, a substantial body of information pointing to thymulin and a synthetic analog as anti-inflammatory and analgesic peptides in the central nervous system brain and other organs will be also reviewed. In recent years, a synthetic DNA sequence encoding a biologically active analog of thymulin, metFTS, was constructed and cloned in a number of adenovectors. These include bidirectional regulatable Tet-Off vector systems that simultaneously express metFTS and green fluorescent protein and that can be down-regulated reversibly by the addition of the antibiotic doxycycline. A number of recent studies indicate that gene therapy for thymulin may be an effective therapeutic strategy to prevent some of the hormonal and reproductive abnormalities that typically appear in congenitally athymic (nude) mice, used as a suitable model of neuroendocrine and reproductive aging. Summing up, this article briefly reviews the publications on the physiology of the thymulin-neuroendocrine axis and the anti-inflammatory properties of the molecule and its analog. 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.

Keywords: Thymulin, neuroendocrine system, anti-inflammatory, nude mice, synthetic gene- gene therapy, regulatable promoters.

RELEVANCE OF THE THYMUS GLAND IN THE IMMUNE-NEUROENDOCRINE AXIS

It is well established that the immune, nervous and endocrine systems are functionally integrated so that they constitute a bidirectional homeostatic network [1]. The neuroendocrine system monitors and regulates the physical and metabolic variables of the organism. The immune system senses, through antigenic recognition, the internal configuration of the macromolecular and cellular components of the body and reacts to abnormal changes of this configuration, thus participating of the "biological" homeostasis of the organism.

In mammals, the interaction of the thymus with the neuroendocrine system is especially important during perinatal life, a period during which the thymus gland and the neuroendocrine system reciprocally influence each other's maturation. This fact was first suggested by early studies demonstrating that in species in which neonatal thymectomy does not impair their immune capacity [2], neuroendocrine physiology is well-developed at birth [3]. In mice, the relevance of the thymus gland for a proper maturation of the neuroendocrine axis is shown by the endocrine alterations induced by congenital absence of the thymus gland or neonatal thymectomy. Thus, in congenitally athymic (nude) female mice reduced levels of circulating and pituitary gonadotropins have been reported, a deficit that is believed to be causally related with a number reproductive derangements described in these mutants [4]. In homozygous nude females the times of first ovulation and vaginal opening are delayed [5], fertility is reduced [4] and follicular atresia is increased which results in premature ovarian failure [6]. Similar derangements are associated with neonatal thymectomy in normal mice [7, 8]. The ovaries of nude mice respond normally to exogenous gonadotropins, suggesting that the defect lies at the level of the hypothalamo-pituitary axis [9, 10]. In homozygous nude CD-1 male mice, the responses of thyrotropin (TSH), prolactin (PRL), somatotropin (GH) and gonadotropin to cold and immobilization stress are reduced as also are serum basal levels of the same peptides when compared with heterozygous counterparts [11-13]. An impairment of the hypothalamo-adrenal axis also occurs in nude mice, an alteration suggesting that thymic and humoral factors are relevant for the maturation of the axis [14].

The influence of the neuroendocrine axis on thymus physiology seems to extend into adult life via a direct action of pituitary hormones or through peripheral hormones, both of which exert their effects on the epithelial cells of the thymus (TEC) and/or on immature intrathymic thymic cells [for a review see, 15].

THYMULIN

Thymulin is a thymus peptide involved in several stages during intra- and extrathymic T-cell differentiation [16]. This peptide, produced by the TEC [17], consists of an inactive nonapeptide component named FTS (an acronym for serum thymus factor in French), coupled in an equimolecular ratio to the zinc ion [18], which endows the molecule with biological activity [19]. The active form of the metallopeptide has a specific molecular conformation that has been demonstrated by nuclear magnetic resonance [20].

NEUROENDOCRINE CONTROL OF THYMULIN PRODUCTION

Thymulin exerts a feedback action on its own secretion both *in vivo* and *in vitro* [21, 22]. The production and secretion of this hormone are also modulated by the neuroendocrine axis [15]. An especially important hormone is somatotropin which influences thymulin secretion and synthesis. GH stimulates thymulin secretion from TEC lines [23] via specific receptors for GH [24]. Animal studies showed that treatment of old dogs with bovine somatotropin partially restored their low serum thymulin levels [25]. Treatment of aged mice with ovine somatotropin increased their low plasma thymulin levels and also raised the concanavalin A (Con A)-dependent proliferative response of their thymocytes as well as interleukin-6 production [26]. Combined treatment of old rats with

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somatotropin and thyroid hormone (T_4) partially restored their reduced thymulin serum concentartion [27]. In congenitally somatotropin-deficient children, who consistently exhibit low circulating thymulin, GH therapy was able to increase thymic hormone levels to near normal values [28]. Acromegalic middle-aged individuals have higher thymulin circulating concentration than age-matched normal subjects [23, 28]. These effects of somatotropin may be mediated, at least in part, by insulin-like growth factor I (IGF-I) as suggested by the fact that the somatotropin-induced increase of thymulin production can be prevented by prior treatment with antibodies against IGF-I or IGF-I receptor [23].

There are also results suggesting the existence PRL-thymulin loop. In effect, TEC have PRL receptors [29] and there is the fact that PRL can stimulate thymulin secretion and synthesis both *in vivo* and *in vitro* [30]. Also, treatment of old mice with PRL mice increased their low serum concentration of thymulin [30].

The thyroid axis also affects on thymulin secretion. Thus, T_4 stimulates thymulin secretion and synthesis in mice [31]. Treatment of mice with triiodothyronine (T_3) enhanced thymulin release while treatment of mice with the thyroid hormone synthesis inhibitor propylthiouracil, reduced their serum thymulin levels [32]. In humans, hyperthyroidism induces a significant increase in serum thymulin levels whereas hypothyroid individuals have reduced levels of thymulin [33]. *In vitro* studies, have demonstrated that thyroid hormones increase thymulin secretion by a direct effect on the TEC [34, 35]. Additionally, it is known that treatment of aged animals with T_4 can correct their serum thymulin deficit [31].

There are no reports revealing a direct effect of gonadotropins or corticotropin (ACTH) on thymulin secretion; nevertheless, gonadectomy or adrenalectomy have been shown to induce a transient reduction of circulating thymulin in mice [36], an effect potentiated by the simultaneous removal of the gonads and adrenals [36]. In TEC lines, exposure to physiological levels of gonadal steroids or glucocorticoids enhanced thymulin levels in the cell supernatants [37].

There is no direct evidence proving the existence of hypothalamic factors able to affect thymulin production by an action on TEC; however, it has been documented that treatment of old mice with hypothalamic extracts from young mice is followed by the reappearance of detectable levels of serum thymulin [38]. Also, pituitary or hypothalamic extracts from young mice induced thymulin secretion from TEC lines. This effect declined when the hypothalamic and pituitary extracts came from aged mice [39].

HYPOPHYSIOTROPIC ACTIVITY OF THE HORMONE THYMULIN

The pleiotropic influence that the neuroendocrine axis exerts on thymulin secretion indicates that the hormone may be part of a feedback loop acting on neuroendocrine organs. This hypothesis is supported by a significant amount of data indicating that thymulin has hypophysiotropic activity. Thus, thymulin stimulates luteinizing hormone (LH) secretion from perfused rat pituitary glands [40] and corticotropin from incubated rat pituitary fragments, the latter constituting an action mediated by cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) accumulation [41]. It has been reported that thymulin modulates the stimulatory activity of gonadotropin releasing hormone (GnRH) on LH and follicle stimulating hormone (FSH) release from pituitary cells from female rats in different days of the estrous cycle [42]. Thymulin also stimulates TSH, PRL, GH and gonadotropin secretion in dispersed rat pituitary cells at doses ranging from 10^{-8} to 10^{-3} M [43-45]. It has been also documented that thymulin at a dose of 10^{-11} M stimulates LH, inhibits PRL release and has no effect on GH release in incubated rat pituitary fragments [41]. Since the stimulatory action of thymulin on hormone release in rat pituitary cells declines with the age of the cell donor [43-45], it is believed that aging causes a desensitization of the pituitary gland to thymic signals.

Thymulin may play a role in the regulation of female spontaneous puberty, possibly via a direct action on pituitary gonadotropin release and ovarian steroidogenesis [40, 42, 46-48]. This thymic peptide also influences gonadotropin-induced testicular steroidogenesis [49].

Immunoneutralization experiments lend support the idea that thymulin may be a physiologic mediator of the perinatal influence of the thymus gland on the maturation of the neuroendocrine axis. Thus, immunoneutralization of serum thymulin from age 1 to 33 days in normal C57BL/6 mice induced clear morphologic changes in most adenohypophysal endocrine cell populations [50-52] as well as a reduction in serum levels of gonadotropins, TSH, PRL and GH (Fig. **1**). When thymulin was immunoneutralized from 1-2 to 9 days of age and excess synthetic thymulin was intraperitoneally (i.p.) administered on day 10 to normalize serum thymulin, circulating TSH and gonadotropin levels were still low at 45 days of age, but serum PRL and GH levels were within the normal range (Fig. **2**). These results suggest that for some pituitary hormones (TSH, FSH and LH) but not for others (GH and PRL) thymulin deficiency during a 9-day postnatal window has irreversible consequences.

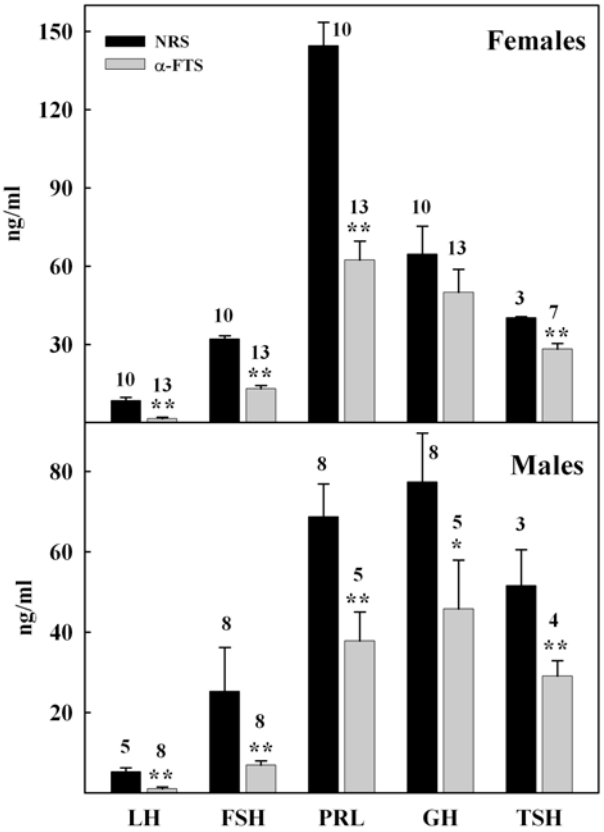

Fig. (1). Effect of long-term thymulin quenching on pituitary hormone serum levels in mice.- Experimental animals (open columns) received weekly i.p. injections of rabbit anti-FTS serum (α-FTS) beginning at birth. Control mice (solid columns) received normal rabbit serum (NRS). All mice were sacrificed at 33 days of age. Numbers over columns represent the number of mice assessed for the corresponding data point. In order to reduce dead space in the graph, PRL values were multiplied by 0.5. Error bars represent SEM. Significance of differences between controls and experimental are indicated by *, P<0.05 or **, P<0.01.

ANALGESIC AND ANTIINFLAMMATORY ACTIONS OF THYMULIN AND ITS ANALOGUE

Among the molecules that interact with thymulin are a set of cytokines that play a major role in the inflammatory response. In

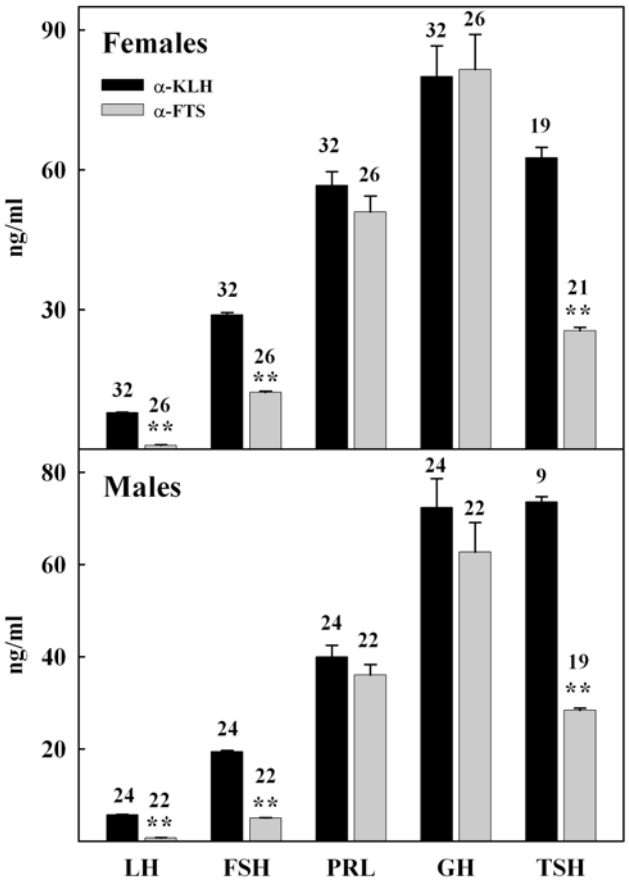

Fig. (2). Effect of short-term thymulin quenching on pituitary hormone serum levels in mice.- Experimental animals (light gray columns) were i.p. injected at postnatal day 1 and 2 with rabbit anti-FTS serum (α-FTS), which was followed by two i.p. injections of synthetic thymulin (60 pg/g BW) on postnatal days 8 and 9. Control mice (solid columns) were submitted to the same treatment except that they received a rabbit anti-keyhole hemocyanin serum (α-KHL) instead of anti-FTS. All mice were sacrificed at 45 days of age for hormone measurement**.** Control mice received anti-KLH rabbit serum while experimental mice received rabbit anti-FTS serum. All mice were sacrificed at 45 days of age**.** Other details are as in Fig. **2**.

this regard, several studies have evidenced the potent effect of thymulin in different animal models of pain. It was first demonstrated that thymulin could modulate in a dose dependent manner some peripheral nervous sensory functions such as those related to pain. We first showed that at low doses (ng), local (intraplantar) or systemic (i.p.) injections of thymulin resulted in hyperalgesia with an increase of the proinflammatory mediators, Interleukin-1ß (IL-1ß), Tumor necrosis factor-alpha (TNF-α) and Nerve growth factor (NGF) and that the peptide could act directly on the afferent nerve terminals through Prostaglandin-E2 (PGE-2) dependent mechanisms [53, 54]. In further experiments, systemic injections of higher doses (1-25μg) of thymulin have been shown to reduce the inflammatory pain and the upregulated levels of cytokines induced by endotoxin injection [55, 56]. The paradoxical effects of thymulin on pain are in keeping with previous data showing that thymulin at low concentrations stimulates IL-1 release from human peripheral blood mononuclear cells, whereas at high concentrations it suppresses the release of IL-1, as well as IL-2, IL-6 and TNF-α [57]. 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 [58].

The dual effect on pain observed for thymulin does not exist for the thymulin related peptide, which is deprived of hyperalgesic effect. This analogue molecule, which differs from thymulin on the N-Terminal and the C-terminal residues, has been proved to be a potent systemic analgesic and anti-inflammatory molecule. In the model of endotoxin-induced systemic inflammation, the pretreatment with this analogue not only induced a down regulation of proinflammatory mediators (IL-1, IL-6, TNF-α and PGE-2) in the liver but also appeared to alleviate the sickness behavior induced by endotoxin. This amelioration took the form of reversal of hyperalgesia, improvement of the motor behavior and prevention of febrile reactions. In addition when compared with the effect of steroidal and non steroidal anti-inflammatory drugs, the thymulin related peptide demonstrated equal or stronger anti-hyperalgesic effects and at much lower concentrations [59]. In the model of neuroinflammation, the 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 [60]. IL-10 itself has been shown to be able to reduce the inflammatory hyperalgesia induced by endotoxin and has a suppressive action on the production of the proinflammatory cytokines [61, 62]. In this model, preliminary results from the same group suggest that the anti-inflammatory effects of the thymulin analogue might be partially mediated through the nicotinic acetylcholine receptor-α7 (α7-nACHR) subtype. The activation of this receptor plays an important role in the inflammatory process and the thymulin analogue has been shown to potentiate its function [63].

Taken together, all the actions of thymulin and its analogue described above suggest that these molecules have potent analgesic and anti-inflammatory properties. The observed antihyperalgesic actions can be attributed to the inhibitory effects of these molecules on the inflammatory cascades through the down-regulation of the levels of proinflammatory cytokines.

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 [64]. A similar protection was observed against thyroiditis caused by reovirus [65], myocarditis induced by encephalomyocarditis virus [66], nephrotoxicity caused by cephaloridine or cisplatin [67, 68], chronic colitis induced by dextran sulphate sodium [69] and pulmonary hypertension induced in rats by monocrotaline [70]. 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 [71]. The protective effect of thymulin against inflammation was further analysed by Lunin and coworkers in an animal model with inflammation induced by lipopolysaccharide (LPS) from gram negative bacteria [72]. These authors showed that the peptide prevented the accumulation of IL-1, IL-6, TNF-α and Interferon-gamma (IFN-γ) in plasma and the LPS-induced upregulation of production of cytokines by splenic lymphocytes and macrophages. Interestingly, this effect was accompanied by a significant decrease in the heat-shock protein HSP70 by splenic lymphocytes induced by LPS treatment. Since they also demonstrated that hyperactivation of immune cells by bacterial toxin was accompanied by increase of HSP70 production, the decrease in HSP70 production induced by thymulin suggests that this peptide reduces stress response to endotoxin, a finding confirmed by the lowering of proinflammatory cytokines in the plasma [72].

In a recent report, Lunin *et al*. studied the modulation of inflammation by thymulin in New Zealand White (NZW) mice with acute autoimmune encephalomyelitis, induced by myelin basic protein [73]. 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-9B) cascade. It was shown that the peptide reduced the level of phosphorylation of the NF-9B signalling protein IKK and the production of HSP72 protein. This modulatory role of thymulin on NF-9B signaling confirms previous data from the same group showing that thymulin decreased LPS-induced NF-9B cascade activation in splenocytes and macrophages [74].

The above data demonstrate that, although the mechanisms of action of these molecules are not yet clearly identified, thymulin and its analogue 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 possibility of implementing gene therapy for thymic hromones constitutes a promising approach for correcting thymic activity when the function of the endocrine thymus is compromised. So far, none of the genes coding for thymic hormones has been cloned. This situation hinders the implementation of gene therapy and other molecular therapies for thymus hormones. Constructing "artificial genes" coding for thymic peptides possessing short amino acid sequences and not requiring posttranslational processing has been proposed as a possible way to overcome this difficulty [75]. This has been done for thymulin where a DNA sequence coding for the peptide was initially inserted in a bacterial plasmid [76]. Later, a DNA sequence encoding a biologically active FTS analog termed metFTS (methionine-FTS), was constructed and cloned in an adenovector, RAd-metFTS [77].

Intramuscular (i.m.) injection of RAd-metFTS to thymectomized (Tx) rats and mice (whose serum levels of thymulin are nondetectable), induced supraphysiological circulating levels of biologically active thymulin which remained elevated for up to 270 days in rats and for at least 112 days in mice [77]. Adenovector-mediated expression of the artificial gene for metFTS in the hypothalamus and substantia nigra of adult Tx rats had a longer duration than adenovirally-mediated expression of the gene for green fluorescent protein (GFP) or *E. coli* β-galactosidase (β-gal) in the same brain regions [78]. These reults are consistent with the anti-inflammatory activity in the brain documented for thymulin and a thymulin analog (see above). This anti-inflammatory activity of the metFTS vector could prevent the immune system from mounting a destructive response against the adenovector-transduced cells. The same mechanism could explain the long-term persistence of high levels of metFTS in the circulation of Tx rodents treated with RAd-metFTS [77].

REGULATABLE ADENOVECTORS FOR THYMULIN

The first adenovector harboring the metFTS sequence (RAd-metFTS) expressed its transgene constitutively [77]. More recently, a Tet-Off regulatable bidirectional first generation and a helper-dependent (HD) system expressing the genes for humanized green fluorescent protein (hGFP) and metFTS were constructed (Fig. **3**). Both systems harbor two expression cassettes: a pCMV-tTA cas-

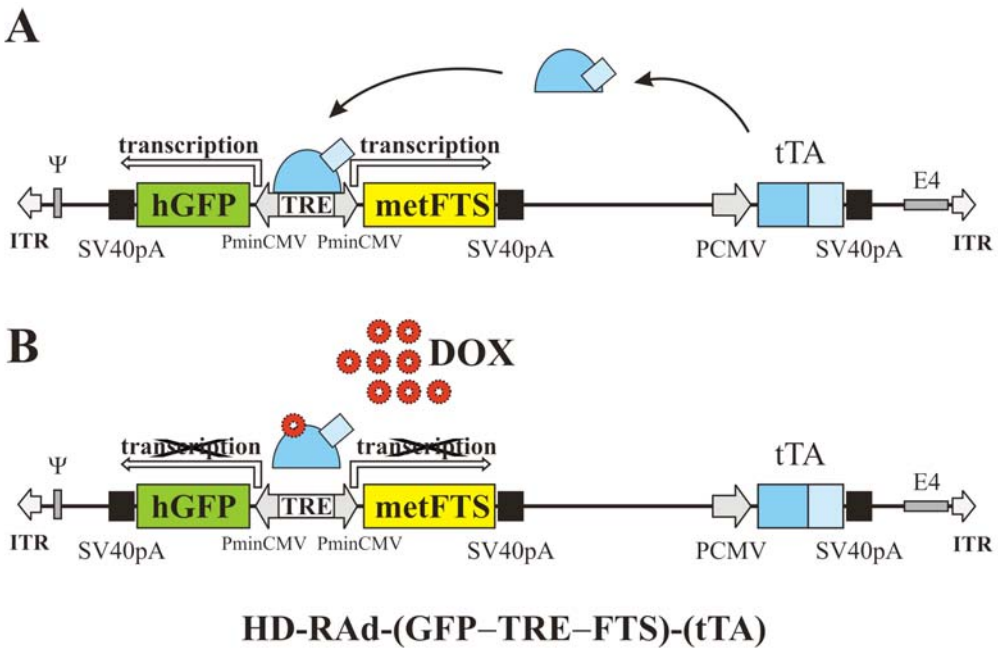

Fig. (3). Diagrammatic representation of the backbone of the genome of a helper-dependent regulatable bidirectional Tet-Off construct expressing the genes for hGFP and metFTS.- It harbors two expression cassettes: The pCMV-tTA cassette (right portion of the genome) expresses the chimeric regulatory protein tTA, which binds to the regulatable bidirectional promoter of the bidirectional cassette hGFP-TRE-metFTS (left portion of the genome) and activates it inducing the expression of the transgenes for hGFP y metFTS. Panel A shows the active system (in the absence of doxycycline, DOX). Panel B depicts the system now inhibited by DOX. hGFP: humanized Green Fluorescent Protein; TRE: Tetracycline responsive element; metFTS: metFTS coding sequence; tTA: chimeric regulatory protein; PminCMV: cytomegalovirus minimal promoter; SV40pA: polyadenylation signal; ITR: inverted terminal repeats; E4: Ad5 early E4 gene, ψ: packaging signal.

sette that expresses the chimeric regulatory protein tTA, which binds to the regulatable bidirectional promoter of the bidirectional cassette hGFP-TRE-metFTS and activates it, thus inducing the expression of the transgenes for hGFP and metFTS. If the antibiotic doxycycline (DOX) is added to the medium it binds to an allosteric site on the tTA protein and causes it to dissociate from the regulatable promoter thus turning off transgene expression (Fig. **3**). A two-viral vector version of this system was also constructed. It consists of two first generation adenoviral vectors, one, RAd-tTA, expresses the tTA regulatory protein and the other, RAd-(GFP-TRE-FTS), expresses the bidirectional regulatable cassette for hGFP and metFTS. When a cell is co-transduced by both vector components, RAd-tTA expresses the tTA regulatory protein, which binds to the regulatable promoter ($PCMV_{min}$-TRE-$PCMV_{min}$) of the second vector and activates the expression of both transgenes. If DOX is added to the medium it inhibits transgene expression by the mechanism described above. The system was successfully tested in cell lines and in brain and muscle (unpublished observations).

These bidirectional vectors allow a ready visualization of metFTS expression in tissues and organs by fluorescence microscopy (if GFP is expressed then metFTS also is because they are under the control of a single promoter). The regulatability by DOX of these vectors allows the experimenter to turn-off and on transgene expression *in vivo* by respectively adding or removing DOX from the drinking water.

NEONATAL GENE THERAPY FOR THYMULIN IN NUDE MICE

A single i.m. administration of RAd-metFTS to newborn nude mice (which have undetectable circulating levels of thymulin) induced long-term restoration of serum thymulin in these mutants. The treatment was able to prevent the slight reduction in the number of gonaddotropin-releasing hormone (GnRH) neurons of these mutants (Fig. **4**) and the deficits in circulating FSH and LH that typically appear in adult female athymic mice [52]. Additionally, neonatal thymulin gene therapy (NTGT) in nude female mice was reported to significantly prevent the ovarian dysgenesis that usually arises in 70-day old female athymic mice [79].

NTGT has also been reported to partially prevent the alterations that occur in some of the endocrine cell populations of nude mice after puberty. Thus, NTGT prevented the reduction in the number of gonadotrophs, thyrotrophs, corticotrophs and somatotrophs in adult nudes [51, 80-82]. NTGT also prevented, to a varying extent, changes in other histomorphometric parameters in the anterior pituitary of adult nudes. Thymulin administration to nudes marginally decreased their hypothalamic content of corticotropin-releasing hormone (CRH) and slightly increased the adrenal content of corticosterone of these mutants [81]. NTGT had the highest histomorphometric impact on the gonadotrophic population of nudes [80].

CONCLUDING REMARKS

Thymulin is the most extensively characterized thymic hormones and appears to be a physiologic mediator of thymus-pituitary communication, particularly during perinatal life. Interest in the therapeutic properties of thymulin was highest during the ’70s and ’80s when research efforts were almost exclusively devoted to using thymulin (and other thymic peptides) for treating autoimmune and other immunopathologies as well as oncologic pathologies [83, 84]. Subsequent studies, most of them carried out during the last twenty years, demonstrated that thymulin is active on the pituitary gland and the CNS. This awareness and the availability of an artificial DNA sequece encoding metFTS as well as sophisticated adenovectors for metFTS have opened new avenues for the exploration and possible exploitation of the therapeutic potential of thymulin.

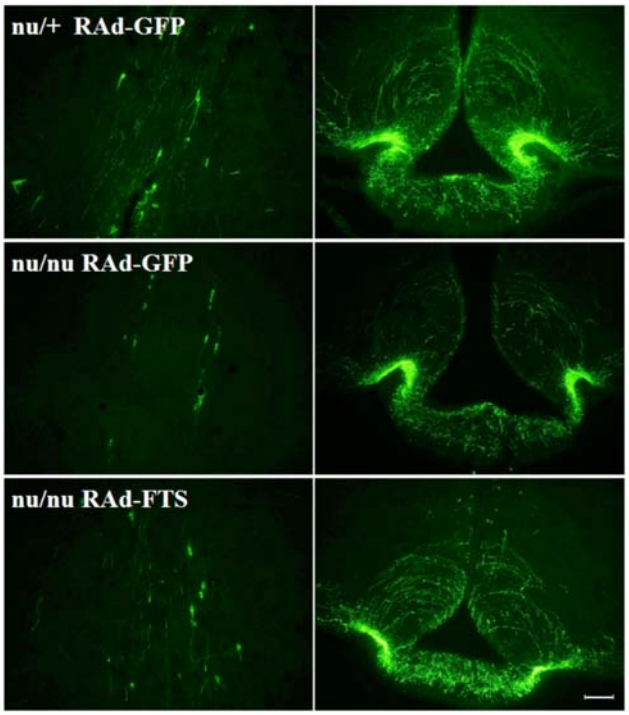

Fig. (4). Hypothalamic GnRH neuron population in control and experimental nude female mice. GnRH perikarya (left panels) and fibers (right panels) in the anterior and mediobasal hypothalamus, respectively, of control hetero and homozygous nude females and in homozygous counterparts submitted to neonatal thymulin gene therapy. Scale bar corresponds to 200 μm (From ref. 79, with permission, Copyright [2012], The Endocrine Society).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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CONTRIBUTION OF THE AUTHORS

PCR and JIS extensively reviewed the literature on thymulin particularly in reference to the adenoviral vectors for metFTS. Their own experimental work on thymulin significantly contributed to this area of research. PCR and RGG prepared the figures. GMC, prepared the portions of the paper dealing with the morphological aspects of thymulin action. EAR, contributed in aspects related to the immunology of thymulin. MD, the co-discoverer of thymulin, reviewed the literature on the anti-inflammatory actions of thymulin. RGG, did the main writing of the mansucript and coordinated the different sections.

DISCLOSURE

Part of the review text in this article has been previously published in Annals of the New York Academy of Sciences Volume 1153, pages 98–106, February 2009. The original experimental

results shown on Figs 1, 2 and 3 have not been previously published.

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