Original Paper

NeuroImmunoModulation

Neuroimmunomodulation
DOI: 10.1159/000346477

Received: October 12, 2012
Accepted after revision: December 12, 2012
Published online: ■■■

The Thymulin-Lactotropic Axis in Rodents: Thymectomy, Immunoneutralization and Gene Transfer Studies

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Key Words
Thymus-lactotropic axis · Thymulin gene therapy · Nude mice · RAd-FTS

Abstract
Objectives: There is clear evidence on the existence of a thymus-pituitary axis which seems to be particularly important during perinatal life. In particular, the thymic peptide thymulin has been shown to be a relevant player in thymus-pituitary communication. Our goal was to explore the effect of thymulin on circulating prolactin (PRL) levels in different animal models. To this end we undertook a series of experiments in rats and mice, implementing adult thymectomy, thymulin immunoneutralization in normal C57BL/6 mice and neonatal thymulin gene therapy in nude mice. ***Methods:*** We assessed the impact of the above manipulations on PRL secretion and lactotrope morphology by measuring serum PRL by radioimmunoassay and by performing morphometric analysis of the lactotropic cell population in the anterior pituitary gland. ***Results:*** Adult thymectomy in female rats slightly increased serum PRL, an effect that was partially reversed by thymulin gene therapy. In mice, thymulin immunoneutralization from birth to age 32 days reduced serum PRL both in males and females. Thymulin immunoneutralization induced a significant ($p < 0.01$) decrease in lactotrope cell density (CD) and volume density (VD) without changes in cell size (CS). Neonatal thymulin gene therapy markedly increased serum thymulin ($p < 0.01$) and lactotrope CD, CS and VD in nude mice of both sexes. ***Conclusions:*** Our findings suggest a modulatory effect of thymulin on the lactotrope cell population and on serum PRL, particularly during early life.

Introduction

There is clear evidence on the existence of a thymus-pituitary axis which seems to be particularly important during perinatal life, when both organs influence their respective maturation [1]. 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 to a number of reproductive derangements described in these mutants [2]. In homozygous adult *nude* CD-1 male mice,

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prolactin (PRL) responses to immobilization and cold stress are reduced as also are serum basal levels of PRL as compared to their heterozygous counterparts [3].

The thymic peptide thymulin seems to play a relevant physiologic role as mediator of the thymus-pituitary axis. This peptide is exclusively produced by the thymic epithelial cells (TEC) [4], and 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 [5], which confers biological activity to the molecule [6].

Thymulin production and secretion are stimulated directly by growth hormone and PRL, and indirectly by thyrotropin through thyroid hormones. Corticotropin and gonadotropins inhibit thymulin secretion via a direct action of adrenal and gonadal steroids, respectively, on thymic epithelial cells [7]. Reciprocally, thymulin is known to possess hypophysiotropic activity in vitro [8–12]. Thus, it was observed that thymulin stimulates PRL release in dispersed rat pituitary cells [9], whereas others have reported that thymulin inhibits PRL release in incubated rat pituitary fragments [8]. Furthermore, we have found in in vivo studies that thymulin is an important player in the communication of the thymus with the gonadotropic, corticotropic and thyrotropic axes [13–16].

Since nude females are known to exhibit a deficient production of milk during lactation we undertook to explore the physiologic relevance of thymulin as a mediator of the thymic-lactotrope axis in rodents. To this end we constructed a synthetic DNA sequence coding for a biologically active FTS analog called metFTS and cloned it in an adenoviral vector [17]. With this vector we assessed the effect of neonatal thymulin gene therapy on the thymus-lactotropic axis in congenitally athymic *nude* mice as well as the effect of thymulin gene therapy in adult female rats. We also evaluated the impact of thymulin immunoneutralization during early life on the lactotrope population of normal mice.

Materials and Methods

Animals

Adult (9 months) female Sprague Dawley rats as well as male and female C57BL/6 mice and neonatal NIH heterozygous (nu/+) and homozygous (nu/nu) *nude* (athymic) mice were used. The parent NIH mice (10-week-old homozygous males and heterozygous females) were purchased from the Ezeiza Atomic Center, Ezeiza, Argentina. Animals were kept in our animal facilities (INIBIOLP) with free access to food and water and were kept at 22°C with a light/dark cycle of 12/12 h. NIH mice were maintained on a γ-irradiated chow diet and sterilized water.

All experiments on animals were done following the Animal Welfare Guidelines of NIH (INIBIOLP´s Animal Welfare Assurance No. A5647-01).

Adenoviral Vectors Used

RAd-FTS. A DNA sequence (5′-ATGCAGGCCAAGTCG-CAGGGGGGGTCG-AACTAGT AG-3′) coding for the biologically active thymulin analog metFTS, here referred to as synthetic gene for thymulin, was constructed as previously reported [17]. A recombinant adenoviral (RAd) vector harboring the synthetic gene for thymulin was constructed by a variant of the two plasmid method, employing the AdMax® plasmid kit (Microbix, Mississauga, Ont., Canada). This kit uses a shuttle plasmid (pDC515) containing an FRT recognition site for the yeast FLP recombinase. This cassette is flanked by sequences of the adenovirus type 5 (Ad5) E1 region. The second plasmid of the kit, the genomic plasmid pBHGfrt(del)E1,3 FLP, consists of the entire genome of Ad5, containing deletions in the regions E1 and E3. Upstream the E1 deletion, the genomic plasmid, contains an expression cassette for the gene of yeast FLP recombinase and immediately downstream the E1 deletion, an FRT recognition site, has been inserted. Once the thymulin synthetic gene was inserted into the shuttle, both plasmids were cotransfected into HEK293 cells. In cotransfected HEK293 cells, FLP recombinase is readily expressed and efficiently catalyzes the site-directed recombination of the expression cassette of pDC515-metFTS into the left end of pBHGfrt(del)E1,3 FLP, thus generating the genome of the desired recombinant adenoviral vector, RAd-FTS. The newly generated RAd was rescued from HEK293 cell lysates and plaque purified. It was further purified by ultracentrifugation in CsCl gradient and titrated by a serial dilution plaque assay.

RAd-GFP. An adenoviral vector termed RAd-GFP was constructed in our laboratory following the general procedures outlined above and was used as a control vector. It harbors a hybrid gene encoding the *Aequorea victoria* enhanced green fluorescent protein (GFP) fused to herpes simplex virus type 1 thymidine kinase (a kind gift from Dr. Jacques Galipeau, McGill University, Montreal, Canada).

RAd-βgal. This RAd was kindly provided by Dr. Michel Perricaudet, Institut Gustave Roussy, NNRS, Villejuif, France. In this vector, the E1 genomic region has been replaced by an expression cassette containing the *Escherichia coli lac Z* reporter gene under the control of the Rous sarcoma virus long terminal repeat. The vector was expanded in 293 cells and purified and titrated as indicated for RAd-FTS.

Experimental Design

Long-Term Thymulin Expression Studies. Adult female rats were thymectomized (TX) following a standard surgical technique [18] and left for 2 weeks in order to ensure absence of circulating thymulin. Intact rats (n = 5) were used as intact controls (intact rat group). TX rats received a single bilateral intramuscular (hind legs) injection of either 10^8 plaque-forming units (pfu) RAd-FTS (n = 6) or RAd-βgal control (n = 6) in 200 μl vehicle (100 μl per side). The three groups of animals were sequentially bled from the tail veins for PRL and thymulin determination. On postinjection day 260 rats were bled and immediately sacrificed.

Thymulin Immunoneutralization Experiments. On the day of birth, C57BL/6 pups received weekly intraperitoneal injections of either normal rabbit serum (NRS group; 20 pups) or anti-thymu-

lin rabbit serum (α-FTS group; 20 pups); the anti-thymulin serum was a kind gift from Dr. Jean-Marie Pléau, CNRS UMR 8147, Hôpital Necker, Paris, France. The α-FTS serum was raised by immunizing rabbits with synthetic FTS coupled to keyhole limpet hemocyanin. The first injection consisted of 20 μl serum per pup, whereas the subsequent injections consisted of doses of 8 μl serum per gram body weight (BW), until the end of the experiment on postnatal day 32. Animals were bled from the retro-orbital plexus and killed by cervical dislocation. The pituitaries were dissected, fixed and processed for immunohistochemical assessment.

Thymulin Gene Therapy Experiments. On postnatal day 1, each experimental heterozygous and homozygous pup received a single bilateral intramuscular (hind legs) injection of 10^8 pfu RAd-FTS or RAd-GFP used as a control vector, in 10 μl vehicle (5 μl per side). On postnatal days 70–71, mice were bled and immediately sacrificed by cervical dislocation. The pituitary glands were immediately dissected, fixed and processed for histology and immunohistochemistry as appropriate.

Thymulin Bioassay

Biologically active thymulin was measured in serum by a rosette bioassay described in detail elsewhere [19]. This method is based on the ability of thymulin to restore the inhibitory effect of azathioprine on rosette formation in spleen cells from thymectomized mice. The inhibitory activity of samples was compared with that of a standard curve using synthetic thymulin. Serum values were expressed as fg/ml bioactive thymulin.

PRL Determination

Serum PRL was determined by radioimmunoassay using the mouse materials provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, UCLA Medical Center, USA. Serum PRL was expressed in terms of mPRL RP-1 or rPRL RP-3 for mice and rats, respectively.

Histology/Immunohistochemistry

Stated in brief, pituitary glands were fixed in Bouin's fluid and embedded in paraffin. Serial sections of 4 μm were obtained at two levels of the blocks following a ventral-to-dorsal sequence. The pituitary sections were immunostained and then incubated for 1 h at room temperature with a primary anti-PRL antibody (Dako, Carpintaria, Calif., USA) diluted 1:100. Thoroughly washed sections were then treated for 30 min with a ready-to-use EnVision reaction system (Dako). The peroxide-sensitive chromogen was diaminobenzidine [20]. In all instances, the specificity of the primary antiserum was monitored either by observing its ability to block the immunocytochemical reaction after its preabsorption with an excess of the related antigen or by its replacement with normal rabbit serum or PBS.

Anterior Pituitary Morphometry

Morphometry was performed as reported in detail previously [21]. Measurements of immunostained lactotrope cells were made by means of an image-analysis system (Imaging Technology, Optimas 5.2). The cells per reference area (RA) were analyzed in each field on an average of ten micrographs taken from two levels (e.g. a and b). These measurements were recorded and processed automatically, and the following parameters were subsequently calculated: cell size (CS, expressed in μm^2), volume density (VD = Σ cell area/RA) and cell density (CD = number of cells/RA). RA represents the total area throughout which the cells were scored. Thus, this area divided into the sum of the individual cell areas yielded VD, a parameter that represents an estimate of cell density according to generally accepted criteria. The CD was calculated by dividing the immunostained area of the pituitary tissue by the mean individual cell area. For this parameter, 100 cells were recorded in each field.

Statistical Analysis

Data are expressed as mean ± SEM, unless otherwise indicated. Statistical comparisons among experimental groups were performed by Student's t test, or by ANOVA followed by Tukey's test when the ANOVA was significant.

Results

Effect of Adult Thymectomy and Thymulin Gene Therapy on Serum PRL Levels in Female Rats

In female rats, adult TX elicited a mild increase in serum PRL levels, an effect that was partially prevented by thymulin gene therapy in the adult animals (fig. 1). Thymectomized rats (which had nondetectable serum thymulin) injected intramuscularly with RAd-FTS showed high levels of serum thymulin until postinjection day 260 (the last day tested), whereas animals that received the adenoviral vector expressing β-galactosidase (RAd-βgal) did not show any reappearance of serum thymulin (fig. 1, inset).

Effect of Thymulin Immunoneutralization on the Morphology of the Lactotrope Population in Normal C57BL/6 Mice

Immunostained PRL-cells of the pars distalis exhibited an ochre definite granular cytoplasmic pattern. The immunolabeled lactotropes showed a reduced number in both males and females treated with the α-FTS serum as compared to the NRS-treated counterparts (fig. 2). Morphometric analysis of the lactotrope population revealed a significant decrease in CD in males and an increase in CS, in both males and females of the α-FTS group as compared to NRS-treated controls (fig. 3a–c).

Impact of Thymulin Immunoneutralization on Serum Thymulin and PRL in C57BL/6 Mice

In preliminary experiments we observed that a single intraperitoneal injection of anti-FTS serum (8 μl/g BW) markedly reduced the serum activity of endogenous thymulin in C57BL/6 infantile mice. This inhibition lasted for at least 10 days (data not shown). Quenching of serum thymulin (antiserum injections done every 7 days) from

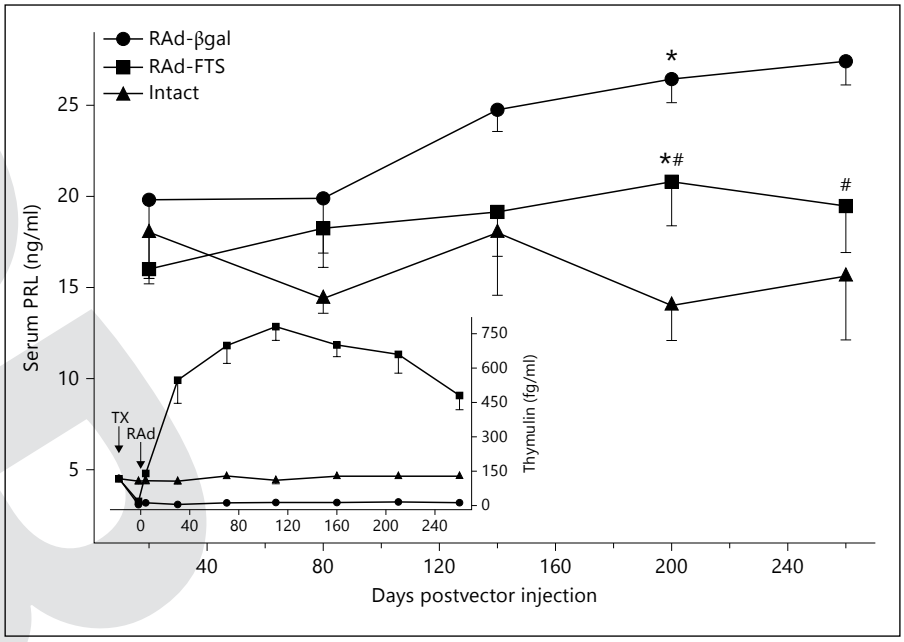

Fig. 1. Effect of adult thymectomy and thymulin gene therapy on serum PRL levels in female rats. TX animals received a single intramuscular (hind legs) injection of either RAd-FTS (squares, n = 6) or RAd-βgal (circles, n = 6). The third group corresponds to intact rats (triangles, n = 5). Animals were bled from the tail veins at the indicated post-injection times. Data are expressed as mean ± SEM. Significant differences are indicated (* and #; $p < 0.05$) from the corresponding time point in the intact or RAd-βgal counterparts, respectively. **inset** Time course of serum thymulin activity after vector injection. Similar results were observed in a previous study [16]. TX arrow indicates day of thymectomy; RAd arrow indicates day of injection of vectors. Some error bars are not visible due to their short length.

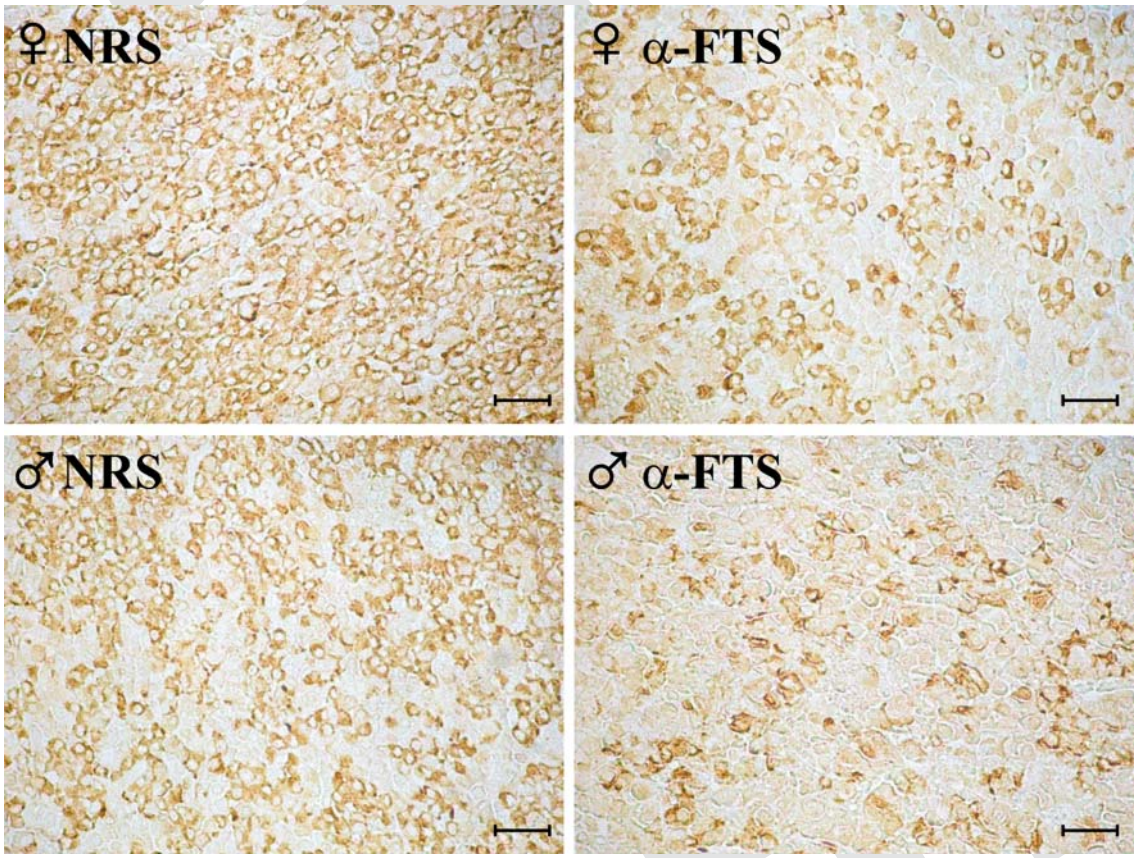

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Fig. 2. Impact of thymulin immunoneutralization on the lactotrope population in C57BL/6 mice. Representative fields of specifically immunostained PRL-cells in the two experimental groups: the α-FTS groups showed a decrease in CD with respect to the NRS groups of both sexes. EnVision system peroxidase. Scale bars = 45 μm.

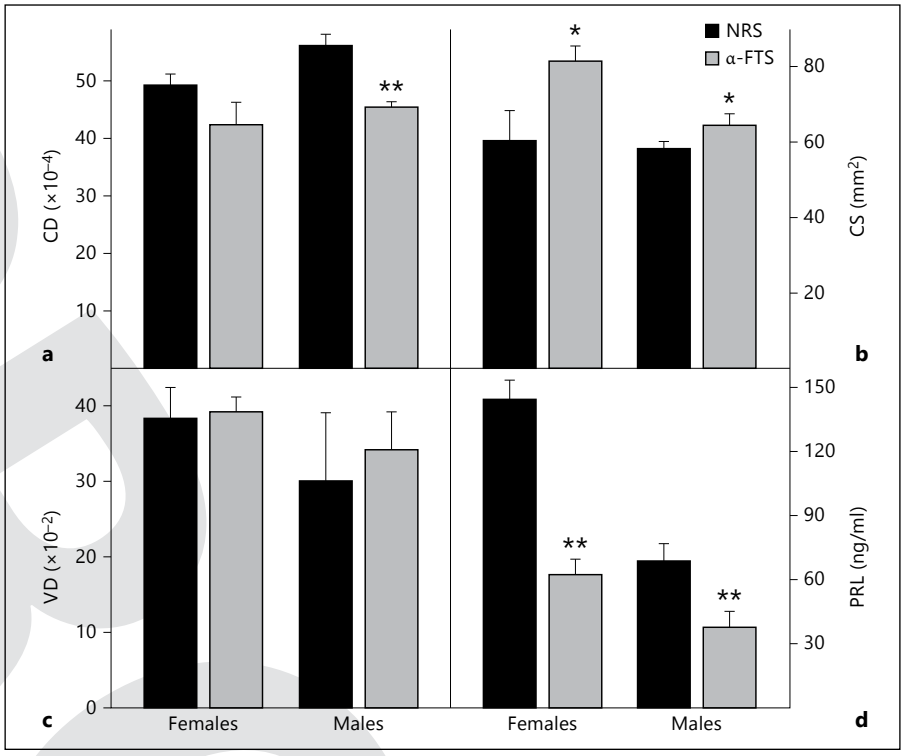

Fig. 3. a–d Effects of thymulin immunoneutralization on the lactotrope cell population and serum PRL. The α-FTS group showed a decrease in CD (**a**) and an increase in CS (**b**) compared with the control group (NRS) in both male and female mice. Serum PRL was significantly lower in the α-FTS groups than in control males and females (**d**). The number of animals per group was 5. Asterisks indicate the level of significance of differences: * $p < 0.05$; ** $p < 0.01$.

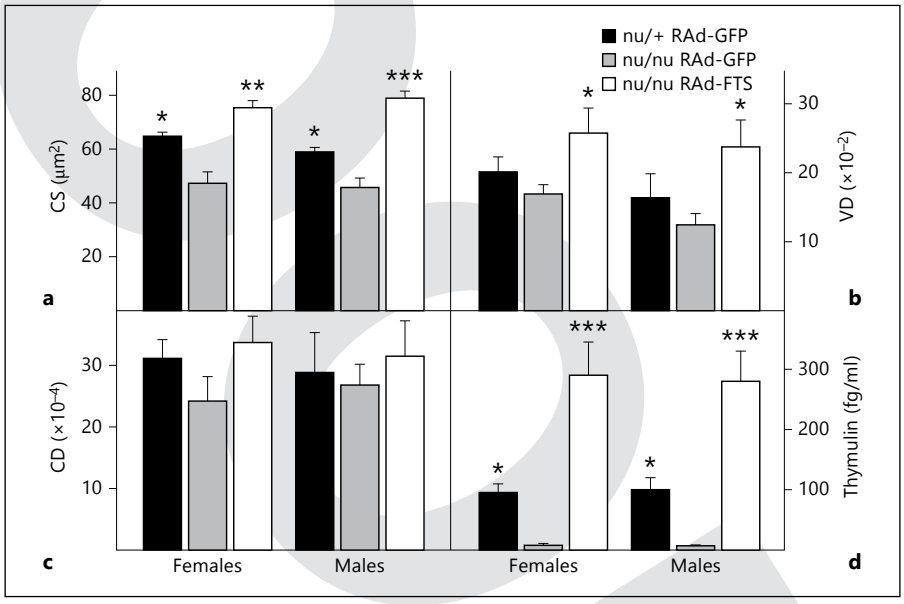

Fig. 4. Morphometric assessment of the effect of neonatal thymulin gene therapy on the lactotrope population in *nude* mice. Lactotrope CS (**a**) and VD (**b**) in the nu/nu RAd-FTS groups showed levels that are comparable with those of normal mice. Five animals in each group were analyzed. Asterisks indicate the level of significance of differences from the corresponding nu/nu RAd GFP group: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

postnatal days 1–2 to 32 induced a significant ($p < 0.01$) fall in the serum levels of thymulin which fell from 70.6 ± 4.6 fg/ml in controls to 15 ± 1.2 fg/ml in the quenched animals at age 32 days. Serum PRL was significantly lower in quenched than in control mice in both males and females (fig. 3d).

Effect of Thymulin Gene Therapy on the Lactotrope Population in Nude Mice

A single neonatal intramuscular injection of RAd-FTS, but not RAd-GFP, increased the circulating levels of biologically active thymulin in homozygous *nude* mice tested at 70 days of age (fig. 4d).

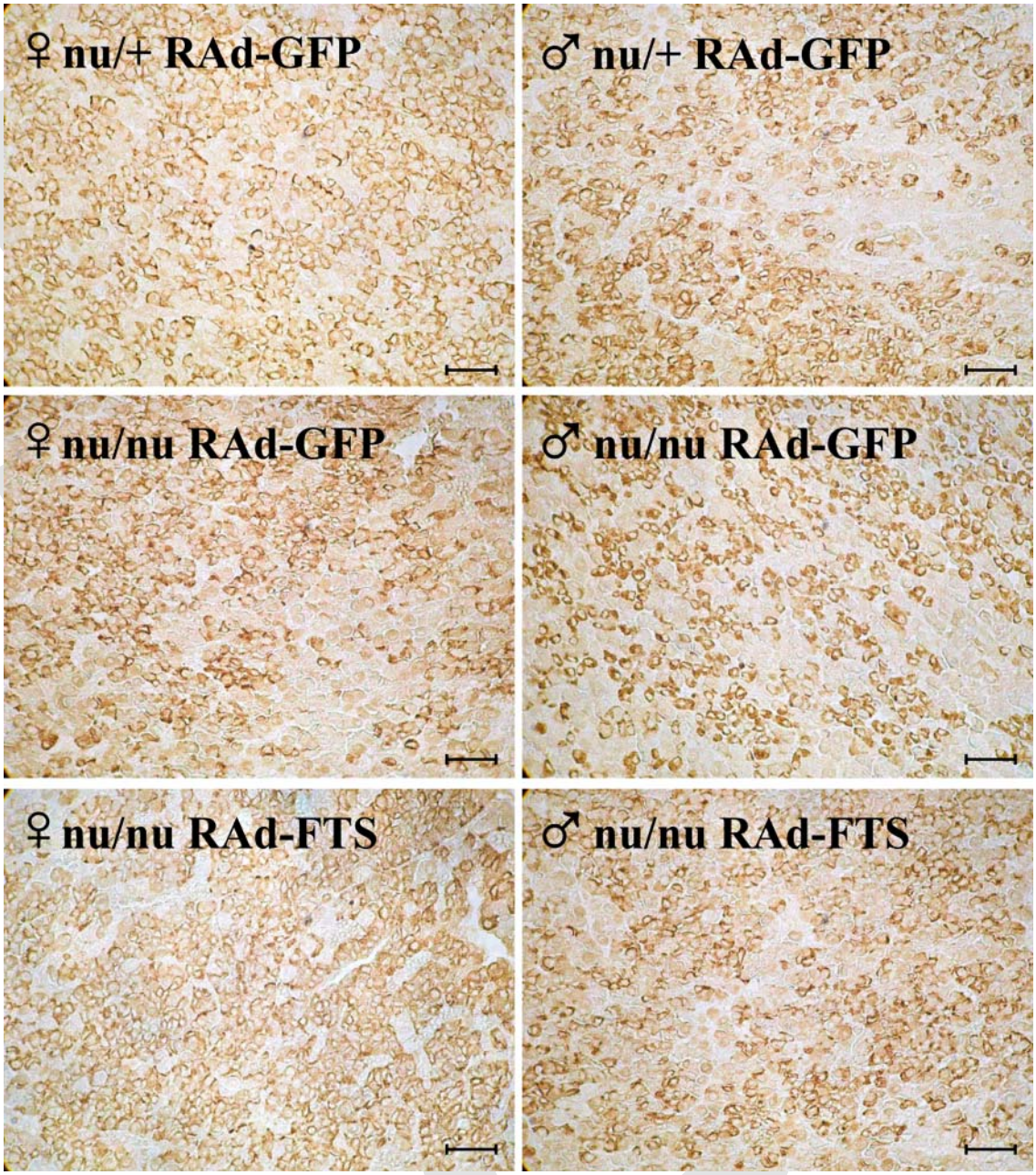

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Fig. 5. Impact of neonatal thymulin gene therapy on the lactotrope cell population in *nude* mice. Representative fields of specifically immunostained PRL cells in the three experimental groups: the nu/nu RAd-FTS groups showed an increase in CS and VD with respect to the nu/nu RAd-GFP groups of both sexes. EnVision system peroxidase. Scale bars = 50 μm.

Neonatal thymulin gene therapy significantly prevented the morphologic alterations in the lactotrope population typically observed in young *nude* mice (fig. 5). In anterior pituitary sections submitted to PRL immunohistochemistry, lactotropes stood out in sharp relief, exhibiting an ochre definite granular cytoplasmic pattern.

Nude mice submitted to neonatal thymulin gene therapy showed significantly higher CS and VD values than control *nudes* (fig. 4a, c). Thus, this intervention prevented the alterations typically observed in the lactotrope population of athymic mice.

Discussion

Our results show that TX or thymulin gene therapy in adult animals has little effect on the PRL axis. For instance, we have routinely observed that adult TX in rats and mice affects neither fertility nor lactation [unpubl. data]. This is consistent with the well-established fact that adult TX in rodents has little impact on immune and neuroendocrine function. Since the thymus is severely involuted in adult male rats due to the inhibitory action of testosterone [22], it is unlikely that TX in adult males has a significant impact on serum PRL levels. Therefore, we only studied adult TX females.

In contrast, manipulation of thymulin levels in early life does have an impact on the lactotropic axis. Although early studies in neonatally TX female mice showed that neonatal removal of the thymus does not affect serum PRL [23], our experiments in normal mice submitted to thymulin quenching from birth to puberty revealed that in both males and females serum PRL levels fall when thymulin deficiency occurs postnatally. This finding is in line with early reports indicating that serum PRL levels and PRL responses to immobilization stress are depressed in adult male *nude* mice [3]. Also, it has been documented that neonatal thymus grafts in *nude* mice increase their circulating PRL levels [24].

Neonatal thymulin gene delivery induced supraphysiologic levels of serum thymulin which resulted in a significant stimulation of lactotrope growth in both homozygous and heterozygous *nudes*. This agrees with previous in vitro studies reporting that thymulin stimulates PRL release in dispersed rat pituitary cells at doses from 10^{-8} to 10^{-3} M [10] but is in contrast with other studies which reported that thymulin doses of 10^{-11} M inhibit PRL release in incubated rat pituitary fragments [9]. The stimulatory effect of thymulin on PRL release in rat pituitary cells has been reported to decline with the age of the cell donor which suggests that aging brings about a desensitization of the pituitary gland to thymic signals [10]. Reciprocally, PRL has been reported to stimulate thymulin synthesis and secretion both in vitro and in vivo [25].

Our immunoneutralization data suggest that a deficiency in serum thymulin during early life may affect the growth, proliferation and secretory activities of lactotrope population in both male and female mice. The present results show that thymulin exerts an influence on the lactotropic axis in rodents. This influence seems to be greater in early than in adult life.

Acknowledgments

The authors are indebted to Mrs. M. Bracamonte for the histological processing of pituitary specimens and to Ms. G. Simonetto and Y.E. Sosa for editorial assistance. G.M.C is a member of the Researcher's Career CICBA; P.C.R, O.A.B, R.G.G are members of CONICET Argentina. This work was supported by UNLP (11/M150), CICBA, Universidad Adventista del Plata, Entre Rios, Argentina, and NIH grant R01AG029798–3 to R.G.G.

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