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Neonatal thymulin gene therapy in nude mice: Effects on the morphology of the pituitary corticotrope population

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Summary. The integrity of the thymus during early life is necessary for a proper maturation of the neuroendocrine system, including the adrenal axis. The thymic metallopeptide thymulin seems to be a central physiologic mediator of thymus-pituitary communication. Furthermore, neonatal thymulin gene therapy has been shown to prevent the typical alterations of gonadotrophic cell number and morphology and serum gonadotropin levels in nude female mice. In the present study we assessed the impact of athymia and the effect of neonatal thymulin gene therapy on the corticotropic cell population in nude mice. The effect of thymulin administration to adult nudes on their hypothalamic content of corticotropin-releasing hormone (CRH) and the adrenal content of corticosterone was also determined. We used an adenoviral vector expressing a synthetic gene for the thymic peptide thymulin (metFTS) termed RAd-FTS. On postnatal day 1 or 2, heterozygous (nu/+) and homozygous (nu/nu) pups of both sexes received a single bilateral i.m. injection of RAd-FTS or RAd-GFP, a control vector. On postnatal day 71, mice were bled and sacrificed, and their pituitaries were immediately dissected, fixed and immunostained for corticotropin. Morphometry was performed by means of an image-analysis system. The following parameters were calculated: volume density (VD: Σ cell area/reference area), cell density (CD: number of cells/reference area), and cell surface (CS: expressed in μm^2). Serum thymulin levels were measured by a bioassay, and CRH as well as corticosterone were determined by IRMA and RIA, respectively. Neonatal thymulin gene therapy in the athymic mice restored their serum thymulin levels and increased corticotrope CD, VD and CS in both control and athymic mice. Athymic mice showed only a marginal reduction in corticotrope CD, VD and CS. In these mutants hypothalamic CRH content was slightly increased, whereas adrenal corticosterone tended to be lower. Thymulin administration to adult mice tended to reverse these changes. Our results suggest a possible modulating effect of thymulin on the corticotrope population and the adrenal gland, confirming the existence of a bidirectional thymus-pituitary-adrenal axis.

Key words: Thymus-pituitary axis, Thymulin gene therapy, Nude mice, Hypophysiotropic activity, RAd-FTS

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Introduction

Important contributions to the study of the relationship between the thymus and the adrenal axis have been made, thus showing that adrenal corticosteroids affect the size and morphology of the thymus gland. In an *in vitro* study, corticotropin (ACTH) increased the release of the thymic metallopeptide thymulin in a dose-dependent fashion (Hadley et al., 1997). *In vivo* ACTH administration or stress conditions result in a marked decrease of T lymphocytes and thymus size. Moreover, adrenalectomy in animals derived in a thymic hyperplasia (Fisher, 1964). Conversely, the integrity of the thymus during perinatal life is necessary for a proper maturation of the thymus-pituitary axis, as revealed by the endocrine alterations caused by neonatal thymectomy (Michael et al., 1980) or congenital absence of the thymus in mice (Rebar et al., 1980). Morover, thymectomy diminished ACTH and corticosterone levels in newborn rats, whereas replacement therapy with thymosin fraction 5 (a partially purified thymus extract) in thymectomized (Tx) animals not only restored the immune response but also

normalized ACTH and corticosterone levels (Deschaux et al., 1979).

Thymulin is the best characterized thymic hormone. It is exclusively produced by thymic epithelial cells (TEC), and consists of a nonapeptide component (facteur thymique sérique or FTS) coupled in an equimolecular ratio to the ion zinc (Dardenne et al., 1982). A number of secretagogues for thymulin have been reported, including dexamethasone, progesterone, testosterone, estradiol (E_2), PRL, GH and thyroid hormones. In TEC cultures, each of these substances had receptors and stimulated thymulin release (Savino and Dardenne, 2000). Moreover, it has been observed that the i.p. treatment of senescent mice with hypothalamic extracts from young mice corrected the deficit of serum thymulin levels (Folch et al., 1986). Exposure to hypothalamic or pituitary extracts derived from young mice in TEC cultures induced an increase in thymulin concentration in the cell supernatants, and this effect was lower when the extracts were obtained from old mice (Goya et al., 1995).

Shortly after birth congenitally athymic (nude) mice, as well as normal mice neonatally thymectomized, develop not only a severe immunodeficiency but also degenerative alterations in the adrenal glands that can be prevented by thymus neonatal grafting (Pierpaoli and Sorkin, 1972). The removal of the adrenal glands in male and female mice produced a temporary decrease (for a month) of serum thymulin, with the consequent increment in the intrathymic content of thymulin (Dardenne et al., 1986).

Recently, a synthetic DNA sequence coding for thymulin has been constructed and used to design a recombinant adenoviral vector termed RAd-FTS. When intramuscularly (i.m.) administered to adult thymectomized (Tx) mice and rats, the vector RAd-FTS induced sustained supraphysiological serum levels of biologically active thymulin which remained high for at least 112 days in mice and for over 320 days in rats (Reggiani et al., 2006; Morel et al., 2008).

In the present study, we assessed the effect of neonatal thymulin gene therapy on the morphology of the corticotropic cell population at puberty in nude mice, as well as the effect of thymulin peptide replacement therapy on hypothalamic CRH content and corticosterone adrenal content in adult nude mice.

Materials and methods

Adenoviral vectors used

RAd-FTS

A DNA sequence coding for the biologically active thymulin analog termed methionine-FTS or metFTS, was constructed and cloned in a recombinant adenoviral (RAd) vector generated by a variant of the two plasmid method (Hitt et al., 1998) employing the AdMax® plasmid kit (Microbix, Canada). This procedure has been described in detail elsewhere (Reggiani et al., 2006).

Briefly, this kit uses a shuttle plasmid (pDC515) and the genomic plasmid pBHGfrt(del)E1,3 FLP. The thymulin synthetic gene (metFTS) was inserted into the shuttle and both plasmids were cotransfected into HEK293 cells. In cotransfected HEK293 cells enzyme-directed recombination generated 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 above general procedures and was used as a control vector in the gene therapy studies. The vector harbours 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). The vector was expanded in HEK293 cells and purified and titrated as indicated above.

Animals and experimental procedures

Gene therapy experiments

The offspring of NIH homozygous (nu/nu) nude male and heterozygous (nu/+) female mice were used. The parent mice were purchased from the Animal Core Facility of the National University of La Plata, Argentina. All mice were maintained on a γ-irradiated chow diet and sterilized water. Animals had free access to food and water and were kept at 22°C with a light/dark cycle of 12/12 h. All experiments in nude animals were done following the Animal Welfare Guidelines of the NIH.

On postnatal day 1 or 2, each experimental heterozygous (nu/+) and homozygous (nu/nu) pup of both sexes received a single bilateral i.m. (hindlegs) injection of 10^8 plaque forming units (pfu) with RAd-FTS or RAd-GFP used as a control vector, in 10 µl vehicle (5 µl per side). On postnatal days 71-72, mice were bled and immediately sacrificed by cervical dislocation, their pituitaries were immediately dissected, fixed and immunostained. The experimental groups analyzed were: nu/nu mice injected with RAd-GFP or RAd-FTS and nu/+ mice injected with RAd-GFP, both females and males. Thus, six groups of mice were studied.

Thymulin replacement therapy experiments

Adult mice received 1 ng synthetic thymulin (FTS) (in 0.1 ml saline containing 1 ng $ZnCl_2$) per day, administered sucbcutaneously (s.c.) for 10 days.

Hypothalamic and adrenal extract preparation

In some adult mice the medial basal hypothalamus (MBH) was immediately dissected after sacrifice and homogenized in 1 ml 0.05M HCl + 1% w/v ascorbic acid per each MBH. After thorough homogenization the extract was frozen and thawed three times and then submitted to three 20-s sonication bursts, leaving 10-s intervals between bursts. Finally, the homogenates were centrifuged at 10,000 g for 15 min and the clear supernatant, considered as undiluted MBH, was aliquoted and stored at - 70°C until use for corticotropin-releasing hormone (CRH) determination.

In the same animals, the adrenals were dissected and homogenized in 0.01M phosphosaline, pH 7.6 (PBS) at a concentration of 10 mg wet tissue/ml PBS and were centrifuged for 10 min at 5,000 rpm and the clear supernatants (typically containing approximately 1 mg PBS-soluble protein per ml) were later used for corticosterone determination.

Hormone assays

Thymulin

Biologically active thymulin was measured in serum by a rosette bioassay described in detail elsewhere (Dardenne and Bach, 1975). This method is based on the ability of thymulin to restore the inhibitory effect of azathioprine (Az) 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.

Two-site CRH-immunoradiometric assay (IRMA)

The human/rat (h/r)CRH-IRMA has been described in detail elsewhere (Linton and Lowry, 1986). Briefly, triplicate 200-µl aliquots of samples or of standard (1-10,000 pg/ml) diluted in 0.05 M sodium phosphate buffer (pH 7.4), 0.25 % (w/v) BSA, were incubated overnight at room temperature with 200:l of the CRH-IRMA reagent mixture. This consisted of ^{125}I-labelled rabbit anti-CRH (36-41)-NH_2 IgG (100,000 cpm/tube) and 1/5,000 guinea pig anti-CRH-(1-20) serum (referred to as "CRH linker") in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.5% (w/v) human serum albumin, 1% (v/v) normal rabbit serum and 0.01% (w/v) sodium azide. Separation of CRH-bound from unbound labelled IgG was performed with sheep anti-guinea pig Fc region IgG coupled to a dynosphere solid phase. The radioactivity of the bound IgG was measured in a γ-counter.

Corticosterone radioimmunoassay (RIA)

The corticosterone RIA was performed according to the method of Gómez-Sánchez et al. (1975). The anticorticosterone-3-bovine serum albumin serum used was kindly provided by Dr. Gordon Niswender and the tracer (1,2,6,7-^{3}H)-corticosterone (75 Ci/mmol) was obtained from Amersham (Arlington Heights, Ill).

Histology-Immunohistochemistry

Stated in brief, pituitary and adrenal tissues from 5 animals of each group 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 the primary antibody, anti-ACTH (Dako, CA, USA), diluted 1:100. Thoroughly washed sections were then treated for 30 min with a ready-to-use EnVision reaction system (Dako, CA, USA). The peroxide-sensitive chromogen was diaminobenzidine (Cónsole et al., 2001). 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 phosphate-buffered saline. The sections of the adrenal glands were stained with hematoxylin-eosin.

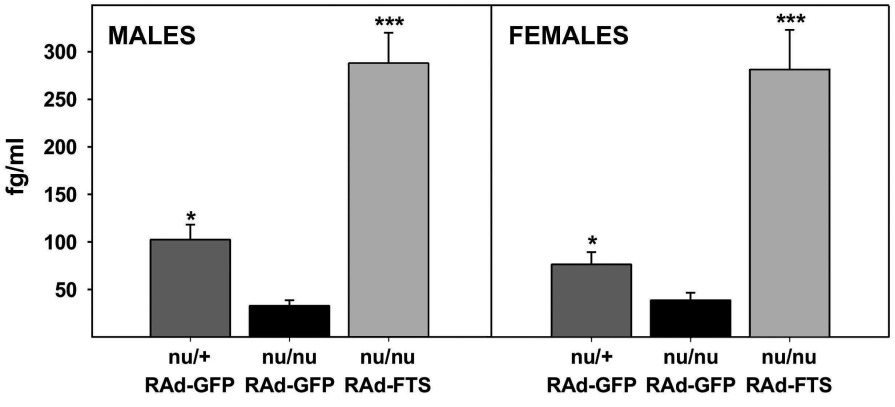

Fig. 1. Effect of neonatal thymulin gene therapy on serum levels of thymulin in female and male nude mice.- Vectors were injected i.m. on the day of birth or the day after. On postnatal day 71-72 the animals were bled and thymulin assayed. Serum thymulin values (fg/ml) are expressed as mean ± SEM; n values are shown in parentheses. Asterisks indicate the level of significance of differences with respect to homozygous nudes injected with RAd-GFP. *: P<0.05; **: P<0.01; ***: P<0.001.

Morphometry

Morphometry was performed as reported in detail previously (Cónsole et al., 2002). Measurements of immunostained pituitary cells were made by means of an image-analysis system (Imaging Technology, Optimas

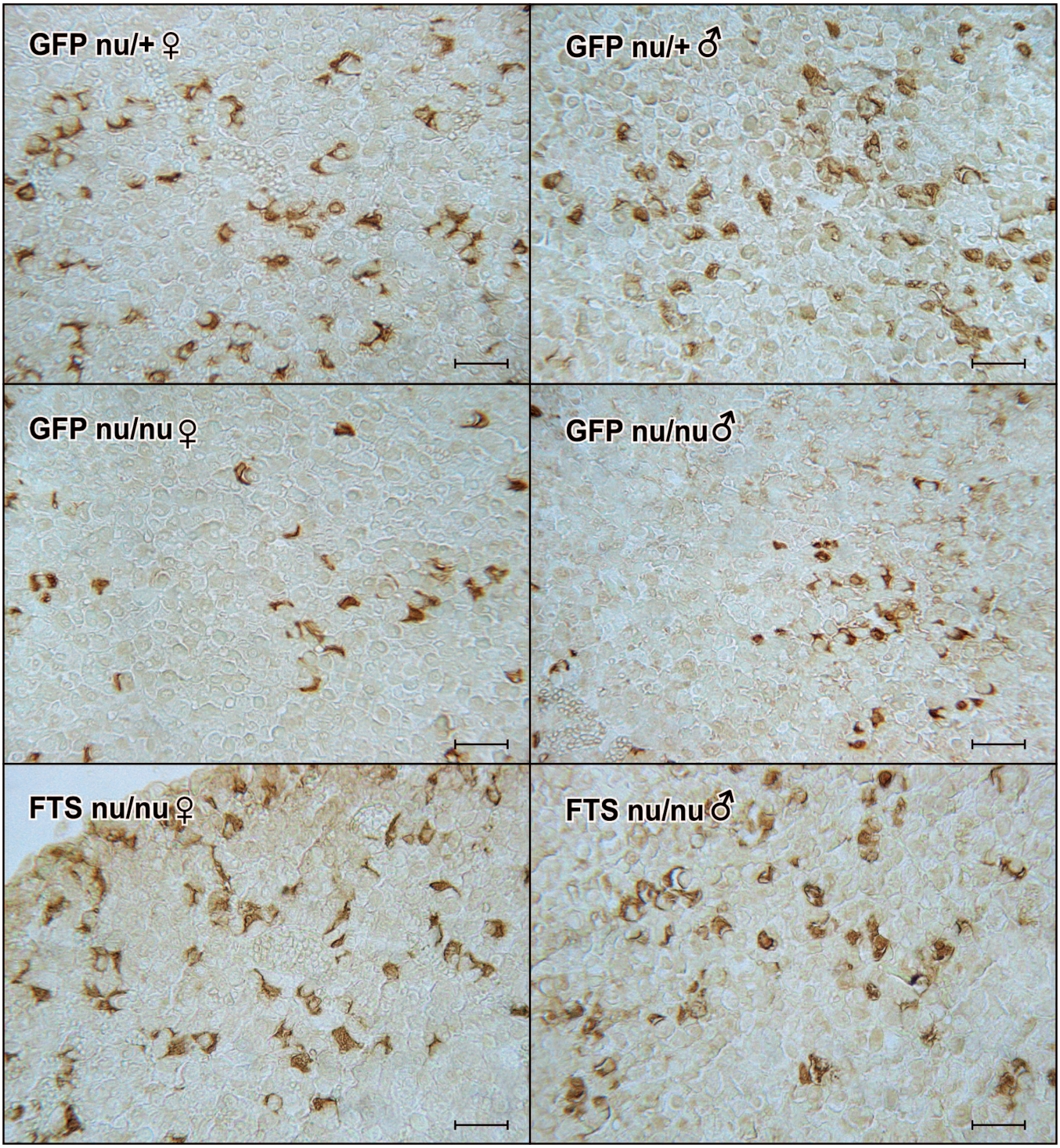

Fig. 2. Effect of neonatal thymulin gene therapy on the corticotropic cell population.- Representative fields of specifically immunostained corticotrope cells in the three experimental groups: nu/+ RAd-GFP, nu/nu RAd-GFP and nu/nu RAd-FTS of both sexes. EnVision system peroxidase. Bar: 45 µm.

5.2, USA). The cells and 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 subsequently calculated: volume density (VD = Σ cell area/RA), cell density (CD = number of cells/RA), and cell surface (CS, expressed in μm^2). RA represents the total area throughout which the cells were scored. Thus, this area divided into the sum (Σ) of the individual cell areas (A) yielded VD, a parameter that represents an estimate of cell mass according to generally accepted criteria. The number of cells (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 the Student's *t*-test or by ANOVA followed by the Tukey test when the ANOVA was significant.

Results

Effect of neonatal thymulin gene therapy on serum thymulin levels

At 71-72 days of age, control nu/nu mice showed low levels of serum thymulin as compared to control nu/+ counterparts. A single neonatal i.m. injection of RAd-FTS markedly increased the circulating levels of biologically active thymulin in athymic female and male nude mice (Fig. 1).

Effect of neonatal thymulin gene therapy on the pituitary corticotropic cell population

Immunostained ACTH-cells stood out in sharp relief, exhibiting an ochre definite granular cytoplasmic

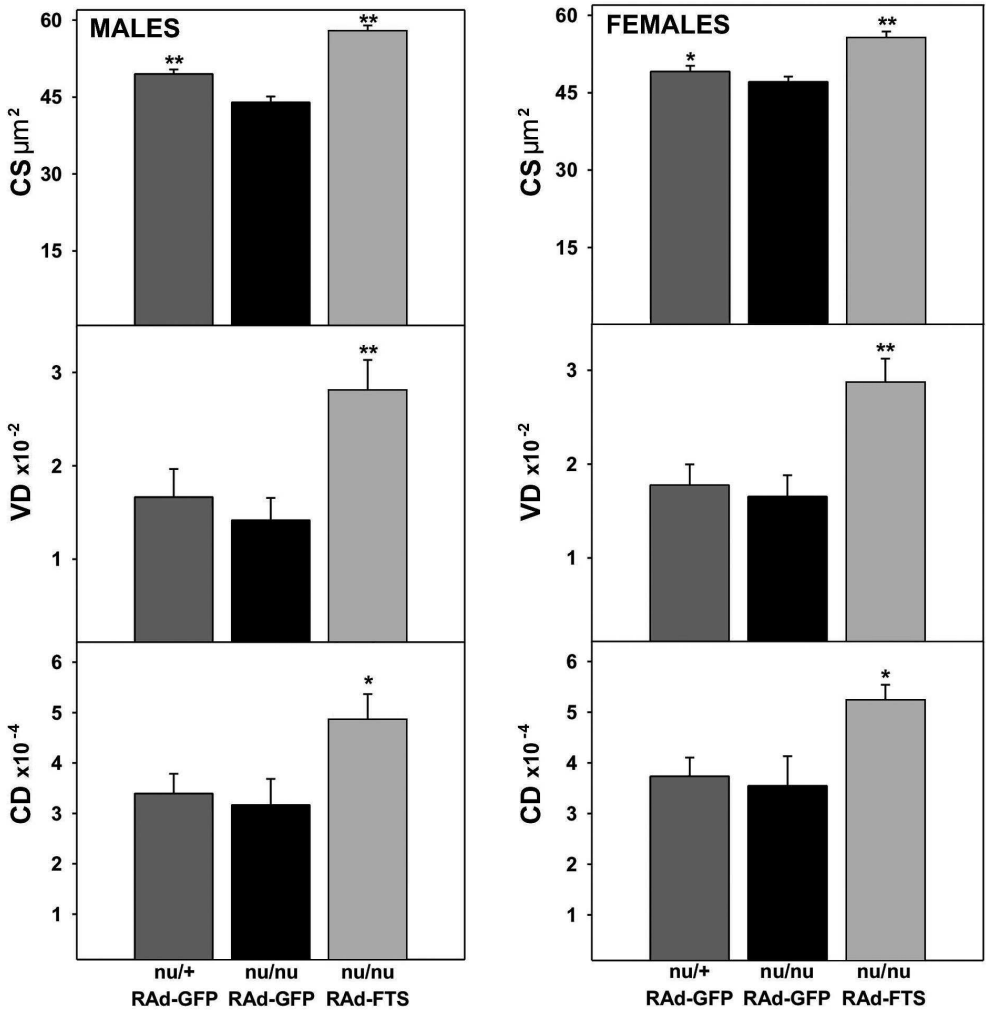

Fig. 3. Effect of neonatal thymulin gene therapy on the cell surface (CS), volume density (VD) and cell density (CD) of ACTH cell population in the three experimental groups.- nu/+ RAd-GFP, nu/nu RAd-GFP and nu/nu RAd-FTS of both sexes. Asterisks indicate the level of significance of differences with respect to homozygous nudes injected with RAd-GFP. *: P<0.05; **: P<0.01; n=5 for all group.

pattern. The immunolabeled corticotrope cells corresponding to representative fields of the histometry performed in the three experimental groups are shown in Fig. 2. The corticotropic cell population showed non significant decrease in cell density (CD), volume density (VD) and cell surface (CS) in control athymic nude mice

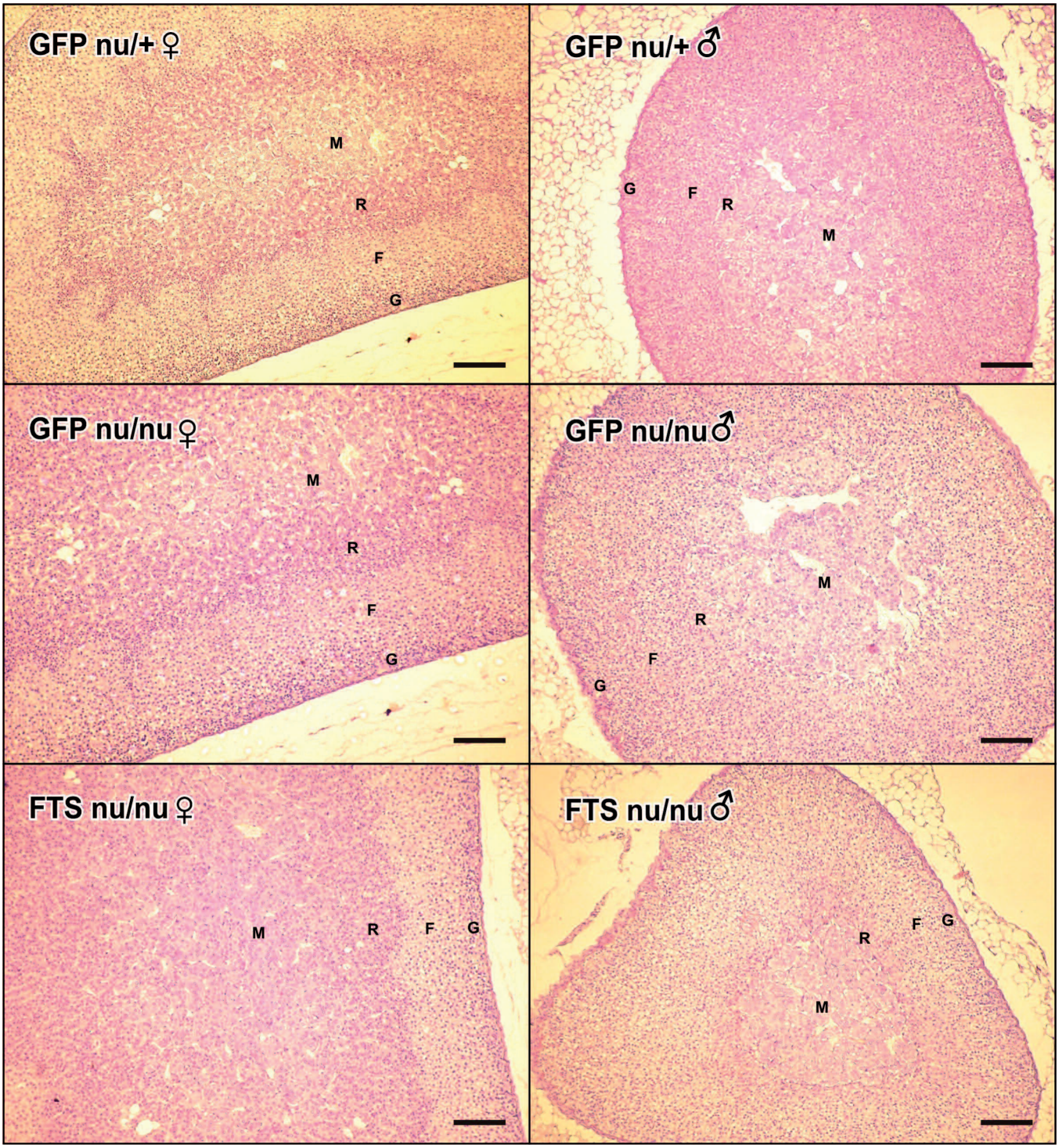

Fig. 4. Effect of neonatal thymulin gene therapy on adrenal morphology.- Representative fields from adrenal glands in the three experimental groups: nu/+ RAd-GFP, nu/nu RAd-GFP and nu/nu RAd-FTS of both sexes. C: cortex. G: glomerulosa. F: fasciculata. R: reticularis. M: medulla. Hematoxylin-eosin.

of both sexes as compared to heterozygous counterparts. The morphometric analysis revealed changes induced by the injection of RAd-FTS on the corticotrope population of the pars distalis in heterozygous nudes. Neonatal thymulin gene therapy significantly increased the CD ($P<0.05$), CS ($P<0.01$) and VD ($P<0.01$) of the corticotrope population in nu/nu mice of both sexes tested (Fig. 3).

The cortical adrenal tissue did not show relevant changes in the different layers, but in the homozygous and heterozygous females an increment in the height of the reticular layer was observed (Fig. 4).

Effect of thymulin therapy on hypothalamic CRH and adrenal corticosterone in adult nude mice

Adult nude mice showed a non significant trend towards an increase in hypothalamic CRH content. Ten-day thymulin peptide therapy in these animals tended to reverse this increase (Fig. 5). Adrenal corticosterone content in the same animals showed a non significant reduction in adrenal corticosterone content whereas 10-day thymulin treatment showed a trend to reverse this reduction (Fig. 6). Bleeding induced artifactually high levels of serum corticosterone thus preventing the comparison of serum corticosterone between nude and heterozygous animals.

Discussion

We have previously shown that neonatal thymulin gene therapy prevents the deficit of circulating gonadotropins as well as the morphological abnormalities of the gonadotrope population that typically occur after puberty in these mutants (Reggiani et al., 2009a,b). The present results suggest that congenital athymia in mice has only a marginal impact on the morphology and numbers of pituitary corticotropes, as well as on the hypothalamic CRH content and the adrenal corticosterone content. It is of interest that thymulin gene therapy significantly increased the number and size of corticotropes in both heterozygous and homozygous nudes. This increase is in line with the ACTH-releasing activity of thymulin *in vitro* (Hadley et al., 1997) as well as with the reports that stress-induced release of ACTH and *in vitro* pituitary response to CRH were significantly lower in Swiss nude than in control mice (Daneva et al., 1995; Gaillard et al., 1998).

We have shown by histometrical assessment of the corticotropic cell population in normal C57BL/6 male mice treated from birth to puberty with anti-thymulin serum that this cell type undergoes a marked decrease in cell density and an increment in cell size, possibly to maintain ACTH levels under basal conditions. These early data and our present results suggest a possible modulating effect of thymulin on the corticotrope population in early life, thus confirming the existence of a bidirectional thymus-pituitary-adrenal axis (Camihort et al., 2006). We have not detected significant differences between homozygous and heterozygous nudes in the cortical layers of the adrenal gland in mice treated with RAd-GFP or RAd-FTS.

Since thymulin possesses hypophysiotropic activity *in vitro* (Zaidi et al., 1988; Brown et al., 1999, 2000; Goya et al., 2004, 2007) it could act directly on the

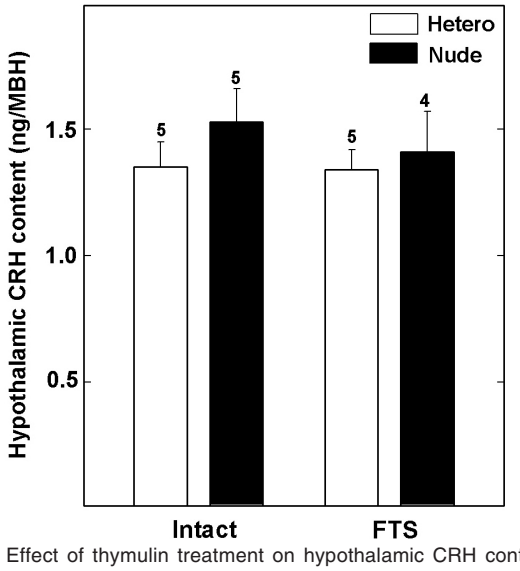

Fig. 5. Effect of thymulin treatment on hypothalamic CRH content in adult nude mice. Heterozygous and homozygous nude males were s.c. injected with synthetic thymulin or saline (controls) for 10 days. Numbers and bars on columns represent number of mice per group and SEM, respectively. No significant differences between groups were detected.

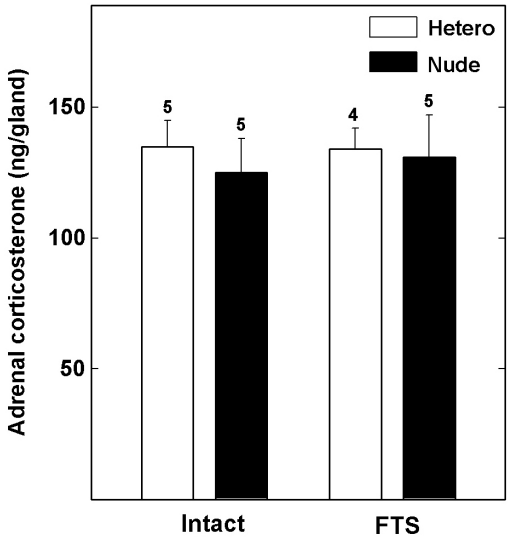

Fig. 6. Effect of thymulin treatment on adrenal corticosterone content in adult nude mice. Heterozygous and homozygous nude males were s.c. injected with synthetic thymulin or saline (controls) for 10 days. Other details are as in Fig. 5.

adenohypophysis *in vivo* modulating the response of the gland to hypothalamic or other secretagogues or inhibitors.

Congenital athymia is known to cause the hyporesponsiveness of adrenocortical cells to serotonin although the adrenal cortex of athymic mice is able to perform its function in response to ACTH (Yang et al. 1995), which suggests that thymulin could also exert a direct action on the adrenal cortex.

Treatment of both heterozygous and homozygous adult nude mice with thymulin did not have a dramatic effect on hypothalamic CRH content or adrenal corticosterone content although the treatment did seem to reverse the marginal differences between normal and nude counterparts (higher CRH and lower corticosterone in the mutants). These results indicate that neuroendocrine sensitivity to thymulin declines after the perinatal period.

In conclusion, the present study suggests a possible modulating effect of thymulin on the corticotrope population, supporting the idea of a bidirectional thymus-pituitary modulatory network for the adrenal axis. Thymulin gene therapy has opened new avenues for the exploration and eventual exploitation of the therapeutic potential of this metallopeptide.

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