Effects of GnRH immunization on the reproductive axis and thymulin

Shiping Su, Xiaoxia Sun[1], Xiuhong Zhou[2], Fuigui Fang and Yunsheng Li

College of Animal Sciences and Technology, Anhui Agricultural University, 130, Changjiang West Road, Hefei, Anhui 230036, People's Republic of China
[1]College of Life Science, Fujian Agriculture and Forestry University, Fuzhou 350002, People's Republic of China
[2]The Biotechnology Center of Anhui Agriculture University, Hefei, People's Republic of China

Correspondence should be addressed to F Fang
Email
fgfang@ahau.edu.cn

Abstract

The bidirectional regulation of thymulin in the reproductive-endocrine function of the hypothalamic–pituitary–gonadal (HPG) axis of rats immunized against *GnRH* remains largely unclear. We explored the alterations in hormones in the HPG axis in immunized rats to dissect the repressive effect of immunization on thymulin, and to clarify the interrelation of reproductive hormones and thymulin *in vivo*. The results showed that, in the first 2 weeks of booster immunization, thymulin was repressed when reproductive hormones were severely reduced. The self-feedback regulation of thymulin was then stimulated in later immune stages: the rising circulating thymulin upregulated LH and FSH, including GnRH in the hypothalamus, although the levels of those hormones were still significantly lower than in the control groups. In astrocytes, thymulin produced a feedback effect in regulated GnRH neurons. However, in the arcuate nucleus (Arc) and the median eminence (ME), the mediator of astrocytes and other glial cells were also directly affected by reproductive hormones. Thus, in immunized rats, the expression of glial fibrillary acidic protein was distinctly stimulated in the Arc and ME. This study demonstrated that thymulin was downregulated by immunization against *GnRH* in early stage. Subsequently, the self-feedback regulation was provoked by low circulating thymulin. Thereafter, rising thymulin levels promoted pituitary gonadotropins levels, while acting directly on *GnRH* neurons, which was mediated by astrocytes in a region-dependent manner in the hypothalamus.

Key Words
- thymulin
- active immunization
- hypothalamic–pituitary–gonadal axis
- gonadotrophin-releasing hormone
- astrocyte

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Introduction

Thymulin consists of a biologically inactive nonapeptide component termed FTS (an acronym for serum thymus factor in French) coupled in an equimolecular ratio to a zinc ion (Gastinel *et al.* 1984), which confers biological activity to the molecule (Dardenne *et al.* 1982). The FTS peptide, whose amino acid sequence is, pyroGlu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-AsnOH, is exclusively produced by thymic epithelial cells (TEC) and is highly conserved in mammals (Dardenne *et al.* 1974, Bach & Dardenne 1989). The secretion of thymulin seems to be dependent on the complex neuro-endocrine–immune (NEI) network (Reggiani *et al.* 2009*a*). Data have now accumulated that demonstrate strongly that several neuropeptides can regulate the endocrine function of the thymus. Opioids can significantly increase the levels of thymulin in the culture supernatants of TEC in a dose-dependent manner, and the effects were abrogated by an opioid receptor antagonist (Savino *et al.* 1990). Two studies showed that treatment of old mice with hypothalamic extracts from young mice resulted in reappearance of detectable levels of

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circulating thymulin, which may represent the evidence that hypothalamic factors influence thymulin production by direct action on TECs (Goya *et al*. 1995). Some pituitary hormones can influence thymulin synthesis and secretion. Prolactin (PRL) can induce a specific increase in thymulin synthesis and secretion *in vivo*, and this stimulatory effect was also observed in primary cultures of human and mouse TECs (Dardenne *et al*. 1989). These data demonstrated that production and secretion of thymulin is influenced directly or indirectly by the hypothalamic–pituitary–gonadal (HPG) axis. However, there are few data about the effect of gonadal steroid hormones on thymulin.

As a key signaling molecule, thymulin has multiple influences in the NEI network, which in turn, produces feedback regulation on the HPG axis. In thymulin deficient nude mice, morphometric analysis revealed that athymic nudes have reduced numbers of brain gonadotropin-releasing hormone (GnRH) neurons and pituitary gonadotropic cells, compared with heterozygotes, and neonatal thymulin gene therapy could prevent these changes (Reggiani *et al*. 2009*b*, 2012, Martines *et al*. 2011). Other studies suggested that thymulin stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) release from perfused rat pituitaries (Brown *et al*. 2000, Hinojosa *et al*. 2004). Moreover, thymulin could augment LH-mediated stimulation of androgen increases *in vitro* and *in vivo* in boar testis (Wise & Ford 1999). These results suggested that thymulin plays a relevant physiological role in the HPG axis.

Generally, glial cells have been viewed as having only structural or support roles in the brain. However, there is increasing evidence that astrocytes also have a neuroregulatory role, as mediators between the signaling molecule and targeting neurons. They also regulate GnRH secretion both by activating the growth factors acting via receptors with tyrosine kinase activity and by inducing plastic rearrangements of glial-GnRH neuron adhesiveness (Ojeda *et al*. 2008). Studies demonstrated that the ability of transforming growth factor α (TGFα) to enhance GnRH release depends on the potentiating interaction of PGE2 with these additional glial-derived molecules. In addition, 17β-estradiol can enhance $TGF\beta_1$ release from hypothalamic astrocytes to increase the secretion of GnRH neurons, which provide further evidence that astrocytes have important neuroregulatory capabilities that are subject to endocrine regulation (Ma *et al*. 1997, Buchanan *et al*. 2000). Perhaps not surprisingly, as an endocrine hormone, thymulin should regulate the GnRH secretion, mediated by glial cells in the hypothalamus.

GnRH produced in the hypothalamus stimulates the release of gonadotropins from the pituitary, thereby controlling steroidogenesis, gametogenesis and other sex-related characteristics (Gore 2002). GnRH appears to not only have a central effect in the process of reproduction, but also is involved in the regulation of the immune response (Marchetti *et al*. 2000). Thus, GnRH is one of the most important signaling molecules in neuroendocrine and immune interactions (Marchetti *et al*. 1998). Recently, with the cloning and sequencing of the GnRH and GnRH receptor (GnRHR), mRNA transcripts encoding GnRH and GnRHR have been detected in rodent thymocytes and TECs (Morale *et al*. 1991, Weesner *et al*. 1997), suggesting that GnRH could regulate T-cell development in an autocrine or paracrine manner, and might influence the endocrine function of TECs, including the production of thymulin.

Active immunization of animals against GnRH directly causes a loss of synthesis and secretion of both pituitary gonadotropins and gonadal steroids in the gonads (Einarsson *et al*. 2009). When used to immunize males, the GnRH vaccine was developed primarily to control immunocastration and to improve the quality of meat (Cook *et al*. 2000, Miller *et al*. 2008), based on the interference of endocrine function. In humans, the GnRH vaccine is likely to impede the growth of androgen-dependent prostatic carcinoma or other hormone-dependent tumors (Ladd *et al*. 1995, Junco *et al*. 2008). Thus, endocrine homeostasis is disrupted in the short or long-term in the GnRH-immunized animal, while this disordered endocrine environment is bound to influence thymulin synthesis and secretion *in vivo*. Conversely, changes in thymulin levels could, in turn, be part of a feedback regulation on the HPG axis. In the present study, we correlated thymulin levels in serum with gonadotropin in GnRH-immunized male rats, and analyzed the thymulin alteration with GnRH and glial fibrillary acidic protein (GFAP) in hypothalamus. We demonstrated the effect of active immunization on the reproductive endocrine and immune endocrine functions of male rats.

Materials and methods

Animals, immunization protocol and sample collection

Three-week-old male Sprague–Dawley rats were purchased from the Experimental Animal Center of Anhui Medical University and kept in the animal house at the Anhui Agriculture University (Hefei, P R China). Rats were housed at three per cage in a controlled temperature

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(22 °C) room and with a 12 h light:12 h darkness cycle. Food and water were available *ad libitum*. The study was conducted strictly in accordance with the guidelines set by the China Council on Animal Care. Three-week-old male rats were divided randomly into two groups ($n=37$ per group). One group received GnRH-tandem-ovalbumin (GnRH-tandem-OVA, ShineGene Molecular Biotech, Shanghai, China) mixed with $Al(OH)_3$ adjuvant (Sigma), and the other group received equivalent $Al(OH)_3$ adjuvant as the control. According to the results of preliminary experiments, rats were injected subcutaneously at four sites on their backs with 500 μl (200 μg peptides/ml) antigen and vehicle, and received boosts with same volume at 5- (300 μg peptides/ml) and 7- (400 μg peptides/ml) weeks-old.

Before immunization, at 3-, 5- and 7-weeks-old, eight rats were randomly selected and 1 ml of blood was sampled from the jugular vein in each group for hormone and antibody analysis. After the final booster immunization, eight rats were randomly taken from each group at 2-week intervals for 8 weeks. Animals were anaesthetized with 1% sodium pentobarbital (1.0 ml/100 g body weight). Blood was then taken for hormone analysis via cardiac puncture, following which the hypothalami were collected for ELISA detection. At the last sample point, another five rats were sacrificed by anesthesia and cardiac perfusion with 4% paraformaldehyde (PFA, Sigma–Aldrich) in 0.1 M PBS (pH=7.4; Invitrogen) was performed in each group. The hypothalami were separated according to Paxinos & Watson (2000), postfixed for 4 h in the same fixative solution, and cryoprotected in 30% sucrose (Sigma–Aldrich) in 0.1 M PBS until tissues sank to the bottom.

RIA

Levels of FSH, LH and testosterone in serum were quantified using iodine [^{125}I]-FSH, [^{125}I]-LH and [^{125}I]-testosterone rat-specific RIA kits (Furui Bioengineering Corporation, Beijing, China), according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation for FSH, LH and testosterone were all 8 and 13% respectively. The cross-reactivity of [^{125}I]-testosterone to dihydrotestosterone and androstenedione was 1.1×10^{-4} and 1.2×10^{-7} respectively.

ELISA

The specific GnRH antibodies in rats immunized with GnRH were tested using a previously published ELISA (Jinshu *et al.* 2005). Briefly, 96-well plates (Thermo Fisher Scientific, Shanghai, China) were coated with 100 μl/well of GnRH-tandem-OVA protein (10 μg/ml) in PBS, and kept at 4 °C overnight. Wells were blocked with 200 μl/well 3% (w/v) BSA (Sigma) in PBS at 37 °C for 1.5 h, washed three times with PBS containing 0.05% Tween-20 (PBST), and incubated with 100 μl a of 1:100 dilution of individual sera obtained from immunized rats for 1 h at 37 °C. The sera were removed, the plates were washed three times, and then incubated with 100 μl/well HRP-conjugated goat anti-rat IgG secondary antibody diluted at 1:3000 (sc-2032, Santa Cruz) with PBST, and incubated for 1 h at 37 °C. After washing, the plates were reacted with 3,3′,5,5′-tetramethyl benzidine (TMB) and hydrogen peroxidase as a substrate. The reaction was stopped with 50 μl/well of H_2SO_4 and absorbance was read at A450 nm by a microplate reader (BioTek, Winooski, VT, USA). All samples were run in triplicate across one assay.

Each hypothalamus was immediately weighed and thawed, and homogenized in chilled 0.01 M PBS, pH 7.4, at a concentration of 100 mg/ml with a micro-glass homogenizer. The tissue was centrifuged at 1200 ***g*** for 20 min in a refrigerated centrifuge. The supernatant was retained and placed at −20 °C until required for the ELISA. Thymulin in the serum and hypothalamus was quantified using an ELISA kit (k9810; ALPCO, Shanghai, China), and GnRH in the hypothalamus and primary cultured supernatant was quantified using a sandwich ELISA kit (orb53025; USCNK, Wuhan, China). These manipulations were carried out according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured using a microplate reader (BioTek). The intra- and inter-assay coefficients of thymulin variation were <7 and <12% respectively. The intra- and inter-assay coefficients of GnRH variation were <9 and <15%, respectively. All samples were run in triplicate across one assay. These two kits assay recognize thymulin and GnRH, thus no significant cross-reactivity or interference was observed.

Immunofluorescent staining

Hypothalami from the two groups rats were embedded in Tissue-Tek OCT compound (Sakura, Japan), and consecutive frontal plane sections were made at a thickness of 40 μm using a frozen microtome (TCS CM1900, Germany). Sections were washed in 0.01 M PBS (pH 7.4) for 30 min and processed for dual immunofluorescent staining.

The dual immunofluorescent staining was processed with a previously described procedure (Brouns *et al.* 2002). Briefly, hypothalamus frozen sections were washed three times for 5 min with 0.01 M DPBS (Sigma). For GnRH and

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GFAP double labeling, hypothalamus tissue sections were incubated in an antibody mixture containing a Rabbit anti-rat GnRH I polyclonal antibody (1:300, sc-20941; Santa Cruz) and a chicken-anti-rat GFAP polyclonal antibody (1:800, ab4674; Abcam, Hangkong, China). These primary antibodies were diluted in 0.01 M DPBS containing 10% normal goat serum and 1% Triton X-100, and incubated overnight at 4 °C with sections.

The hypothalamus tissue reactions were developed by incubation for 2 h at room temperature with Goat anti-rabbit IgG-FITC (1:300, sc-2011; Santa Cruz) and Cy5-AffiniPure Goat Anti-Chicken IgY (1:500, ab97147; Abcam). The sections were washed and mounted on poly-L-lysine (Boster Company, Wuhan, China)-coated glass slides. To determine the specificity of the primary antibodies, negative staining controls were performed by consecutively incubating the sections with the normal serum that was homogenous with primary antibody (Burry 2000). The dual-labeling results were analyzed using a confocal laser scanning microscopy (Olympus, FV1000, Japan) equipped with an IX2-UCB/U-HSTR2 control systems FV10.ASW 3.0 software (Olympus, Europa SE, Japan). An argon ion laser producing light at 467 and 488 nm, and an HeNe laser for 543 and 633 nm measurements, were used for the excitation of FITC and Cy5. GnRH and GFAP fluorescence intensities were calculated and analyzed according to a previously published method (Wang *et al.* 2009, Lim *et al.* 2014).

Statistical analysis

All data were expressed as the mean ± S.E.M. and statistical analysis was carried out using SPSS 18.0 statistic analysis software. Statistical significance was determined using Student's paired *t*-test or one-way ANOVA with LSD. A value of $P<0.05$ was considered significant.

Results

The GnRH antibody titer changed after immunization

Rats injected with GnRH-tandem-OVA showed increasing titers of anti-GnRH antibodies after the last booster immunization and titers remained high until the end of the experiment. The antibody titers in rats immunized with GnRH-tandem-OVA appeared to be significantly higher than in $Al(OH)_3$ adjuvant groups (Fig. 1), which produced very low antibody titers during the inoculation period. These findings suggested that the use of GnRH-tandem-OVA as an antigen was effective in stimulating an immune response.

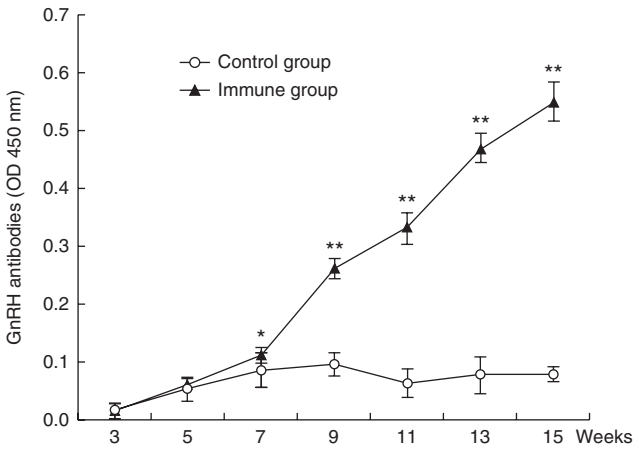

Figure 1
Detection of GnRH antibody titers. Measurements were taken every 2 weeks from the first immunization. Absorbance readings taken at 450 nm showed increased antibody titers from representative sera taken from male rats immunized with GnRH-tandem-OVA compared with $Al(OH)_3$ adjuvant control ($n=8$). *$P<0.01$, **$P<0.001$.

The hormone levels in serum changed after immunization

We investigated the testosterone, LH, FSH and thymulin levels in the serum of immunized and control rats respectively (Fig. 2A, B, C, and D). After the last booster immunization, serum testosterone concentrations were significantly reduced in the rats immunized with GnRH-tandem-OVA compared with the controls (Fig. 2A). Before the second immunization, there was no significant difference in serum testosterone levels between the immune and control groups (Fig. 2A). With age, serum testosterone levels showed a distinct increase in the control rats, but significantly decreased in the immunized rats. Moreover, testosterone concentrations decreased to below the detection limit of the assay in several immunized sera from 13- to 15-week-old rats. These sample values were counted as zero and were placed on *x*-axis in Fig. 2A.

Generally, serum LH and FSH concentrations gradually increased with age in the control group, but were significantly suppressed by immunization against GnRH, although their levels increased before 9-weeks-age in the immunized group (Fig. 2B and C). LH was more sensitive to active immunization compared with FSH, because the obvious suppression of LH occurred at the second immunization (Fig. 2B). By contrast, the FSH concentration was significantly reduced at 11 weeks in the immunized rats and remained at lower levels thereafter (Fig. 2C), while LH concentration significantly decreased to its lowest level compared with other time points (Fig. 2B).

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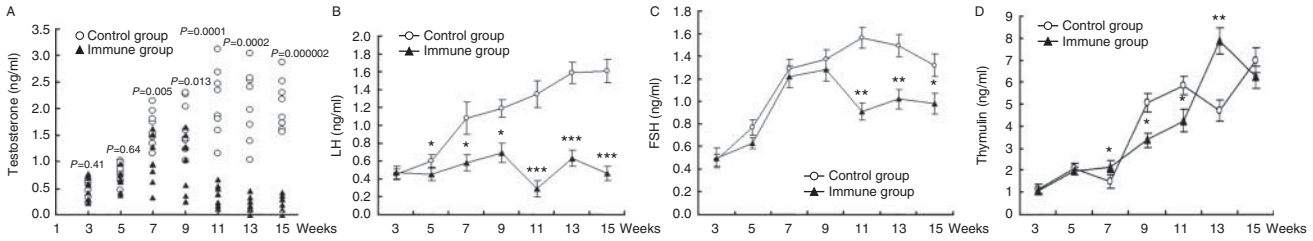

Figure 2
Levels of several hormones in serum of control and immunized rats ($n=8$). Measurements were taken every 2 weeks from the first immunization. (A) Testosterone levels in serum. (B) LH levels in serum. (C) FSH levels in serum. (D) Thymulin levels in serum. The levels of detection for testosterone, LH and FSH were all 0.02 ng/ml. The sensitivity of thymulin was 10.0 pg/ml. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

The variation in thymulin levels in the immunized rats was obviously different from that observed for the reproductive hormones (Fig. 2D). At the last booster immunization, the levels of thymulin were significantly increased by immunization with GnRH (Fig. 2D). Thereafter, they were significantly lower than in the controls until the end of 11 weeks-of-age. Meanwhile, the thymulin levels of the controls showed a sudden rise. Over the next 2 weeks, the thymulin concentration of the immunized rats abruptly increased and reached its highest level compared with other time points and was significantly higher than in the control group (Fig. 2D). At the end of experiment, there was no significant difference of serum thymulin levels between immunized and control rats (Fig. 2D).

GnRH and thymulin levels in hypothalamus changed after immunization

Active immunization against GnRH obviously suppressed the secretion of GnRH and stimulated the accumulation of thymulin in the hypothalamus (Fig. 3A and B). The GnRH concentrations in the hypothalamus were significantly lower in immunized rats compared with control rats, except at 11-weeks-old (Fig. 3A). In the immunized rat hypothalamus, thymulin levels significantly increased from 11 to 13 weeks (Fig. 3B). There were no significant differences within groups in the immunized cohort after 9 weeks and in the control group at the period of experiments (Fig. 3B). These data showed that the level of thymulin was negatively correlated with that of GnRH in the immunized rat hypothalamus.

GnRH and GFAP expressions changed in the hypothalamus

Immunofluorescent staining of GnRH neurons was mainly found in the paraventricular nucleus (Pa, Fig. 4A and B), medial preoptic nucleus (MPN, Fig. 4C and D) and the arcuate nucleus (Arc, Fig. 4E and F). Moreover, the immunoreactive nerve fibers of GnRH were mainly a large gathering at the median eminence (ME, Fig. 4G and H) of the rat hypothalamus, and part of the peripheral side around the third ventricle. GFAP immunoreactivity was also found in the preceding hypothalamic areas. Furthermore, the GnRH and GFAP average fluorescence intensities (AFIs) were analyzed statistical in each area of the hypothalamic nucleus (Fig. 4I and J). The AFIs of GnRH were all distinctly reduced in four hypothalamic nuclei of immunized rats (Fig. 4I). By contrast, the positive immunoreactivity of GFAP in the four areas was not consistent with that of GnRH in the immune groups. The intensity of GFAP immunostaining in the Pa and MPN showed no difference between the immune and control groups (Fig. 4J). GFAP immunoreactivity was significantly increased in the Arc and the ME of the immunized rats (Fig. 4J).

Discussion

GnRH and thymulin are the two keys signaling molecules that link the reproductive axis and the immune axis.

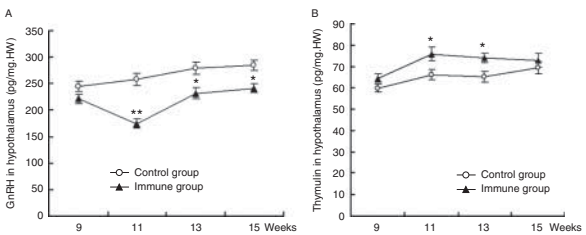

Figure 3
The levels of thymulin and GnRH in the hypothalami of control and immunized rats ($n=8$). Measurements were taken every 2 weeks from the final booster immunization. (A) GnRH levels in homogenized hypothalamus tissue of control and immunized rats. (B) Thymulin levels in homogenized hypothalami. The sensitivities of GnRH and thymulin were 5.2 and 10.0 pg/ml respectively. HW, hypothalamus weight. *$P<0.05$; **$P<0.01$.

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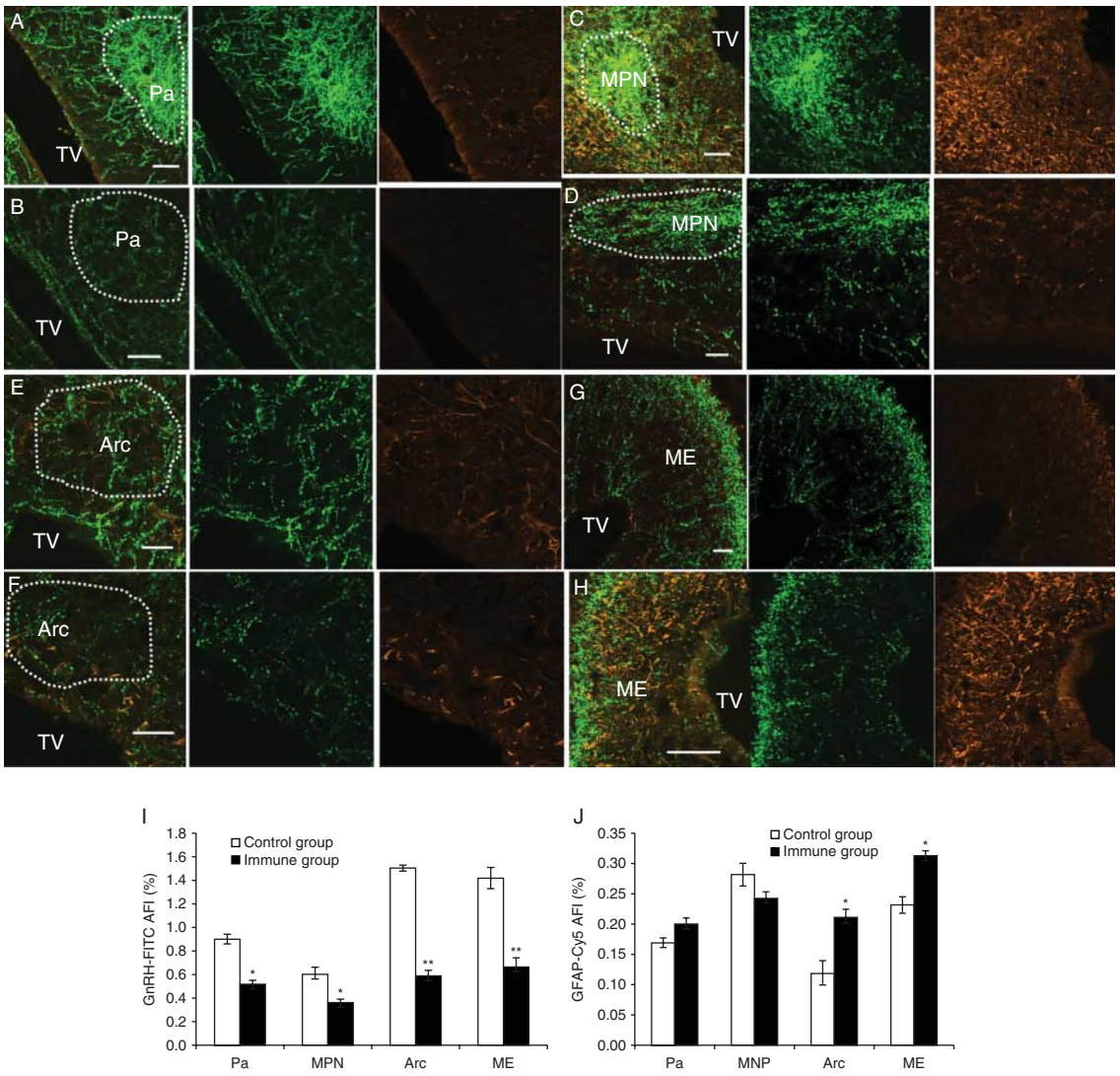

Figure 4

Protein expression of GnRH and GFAP in hypothalamic nuclei of control and immunized rats ($n=5$). (A and B) Paraventricular nucleus (Pa) of control and immune rats respectively; (C and D) medial preoptic nucleus (MPN) respectively; (E and F) arcuate nucleus (Arc) respectively; (G and H) median eminence (ME) respectively. (I and J) Average fluorescence intensities (AFIs) of GnRH and GFAP in different areas of the hypothalamus respectively. A dotted line indicated the nuclei of the hypothalamus. Green indicates a positive reaction of GnRH with IgG-FITC, orange a positive reaction of glial fibrillary acidic protein (GFAP) with IgY-Cy5, and their merge image was marked. TV: third ventricle, bar = 50 μm. AFIs of GnRH (E) and GFAP (F) in nuclei of rat hypothalami were analyzed. AFI was obtained and analyzed in two 40× object lens microscopic fields of every nucleus per hypothalamus of five rats in two treatments using the FV10.ASW 3.0 software and Image Pro-Plus 6.0 (Media Cybernetics Co., Rockville, MD, USA). Significant differences between groups were determined using an unpaired Student's *t*-test ($n=10$; two slices per hypothalamic nuclei). $^{*}P<0.05$, $^{**}P<0.01$.

Immunization against GnRH is an effective method of limiting reproduction in animals (Einarsson *et al.* 2009, Fang *et al.* 2010). The method is not often used in humans, because the balance between reproduction and immunity is damaged, and the side effects remain unclear. Although there have been some studies on the mechanism of immunization, studies of the integrative mechanism are scarce.

With the increasing titer of specific antibodies produced in the immune system by immunization against GnRH, the concentration of gonadal steroids declined to a very low level, except in the first 2 weeks. The levels of gonadotropins (LH and FSH) decreased sharply in the first 4 weeks, and then in the ensuing 2 weeks, their levels significantly increased, especially LH, although their levels were very low compared with the control rats.

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These variations of antibody and reproductive hormones were consistent with the results of previous studies (O'Leary *et al*. 2008), indicating that active immunization in an animal model was successful. Meanwhile, the serum thymulin was also reduced sharply. However, the concentration increased to a high level in the ensuing 2 weeks. The present findings suggest strongly that the primary immunization effect was caused entirely by suppression of reproductive hormones, and in such a disordered endocrine internal environment, thymulin was also suppressed for about 4 weeks. The production and secretion of thymulin are stimulated directly by growth hormones and PRL, and thymulin exerts a controlling feedback effect on its own secretion (Savino *et al*. 1983, Goya *et al*. 2004). I.p. injection of anti-FTS serum markedly reduced the serum activity of endogenous thymulin. Moreover, this inhibition lasted for at least 10 days (Goya *et al*. 2007). The effective cycle was shorter than in rats actively immunized with GnRH, probably because of the difference in the reaction times of the thymus to passive immunization (antibody immunoneutralization) and to active immunization (antigen vaccination). Considering the multihormone control effect by the pituitary–gonadal axis on thymulin secretion, gonadotropins inhibition of thymulin secretion might act via direct action of gonadal steroids in immunized rats.

When immunization against GnRH occurred *in vivo*, the neuroendocrine axis was disordered and suppressed, and the gonadotropins and steroid hormones were significantly inhibited. Thus, with the reduced levels of gonadotropins and gonadal steroid, the circulating levels of thymulin also decreased in the first 4 weeks. Although, there is no direct evidence proving that gonadotropins affect thymulin secretion, gonadectomy induces a transient reduction in thymulin levels in serum (Dardenne *et al*. 1986). Moreover, TEC lines cultured with physiological levels of gonadal steroids showed enhanced thymulin levels in the cell supernatants (Savino *et al*. 1988, Goya *et al*. 1995). Our results and the previous data, demonstrated that circulating thymulin was directly downregulated by the repressed gonadal steroid in rats actively immunized with GnRH in the early immune period. On the other hand, testosterone is an immunosuppressant (Bilbo & Nelson 2001) and was present at a very low levels in immunized rats. Also, the thymus endocrine function was enhanced and the secretion of thymulin in thymus was strengthened, as well as that of GnRH (Su *et al*. 2013). Consequently, our results showed that the level of thymulin was upregulated in the subsequent immunization period (about 2 weeks). Moreover, the effect might be in part because of the self-feedback regulation of thymulin (Cohen *et al*. 1986). The inhibitory effect of gonadotropins that produced the decline of thymulin in the immunized rat might be mediated by gonadal steroids, the exact mechanism of which should be determined.

As mentioned previously, in the early immunization period, the alterations in hormone the levels showed that the reproductive axis regulated thymulin production and secretion. In successive weeks (from the 4th to the 8th week), our data showed that thymulin feedback regulated the reproductive axis, especially the hypothalamic–pituitary axis. Thus, the gradually recuperation of circulating thymulin upregulated the levels of LH and FSH, but not the testosterone level, although gonadotropins levels were also at a quite low level compared with controls. These results were consistent with studies that demonstrated that the thymulin exerted positive feedback regulation on LH and FSH in immunized rats during the late period (Reggiani *et al*. 2009*a*). However, the effect of stimulation was slight. By contrast, the accumulation of thymulin in the hypothalamus was more notable than in the serum. The concentration of thymulin in the hypothalamus of immunized rats was significantly higher than that of the control rats, except at the 8th week. Meanwhile, the GnRH concentration in the hypothalamus of immunized rats also increased significantly. These results suggested that thymulin could exert positive feedback regulation on the secretion of GnRH in the hypothalamus of rats actively immunized with GnRH. This is consistent with the hypothesis that thymulin may be part of a feedback loop acting on neuroendocrine organs, such as the hypothalamus, and modulates the stimulatory activity of GnRH on LH and FSH release from pituitary cells (Hinojosa *et al*. 2004, Siemion *et al*. 2005). In addition, thymulin plays a neuroprotective role in different areas of the brain that interact with a set of cytokines (Safieh-Garabedian *et al*. 2011). Thus, the effect of upregulation on GnRH in hypothalamus may be, at least in part, explained by the neuroprotective role of thymulin. Indeed, active immunization against GnRH disordered the function and even remodeled the structure of hypothalamus. Furthermore, the average immunofluorescence density showed that the synthesis and secretion of GnRH neurons were suppressed in the immune hypothalamus. However, it is unclear by what mechanism thymulin upregulates GnRH in the immune hypothalamus: whether it involves plastic rearrangements of glial-GnRH neurons adhesiveness or the production of growth factors acting via receptors with tyrosine kinase activity remains to be determined (Mullier *et al*. 2010, Heja *et al*. 2012).

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Hypothalamic astrocytes release a variety of neuroactive factors, including TGFα, prostaglandin E2 and their receptors forming a signaling pathway that is essential for the glial mediation in GnRH release (Buchanan *et al*. 2000, Clasadonte *et al*. 2011). The AFIs of GnRH were all low in four areas of the hypothalamus from rats immunized with GnRH. Moreover, the inhibitory effect was more significant in the Arc and ME. The regional discrepancy in immunoactive GnRH may be largely explained by the fact that the Arc that is the key area of the steroid hormone feedback regulation on GnRH (Yeo & Herbison 2014). In immunized male rats, the fairly low level of testosterone negatively feedback regulates the production of GnRH neurons in the Arc instead of the Pa and MPN, which contain many GnRH cell bodies to regulate other hypophysiotropic hormone releasing hormones besides GnRH (Van Vugt *et al*. 1997, Rivalland *et al*. 2006). Likewise, the release of GnRH was significantly suppressed via reduction in the conveyance of GnRH to its nerve terminals in the ME of immunized rats (Glanowska & Moenter 2015).

Interestingly, regional specificity was also shown by GFAP positive immunoreaction in the immune hypothalamus. In the Arc and ME, the AFIs of GFAP were significantly higher than in the control rats. By contrast, AFIs were similar in the Pa and MPN between the two treatments. These changes were associated with altered astrocyte-neuron contacts and synaptic remodeling in immunized rats. The stimulation of hormones, especially gonadal steroids, on the activities of astrocytes in the Arc and ME (Blutstein *et al*. 2009, Yin *et al*. 2009, Yeo & Herbison 2014), at least demonstrated that testosterone reduced plastic rearrangements of glial-GnRH neurons adhesiveness. By contrast, testosterone increased the number of astrocytes in the hippocampus reduced GFAP expression and stimulated the reactive astrocyte hypertrophy in the infarct area of the rat brain (Pan *et al*. 2005, Emamian *et al*. 2010). The results confirmed that the effect of testosterone on astrocytes is independent of the location and the physiological status. The activities of astrocytes in the Arc and ME may be also be regulated by other signaling pathways of peripheral small-molecules, such as thymulin. After all, without the blood–brain barrier, circulating molecules were taken up by astrocytes in Arc and ME (Cheunsuang & Morris 2005, Morita & Miyata 2013). Thus, it is unclear whether the effect and regulation of thymulin on GnRH is direct or mediated by astrocytes. These results suggested that the secretion and release of GnRH to modulate reproduction in the Arc and ME was regulated not only via the synaptic connectivity between astrocytes and GnRH neurons modulated by the feedback regulation of reproductive hormones, but also via a specific molecular signaling pathway.

In summary, the current study demonstrated that reproductive hormones and thymulin were distinctly suppressed by immunization against GnRH. In the late period of immunization, the immunosuppression of androgens and the increase of LH were abolished, and the circulating thymulin level increased significantly. We showed that thymulin could stimulate GnRH neurons development and secretion, not only by direct contact with GnRH, but also mediated via astrocytes in different regions of the hypothalamus.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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