Related Research Paper

Catecholaminergic signalling through thymic nerve fibres, thymocytes and stromal cells is dependent on both circulating and locally synthesized glucocorticoids

I. Pilipović[1], K. Radojević[1], M. Perišić[1], D. Kosec[1], M. Nacka-Aleksić[2], J. Djikić[2] and G. Leposavić[2]

[1]*Immunology Research Centre 'Branislav Janković', Institute of Virology, Vaccines and Sera 'Torlak', Belgrade, Serbia*
[2]*Department of Physiology, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia*

Glucocorticoids have been shown to modulate the expression of noradrenaline metabolizing enzymes and β_2- and α_{1B}-adrenoceptors in a tissue- and cell- specific manner. In the thymus, apart from extensive sympathetic innervation, a regulatory network has been identified that encompasses catecholamine-containing non-lymphoid and lymphoid cells. We examined a putative role of adrenal- and thymus-derived glucocorticoids in modulation of rat thymic noradrenaline levels and adrenoceptor expression. Seven days postadrenalectomy, the thymic levels of mRNAs encoding tyrosine hydroxylase, dopamine β-hydroxylase, monoamine oxidase-A and, consequently, noradrenaline were decreased. Catecholamine content was diminished in autofluorescent nerve fibres (judging by the intensity of fluorescence) and thymocytes (considering HPLC measurements of noradrenaline and the frequency of tyrosine hydroxylase-positive cells), while it remained unaltered in non-lymphoid autofluorescent cells. In addition, adrenalectomy diminished the thymocyte expression of β_2- and α_{1B}-adrenoceptors at both mRNA and protein levels. Administration of ketoconazole (an inhibitor of glucocorticoid synthesis/action; 25 mg kg^{-1} day^{-1}, s.c.) to glucocorticoid-deprived rats increased the thymic levels of tyrosine hydroxylase, dopamine β-hydroxylase and, consequently, noradrenaline. The increased intensity of the autofluorescent cell fluorescence in ketoconazole-treated rats indicated an increase in their catecholamine content, and suggested differential glucocorticoid-mediated regulation of catecholamines in thymic lymphoid and non-lymphoid cells. In addition, ketoconazole increased the thymocyte expression of α_{1B}-adrenoceptors. Thus, this study indicates that in the thymus, as in some other tissues, glucocorticoids not only act in concert with cateholamines, but they may modulate catecholamine action by tuning thymic catecholamine metabolism and adrenoceptor expression in a cell-specific manner. Additionally, the study indicates a role of thymus-derived glucocorticoids in this modulation.

(Received 1 February 2012; accepted after revision 3 May 2012; first published online 4 May 2012)
Corresponding author G. Leposavić: Department of Physiology, Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe, 11221 Belgrade, Serbia. Email: gordana.leposavic@pharmacy.bg.ac.rs

The thymus is the primary lymphoid organ required for the development of immunocompetent T cells and the establishment of an efficient immune response. Development of T cells is modulated by many extrinsic factors, directed by the CNS. The brain delivers information to the thymus by two major channels, namely the hypothalamic–pituitary–adrenal axis hormones and sympathetic fibres of the autonomic nervous system (Weigent & Blalock, 1997). Although the sympathetic nerve fibres are the main source of catecholamines (CAs) in rodent thymus (Felten *et al.* 1985; Leposavić *et al.* 1992; Elenkov *et al.* 2000), their synthesis in thymocytes and thymic non-lymphoid cells has also been demonstrated (Pilipović *et al.* 2008). Moreover, it has been shown that CAs, acting on thymic lymphoid and non-lymphoid cell β_2- and α_{1B}-adrenoceptors (ARs) provide fine-tuning of thymocyte differentiation/maturation and, consequently, the maintenance of the peripheral T-cell

DOI: 10.1113/expphysiol.2012.064899

repertoire and self-tolerance (reviewed by Leposavić *et al.* 2011). Additionally, CAs released from thymic cells are also supposed to act as immunotransmitters, transferring information to nerve fibre endings (reviewed by Leposavić *et al.* 2011).

When stability of the internal milieu is disturbed or threatened by internal or external challenges, both the sympathetic nervous system and hypothalamic–pituitary–adrenal axis become activated, resulting in increased circulating levels of CAs and glucocorticoids (GCs). They act in concert to maintain the steady state of the internal milieu (Chrousos & Gold, 1992). In addition, GCs have been shown to influence bioavailability of CAs in the tissues by modulating the expression of enzymes involved in CA biosynthesis and degradation in a tissue- and cell-type-specific manner (Lucas & Thoenen, 1977; McMahon & Sabban, 1992; Hiwatashi *et al.* 2002; Kano *et al.* 2005; Lindley *et al.* 2005; Kvetnansky *et al.* 2009). Moreover, GCs influence β- and α_1-AR gene expression in a tissue- and cell-type-specific manner (Davies & Lefkowitz, 1980; Kavelaars, 2002; Abraham *et al.* 2003).

Thymocytes (Wiegers *et al.* 2001; Boldizsar *et al.* 2010), thymic epithelial cells (TECs) and dendritic cells express the classical cytosol glucocorticoid receptor (GR; Sacedón *et al.* 1999; Talaber *et al.* 2011). In addition, local GC synthesis has been found in thymocytes and TECs (Qiao *et al.* 2009; Taves *et al.* 2011), as in some other cell types (Cima *et al.* 2004). The thymic GC synthesis is influenced by the circulating GC level in a cell-type-specific manner (Qiao *et al.* 2009; Taves *et al.* 2011). Glucocorticoids in the thymus act as important regulators of thymocyte survival and differentiation (Ashwell *et al.* 1996; Chung *et al.* 2002; Stojić-Vukanić *et al.* 2009).

Considering all the aforementioned information, it may be supposed that GCs modulate CA-mediated tuning of thymopoiesis by influencing neural and/or non-neural thymic CA synthesis and/or AR expression. This hypothesis is supported by a study showing that the effects of β-AR blockade on thymopoiesis differ substantially between adrenalectomized (Adx) and control non-Adx rats (Pilipović *et al.* 2010).

To further confirm this hypothesis in thymi and thymocytes from Adx rats, in the present study we measured the following parameters: (i) noradrenaline (NA) level; (ii) the expression of mRNAs encoding major enzymes involved in NA metabolism; (iii) the density of CA-containing autofluorescent nerve fibres and cells and the intensity of their fluorescence; and (iv) the expression of β_2- and α_{1B}-ARs at both the mRNA and the protein level. To elucidate the putative influence of intrathymically synthesized GCs on thymic NA bioavailability and AR expression, we examined the same thymic and thymocyte parameters in adrenal GC-deprived rats treated with ketoconazole (KET), a steroid hormone synthesis blocker and GC receptor antagonist (Loose *et al.* 1983; Deuschle *et al.* 2003). To exclude possible effects of KET-induced testicular steroid deprivation on these parameters (Pilipović *et al.* 2008; Leposavić *et al.* 2011), the rats treated with KET were bilaterally orchidectomized.

Methods

Ethical approval

The animals used in this study were handled in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (revising Directive 86/609/EEC), and the experimental protocol was approved by the Experimental Animal Committee of the Immunology Research Centre 'Branislav Janković'.

Experimental design

Two sets of experiments were performed using male 75-day-old Dark Agouti rats ($n = 74$).

In the first experiment, rats were subjected to bilateral adrenalectomy via a single dorsal incision under general anaesthesia. The anaesthetic solution (0.8 ml (100 g body weight)$^{-1}$, I.P.) consisted of ketamine (100 mg ml^{-1}; Ketamidor®; Richter Pharma AG, Wels, Austria), xylazine (20 mg ml^{-1}; Xylased®; Bioveta, Ivanovice na Hané, Czech Republic) and saline, in a 1:0.5:8.5 ratio. Control rats were sham adrenalectomized using the same surgical procedure, but without removal of the adrenals. Both Adx and sham-Adx animals were provided with 0.9% saline in drinking water. Completeness of adrenalectomy was confirmed by histological examination of removed tissue, checking for residual adrenal tissue at post-mortem examination, and measuring the blood corticosterone concentration.

In the second experiment, following adrenalectomy the rats were bilaterally orchidectomized through a transverse scrotal incision. From the following day, the rats were subcutaneously administered with 25 mg kg^{-1} day^{-1} KET (Sigma-Aldrich Chemie Gmbh, Tufkirchen, Germany). Animals administered with vehicle (polyethylene glycol; PEG) served as controls.

In both sets of experiments, 7 days postsurgery the rats were killed by exposure to an increasing concentration of CO_2, followed by exsanguination. Each group consisted of six animals.

Chemicals, antibodies and immunoconjugates

Tween 20 and L-noradrenaline hydrochloride were purchased from Sigma-Aldrich Chemie Gmbh. Target Retrieval Solution and LSAB+ System were obtained from DakoCytomation (Glostrup, Denmark). Gene Expression Master Mix and commercial TaqMan Gene Expression

Assays for rat tyrosine hydroxylase (TH; Rn00562500_m1), dopamine β-hydroxylase (DBH; Rn00565819_m1), phenylethanolamine *N*-methyltransferase (PNMT; Rn01495588_m1), monoamine oxidase-A (MAO-A; Rn01430950_m1), β_2-AR (Rn00560650_s1), α_{1B}-AR (Rn01471343_m1) and hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1; Rn01527840_m1) were obtained from Applied Biosystems (Foster City, CA, USA).

Monoclonal antibody specific to TH (clone TOH A1) were purchased from BD Biosciences Pharmingen (San Jose, CA, USA), polyclonal rabbit anti-β_2-AR and polyclonal goat anti-α_{1B}-AR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Second-step reagents were obtained from BD Biosciences Pharmingen (goat anti-mouse IgG fluorescein isothiocyanate, goat anti-rabbit IgG fluorescein isothiocyanate) and Sigma-Aldrich Chemie Gmbh (rabbit anti-goat IgG fluorescein isothiocyanate).

Measurement of CAs by HPLC

Briefly, one lobe of each thymus was frozen at −70°C for whole thymic tissue CA analyses, while the other was passed through a stainless-steel mesh into ice-cold PBS to obtain a thymocyte suspension. The thymocyte suspension (1×10^8 cells) was centrifuged at 250*g* and the pellet resuspended in 200 μl of cold 0.1 M perchloric acid solution containing 0.15% $Na_2S_2O_5$ and 0.05% Na_2EDTA (HPLC solution) and stored at −70°C until analysis. Before analysis, the thymic tissue homogenized in 0.4 ml of HPLC solution and thawed thymocyte suspensions were centrifuged at 30,000*g* for 20 min. The analytical system consisted of a Waters high-performance liquid chromatograph (E600 pump; Waters Corp., Milford, MA, USA) with an autosampler (Waters 717 Plus) and detector (GBC amperometric ECD 1260) and a Chromsystems column for Catecholamines (p/n 6100, 150 mm; Chromsystems Instruments & Chemicals GmbH, Gräfelfing/München, Germany). A mobile phase of Chromsystems Mobile phase for Catecholamines (p/n 5001) was used. All separations were performed at flow rate of 1 ml min^{-1}. The detector potential was maintained at +0.65 V. The electrochemical detector sensitivity for thymic and thymocyte samples was 2 and 1 nA, respectively. For the quantification of NA content, the software Empower CS (Empower Masterpay Pty Ltd, Kent Town, SA, Australia) was used.

Fluorescence histochemistry

To visualize CA-containing nerve fibres and cells, serial 20-μm-thick thymic cryosections were treated by a modification of the sucrose phosphate–glyoxylic acid method (Radojević *et al.* 2011). Briefly, the slides were dipped in a solution containing 1% glyoxylic acid, 0.2 M sucrose and 0.236 M KH_2PO_4 (pH 7.4), drained, covered with non-autofluorescent immersion oil and heated at 95°C for 2.5 min, and then coverslipped. The sections were photographed on the same day using an Olympus BH 2 fluorescence photomicroscope (Olympus Optical Co., Ltd, Tokyo, Japan) and digital camera (Color View III; Olympus Soft Imaging Solutions, Münster, Germany) and AnalySIS FIVE software (Olympus Soft Imaging Solutions). For digital quantification of fluorescence intensity and density, 15 test areas selected at random from five sections of each thymus were captured. The digital outline of each autofluorescent profile/large autofluorescent cell was traced, and the fluorescence intensities were measured digitally using ImageJ software, yielding values of pixel intensity per unit area, as previously described (Radojević *et al.* 2011). Fluorescent nerve fibre density was measured using a stereological grid point-counting approach and expressed as the percentage of field area occupied by the fluorescent nerve profiles (Radojević *et al.* 2011). Large autofluorescent cell density was counted using a 0.027 mm^2 quadrant lattice by an observer with no previous knowledge of which slide was being analysed.

Immunofluorescence staining and flow cytometric analysis

A single-cell suspension was obtained by grinding the thymic tissue on a 60 μm sieve screen submerged in ice-cold PBS containing 0.09% NaN_3 and 2% fetal calf serum (FACS buffer), washed and enumerated using an improved Neubauer haemocytometer.

For analysis of TH and β_2-AR expression, 1×10^6 thymocytes were fixed (30 min) with 0.25% paraformadehyde (Sigma-Aldrich Chemie Gmbh) in PBS at 4°C, permeabilized with PBS containing 0.2% Tween 20 and 0.09% NaN_3 for 15 min at 4°C and washed twice in ice-cold washing solution. Permeabilized and non-permeabilized cells (for α_{1B}-AR expression analysis) were incubated with primary antibody. After incubation (30 min, 4°C) cells were washed twice, and appropriate secondary fluorescein isothiocyanate-conjugated antibody was added. Following incubation and washing, the cells were analysed on FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using CellQuest software (Becton Dickinson Immunocytometry Systems).

Immunocytochemical staining and quantification

To identify the cells expressing TH, β_2- and α_{1B}-ARs, thymic tissue was fixed in IHC Zinc fixative (BD Biosciences Pharmingen), embedded in paraffin and cut into 5-μm-thick serial sections. These sections were subjected to heat-induced epitope retrieval in Target

retrieval solution. The staining procedure was performed using LSAB+ System according to the manufacturer's manual. Anti-rat TH (1:200 dilution), β_2-AR (1:40 dilution) and α_{1B}-AR (1:100 dilution) were used as primary antibodies. Upon completion of the reaction, thymic sections were counterstained with Haematoxylin and analysed using an Olympus BH 2 microscope equipped with a digital colour camera (Color View III; Olympus Soft Imaging Solutions). Approximately every 40th section was subjected to stereological measurements of immunoreactive (ir) cell numerical density (Nv; number of cells per tissue volume unit) by the point and intersection counting method (Karapetrovic *et al.* 1995) using the AnalySIS FIVE software.

Quantification of mRNA using quantitative PCR

Total RNA was isolated from freshly harvested thymic tissue and thymocytes, using ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems) and Total RNA Chemistry (Applied Biosystems). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and 5 μl cDNA was used for real-time PCR. Triplicate 25 μl reactions were run in Applied Biosystems 7500 universal cycling conditions. All procedures were performed according to manufacturer's manuals. Hypoxanthine guanine phosphoribosyltransferase 1 was selected as the internal standard, to normalize for input cDNA variations, because it displayed an optimal stability among various samples tested. Quantitative differences in gene expression levels were assessed using Applied Biosystems SDS software (version 1.4.0.) and the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data are presented as means ± SEM. To determine the significance of differences between groups, depending on the F test for unequal variance, Student's unpaired t test was applied without or with Welch's correction. Statistical analysis of the quantitative PCR ΔCt values was performed using the Mann–Whitney rank sum test. All analyses were performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), with the significance level set at $P < 0.05$.

Results

Effect of surgical removal of circulatory adrenal hormones on thymic CA-containing nerve fibres and cells and AR expression in Adx and male Dark Agouti rats

Thymic NA level and NA-synthesizing and -degrading enzyme mRNA expression. In hypertrophic ($P < 0.01$) thymi of Adx rats (Fig. 1*Aa*) the concentration of NA was diminished ($P < 0.05$) compared with sham-Adx control animals (Fig. 1*Ab* and Fig. S1). To elucidate mechanisms underlying this decrease, the thymic expression of mRNAs encoding enzymes involved in NA biosynthesis and degradation was examined. Adrenalectomy diminished ($P < 0.05$) the expression of mRNAs for the following enzymes: (i) TH, which catalyses the rate-limiting step in CA synthesis (conversion of tyrosine to 3,4-dihydroxyphenylalanine); (ii) DBH, which converts dopamine to NA; and (iii) MAO-A, which catalyses monoamine oxidation. The amount of mRNA encoding PNMT (which converts NA to adrenaline) remained unaltered in Adx compared with sham-Adx rats (Fig. 1*Ac*).

Fluorescence histochemistry and TH immunocytochemistry. To estimate the contribution of distinct thymic NA sources to changes in this neurotransmitter/hormone concentration in the thymus, we analysed the density of CA-containing nerve fibres and cells. Adrenalectomy diminished ($P < 0.05$) the density of autofluorescent nerve fibres and the intensity of their fluorescence (Fig. 2*A*); however, it did not affect either the density of large autofluorescencent cells or the intensity of their fluorescence (Fig. 2 A).

Furthermore, no difference was observed in either Nv of TH-ir thymocytes or that of TH-ir thymic non-lymphoid cells between Adx and control rats (Fig. 3*B*). The unchanged Nv of TH-ir non-lymphoid cells, in conjunction with the unaltered density of autofluorescent cells and the intensity of their fluorescence (Fig. 2*A*), suggested that adrenalectomy did not affect the contribution of the non-lymphoid cellular compartment to the overall thymic NA concentration.

Thymocyte NA content and expression of TH mRNA. To estimate the putative contribution of the numerically enriched ($P < 0.01$) thymocyte compartment (Fig. 4*Aa*) to the decrease of thymic NA concentration in Adx rats, the thymocyte NA content and expression of TH were examined. Adrenalectomy diminished (by ∼44%; $P < 0.05$) the NA content in thymocyte suspensions (Fig. 4*Ab* and Fig. S1). This decrease was more pronounced than that (by ∼33%; $P < 0.01$) in the frequency of tyrosine hydroxylase-positive (TH+) cells in the same thymocyte suspensions (Fig. 4*Ac* and Fig. S2). Thus, it may be assumed that adrenalectomy diminished the amount of NA per thymocyte. Consistent with this view, a corresponding decline ($P < 0.05$) was observed for mean fluorescence intensity (MFI; reflecting decrease in the protein density) of TH (an enzyme with a major regulatory role in CA biosynthesis) in TH+ thymocytes from Adx rats (Fig. 4*Ad* and Fig. S2). In addition, the TH mRNA expression was also decreased ($P < 0.05$) in the thymocyte

suspensions from Adx rats (Fig. 4*Ae*). Given that this decrease (by ∼47%) was also more pronounced than that in the frequency of TH+ cells, a diminished expression of TH mRNA per thymocyte in Adx rats was strongly indicated.

Thymic and thymocyte expression of ARs. Adrenalectomy decreased ($P < 0.05$) β_2-AR mRNA expression in thymic tissue (Fig. 5*Bb*). However, treatment did not affect Nv of either β_2-AR-ir thymocytes or β_2-AR-ir thymic non-lymphoid cells (Fig. 5*Ba*).

Analysis of the β_2-AR mRNA expression in thymocyte suspensions showed that adrenalectomy downregulated ($P < 0.05$) β_2-AR mRNA expression (Fig. 5*Be*). This finding, together with the increased ($P < 0.05$) frequency of β_2-AR-positive cells in thymocyte suspensions from Adx compared with control rats (Fig. 5*Bc*), indicated a decreased β_2-AR mRNA expression per thymocyte. In addition, the diminished ($P < 0.05$) β_2-AR MFI on β_2-AR-positive thymocytes indicated a decrease in the receptor surface density on thymocytes from Adx compared with sham-Adx rats (Fig. 5*Bd* and Fig. S2).

Adrenal gland ablation decreased ($P < 0.05$) α_{1B}-AR mRNA expression in thymic tissue (Fig. 6*Bb*). In thymi of these rats, Nv of α_{1B}-AR-ir thymic non-lymphoid cells was substantially increased ($P < 0.01$), while that of α_{1B}-AR-ir thymocytes was unaltered when compared with control animals (Fig. 6*Ba*).

Adrenalectomy reduced both α_{1B}-AR mRNA expression ($P < 0.05$; Fig. 6*Be*) in thymocyte suspensions and the frequency of α_{1B}-AR+ cells ($P < 0.01$; Fig. 6*Bc*). A more pronounced adrenalectomy-induced decrease (by ∼67%) in the α_{1B}-AR mRNA expression than that in the frequency of α_{1B}-AR-positive cells (by ∼30%; $P < 0.01$) indicated the diminished α_{1B}-AR mRNA expression per thymocyte. Consistent with this view was a corresponding decline ($P < 0.01$) in α_{1B}-AR MFI on α_{1B}-AR-positive thymocytes from Adx rats (Fig. 6*Bd* and Fig. S2).

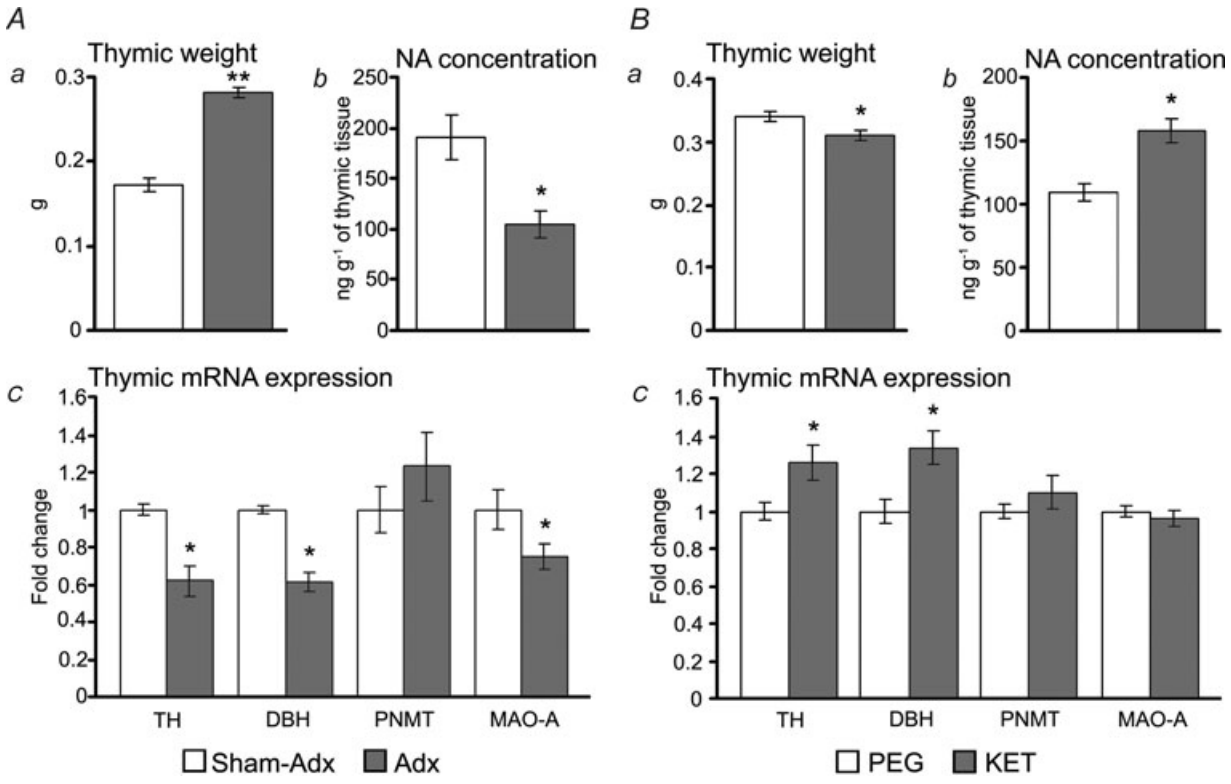

Figure 1. Influence of adrenal- and thymus-derived glucocorticoids on thymic weight (a), thymic noradrenaline (NA) concentration (b) and the mRNA expression of NA biosynthetic and metabolizing enzymes (c) in thymi of adrenalectomized (Adx) and sham-Adx rats (*A*) and in Adx and orchidectomized rats treated with ketoconazole (KET) and polyethylene glycol (PEG; *B*)
Abbreviations: DBH, dopamine β-hydroxylase; MAO-A, monoamine oxidase-A; PNMT, phenylethanolamine *N*-methyltransferase; and TH, tyrosine hydroxylase. Results are expressed as means ± SEM ($n = 6$). $^*P < 0.05$, $^{**}P < 0.01$.

Effect of inhibition by ketoconazole of thymic steroid synthesis and action on thymic CA-containing nerve fibres and cells and AR expression in Adx and orchidectomized male Dark Agouti rats

Thymic NA level and NA-synthesizing and -degrading enzyme mRNA expression. Inhibition of local GC synthesis in Adx and orchidectomized rats by KET reduced ($P < 0.05$) thymic weight (Fig. 1*Ba*). Compared with vehicle-treated control animals, in KET-treated rats the thymic NA concentration was increased ($P < 0.05$; Fig 1*Bb* and Fig. S1). In addition, in these rats the thymic expression of TH ($P < 0.05$) and DBH mRNAs ($P < 0.05$) were augmented (Fig. 1*Bc*). The thymic expression of

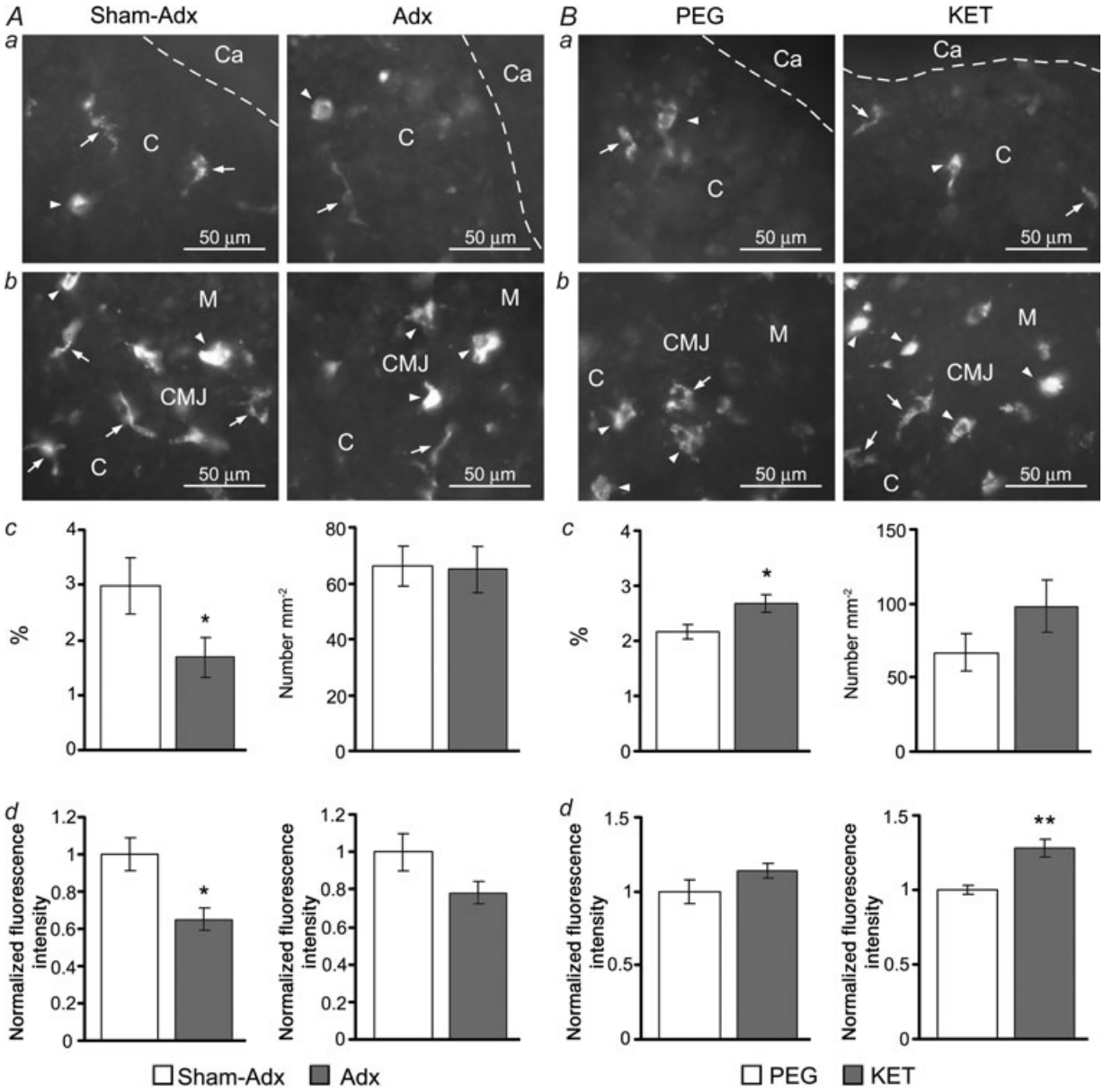

Figure 2. Influence of adrenal- and thymus-derived glucocorticoids on the density of thymic catecholamine-containing autofluorescent nerve fibres and cells and the intensity of their fluorescence
The photomicrographs show catecholamine-containing nerve profiles (arrows) and cells (arrowheads) visualized using sucrose phosphate glyoxylic acid in the outer thymic cortex (*a*) and the deep cortex and medulla (*b*) of adrenalectomized (Adx) and sham-Adx rats (*A*) and KET- and PEG-treated Adx and orchidectomized rats (*B*). Original magnification ×400. Abbreviations: C, cortex; Ca, capsule; CMJ, cortico-medullary junction; and M, medulla. Dashed white lines indicate thymic capsule-cortex borders. The histograms (*c* and *d*) represent the density (*c*) of autofluorescent nerve profiles (left panels) and cells (right panels) and the normalized intensity of their fluorescence (*d*) in Adx and sham-Adx rats (*A*) and in KET- and PEG-treated Adx and orchidectomized rats (B). Results are expressed as means ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$.

PNMT and MAO-A mRNAs remained unchanged in KET-treated rats compared with control animals (Fig. 1*Bc*).

Fluorescence histochemistry and TH immunocytochemistry. Ketoconazole increased ($P < 0.05$) the density of thymic autofluorescent nerve fibres, without affecting the intensity of their fluorescence (Fig. 2*B*). On the contrary, KET did not affect fluorescent cell density, but increased ($P < 0.01$) the intensity of their fluorescence (Fig. 2*B*).

The immunocytochemical visualization of TH+ cells demonstrated no difference in Nv of TH-ir non-lymphoid cells or TH-ir thymocytes between KET-treated rats and control animals (Fig. 3*C*).

Thymocyte NA content and expression of TH mRNA. Compared with PEG-treated control rats, KET diminished ($P < 0.01$) the total number of thymocytes (Fig. 4*Ba*), as well as NA content ($P < 0.05$; Fig. 4*Bb* and Fig. S1) and amount of TH mRNA ($P < 0.05$; Fig. 4*Be*), in thymocyte suspensions. The unaltered frequency of TH+ cells in thymocyte suspensions from KET-treated rats compared with PEG-treated rats (Fig. 4*Bc* and Fig. S2) suggested a decrease in the amount of NA and TH mRNA per thymocyte. In addition, judging by TH MFI, TH expression at protein level (Fig. 4*Bd* and Fig. S2) was decreased ($P < 0.05$) in thymocyte suspensions from KET-treated rats compared with control animals.

Thymic and thymocyte expression of ARs. The inhibition of local GC synthesis did not affect thymic β_2-AR mRNA expression (Fig. 5*Cb*). In addition, Nv of both β_2-AR-ir thymic non-lymphoid cells and thymocytes remained unaltered in KET-treated rats compared with PEG-treated control animals (Fig. 5*Ca*).

The frequency of β_2-AR-positive cells, the amount of thymocyte β_2-AR mRNA and β_2-AR MFI on β_2-AR-positive thymocytes remained unaltered in thymocyte suspensions from KET-treated rats compared with PEG-treated control rats (Fig. 5*Cc–e* and Fig. S2).

In thymi from KET-treated rats, the α_{1B}-AR mRNA expression was almost twofold higher ($P < 0.05$) than in thymi from vehicle-administered control animals (Fig. 6*Cb*); however, neither Nv of α_{1B}-AR-ir thymic non-lymphoid cells nor that of α_{1B}-AR-ir thymocytes was affected by KET administration (Fig. 6*Ca*).

Ketoconozole decreased ($P < 0.05$) the frequency of α_{1B}-AR-positive cells in thymocyte suspensions, but it increased ($P < 0.05$) α_{1B}-AR MFI on α_{1B}-AR-positive thymocytes (Fig. 6*Cc* and *d* and Fig. S2). The decreased (by ~30%) frequency of α_{1B}-AR-positive cells in thymocyte suspensions, coupled with the unaltered amount of α_{1B}-AR mRNA (Fig. 6*Ce*), suggested an increased α_{1B}-AR mRNA expression per thymocyte.

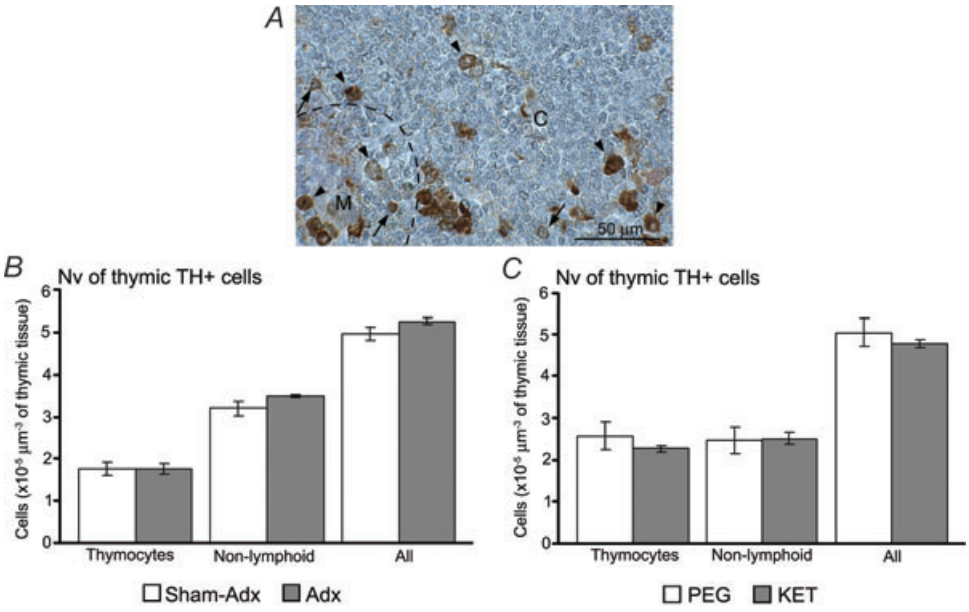

Figure 3. Influence of adrenal- and thymus-derived glucocorticoids on the density of thymic tyrosine hydroxylase-positive (TH+) cells
A, representative photomicrograph shows TH-immunoreactive thymocytes (arrows) and non-lymphoid cells (arrowheads) in thymus from sham-adrenalectomized (Adx) rats. Original magnification ×400. Abbreviations: C, cortex; and M, medulla. Dashed black line indicates the cortico-medullary border. The histograms show numerical density (Nv) of thymocytes, thymic non-lymphoid cells and all thymic cells expressing TH in thymi from Adx and sham-Adx rats (*B*) and KET- and PEG-treated Adx and orchidectomized rats (*C*). Results are expressed as means ± SEM ($n = 6$).

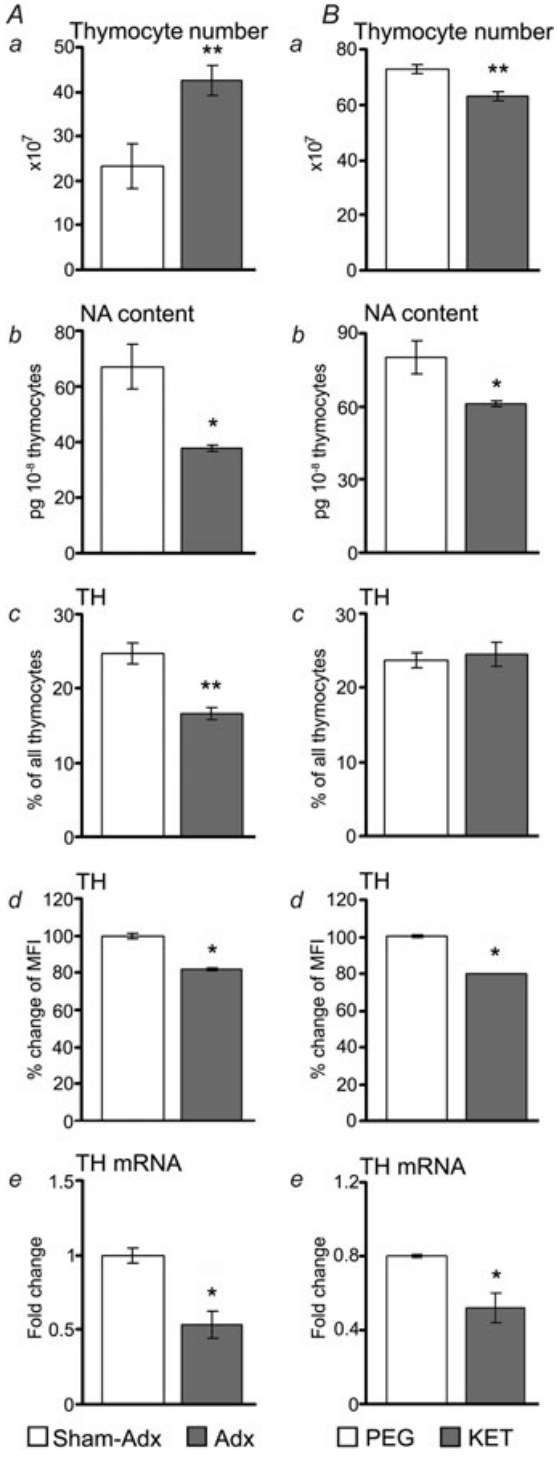

Figure 4. Influence of adrenal- and thymus-derived glucocorticoids on the overall number of thymocytes (*a*), thymocyte noradrenaline (NA) content (*b*), the percentage of TH+ cells (*c*), TH mean fluorescence intensity (MFI) on TH+ thymocytes (*d*) and TH mRNA level (*e*) in thymocyte suspensions from Adx and sham-Adx rats (*A*) and from KET- and PEG-treated Adx and orchidectomized rats (*B*)
Results are expressed as means ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$.

Discussion

The present study demonstrated that lack of adrenal GCs, as well as inhibition of local GC synthesis in the absence of adrenal GCs, influences the thymic NA concentration by modulating the abundance and/or synthetic capability of distinct thymic sources of NA and influences thymocyte and possibly thymic non-lymphoid cell AR expression.

Effect of surgical removal of circulatory adrenal hormones on thymic CA-containing nerve fibres and cells and AR expression in male Dark Agouti rats

The first set of our experiments indicated that adrenalectomy decreased thymic NA concentration by diminishing the expression of TH and DBH mRNAs. The decrease in TH mRNA expression was consistent with data showing that GCs upregulate TH gene transcription in some non-immune system cells (Lucas & Thoenen, 1977; Sabban, 1997). The decrease in the DBH mRNA level in thymi from Adx rats was in accordance with the finding that GCs enhance DBH mRNA expression in PC12 cells (McMahon & Sabban, 1992). Furthermore, it seems obvious that these changes were sufficient to provide the reduction in the NA level, notwithstanding the decline in the level of mRNA encoding MAO-A (a key enzyme for the degradation of NA, dopamine and serotonin) in thymi from Adx rats. In support of this finding are data showing that GCs enhance MAO-A gene expression in many cell types (Manoli *et al.* 2005; Ou *et al.* 2006). Subsequent analyses showed that the NA content in thymi from Adx rats was diminished in the autofluorescent noradrenergic nerve fibres (judging by the reduced intensity of autofluorescence) and thymocytes (as concluded from the findings indicating changes in the amount of NA and TH, the major regulator of CA synthesis, per thymocyte). There are several lines of evidence to explain the reduced fluorescence in autofluorescent nerve fibres. Firstly, it was shown that 15 days postadrenalectomy the concentration of NA was decreased in the rat hypothalamus (Rastogi & Singhal, 1978), which is the site of the group of neurons involved in the regulation of thymic sympathetic nervous fibre activity (Trotter *et al.* 2007) and that administration of corticosterone to Adx rats effectively reversed the decrease in the concentration of this neurotransmitter (Rastogi & Singhal, 1978). Secondly, there is evidence that in the rat superior cervical ganglion, where noradrenergic fibres innervating the thymus originate (Tollefson & Bulloch, 1990), preganglionic nerve stimulation elicited an increase in TH expression and activity (Biguet *et al.* 1989). Furthermore, there are data suggesting a direct stimulatory influence of dexamethasone on the TH synthesis in organ

cultures of rat superior cervical ganglia (Nagaiah *et al.* 1977).

The decreased density of autofluorescent nerve fibres also contributed to the decrease in NA concentration in large thymi from Adx rats. This finding suggested that thymic hypertrophy was not followed by proportional growth of autofluorescent CA-containing nerve fibres. This could be related to data showing

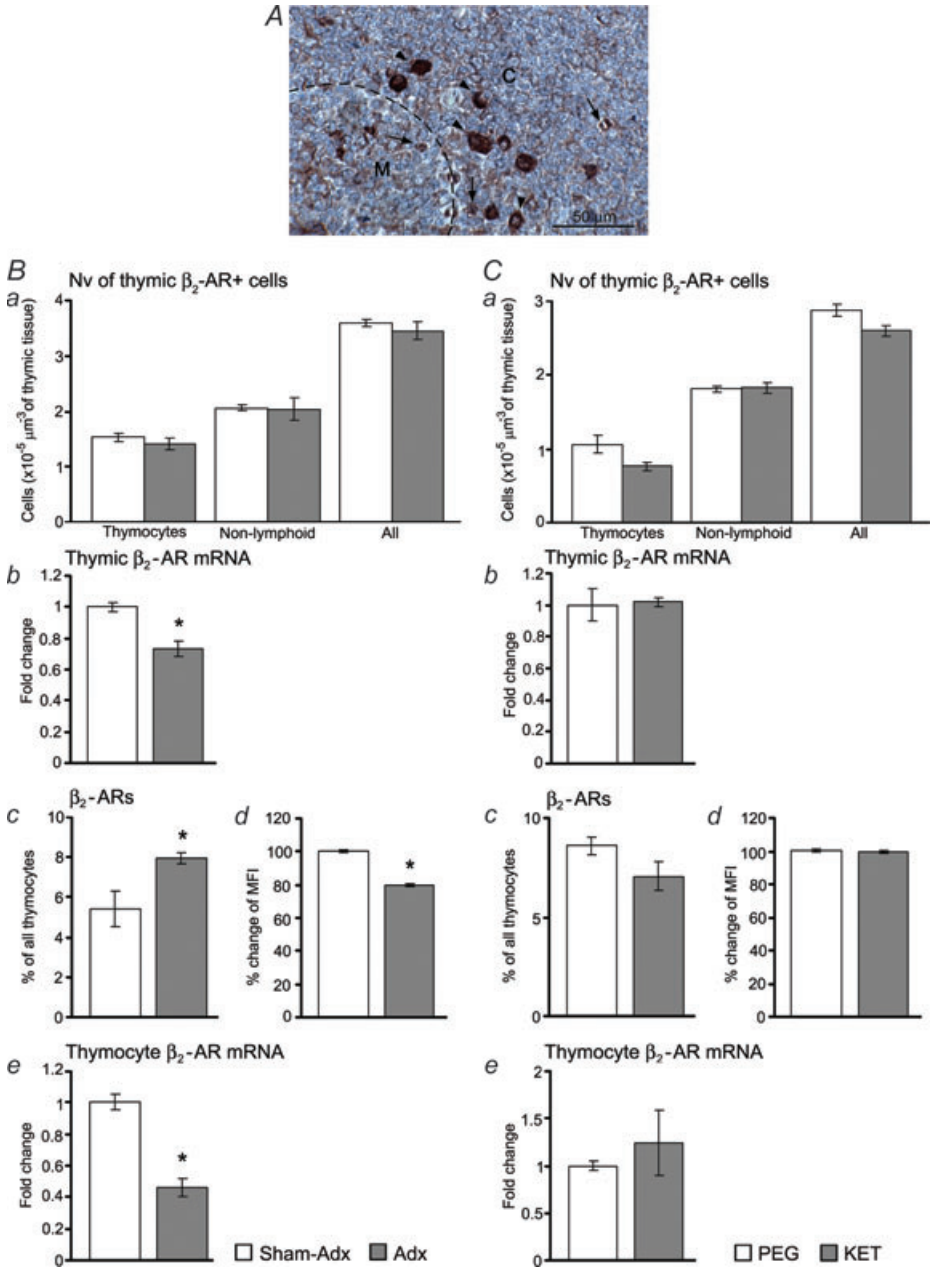

Figure 5. Influence of adrenal- and thymus-derived glucocorticoids on β_2-adrenoceptor (AR) expression in thymus and thymocytes

A, representative photomicrograph illustrates β_2-AR-positive thymocytes (arrows) and thymic non-lymphoid cells (arrowheads) in thymi from sham-adrenalectomized (Adx) rats. Original magnification ×400. Abbreviations: C, cortex; and M, medulla. Dashed black line indicates the cortico-medullary border. The histograms indicate the numerical density (Nv) of thymocytes, thymic non-lymphoid cells and all thymic cells expressing β_2-AR (*a*), the thymic β_2-AR mRNA expression (*b*), the percentage of β_2-AR-positive thymocytes (*c*), the β_2-AR mean fluorescence intensity (MFI) on β_2-AR-positive thymocytes (*d*) and the β_2-AR mRNA level (*e*) in thymocyte suspensions from Adx and sham-Adx rats (*B*) and KET- and PEG-treated Adx and orchidectomized rats (*C*). Results are expressed as means ± SEM ($n = 6$). $^*P < 0.05$.

that: (i) adrenalectomy decreases the level of nerve growth factor, a potent axon growth factor for neuronal populations in the peripheral nervous system, in adult rats (Aloe, 1989); and (ii) rat thymic cells express nerve growth factor (Lee *et al.* 2007).

We found an unaltered intensity of fluorescence of large subcapsular and medullar autofluorescent cells, which are most likely to represent subsets of CA-synthesizing TECs and macrophages (Pilipović *et al.* 2008). Although thymic macrophages, in particular those at the

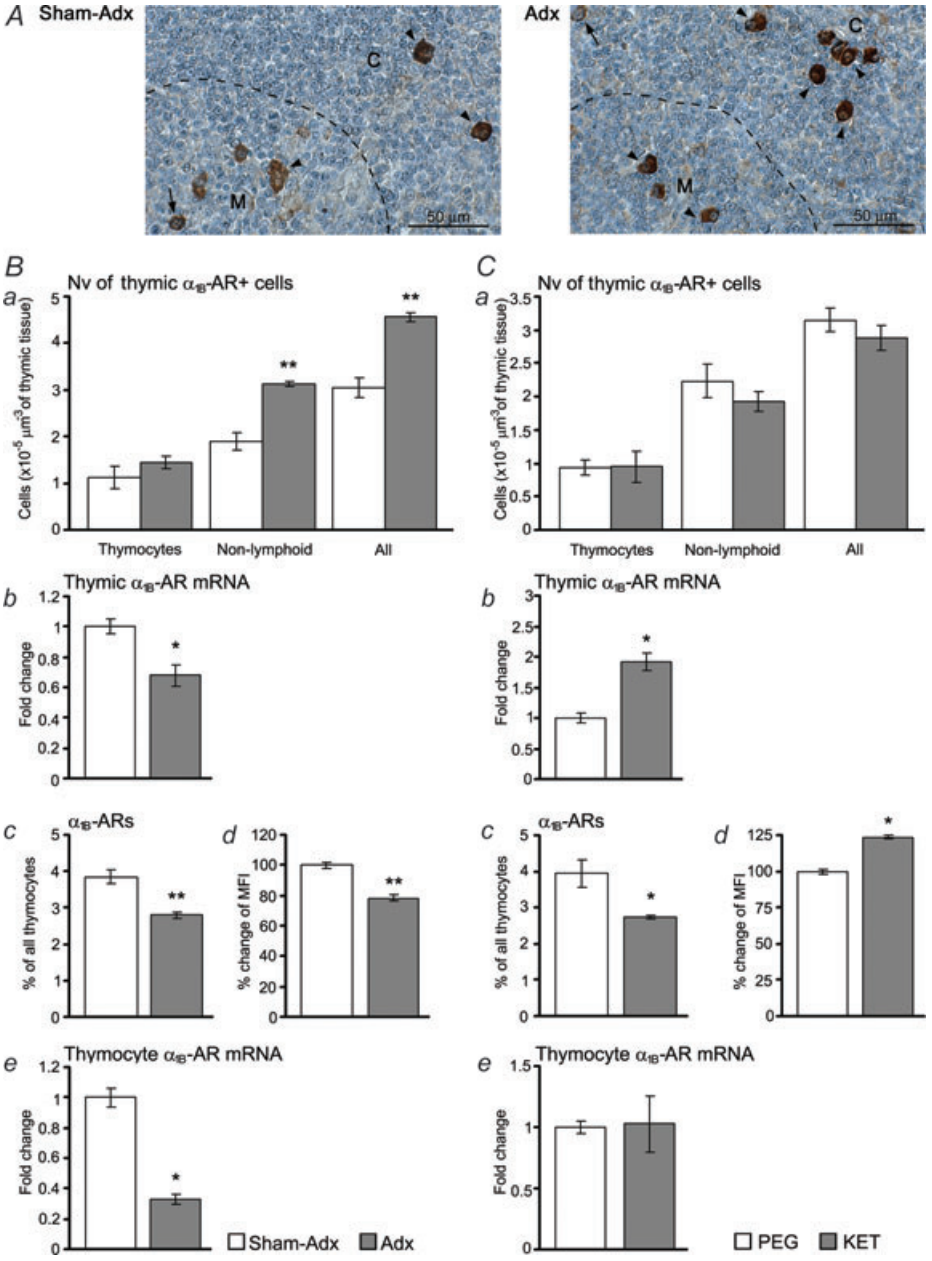

Figure 6. Influence of adrenal- and thymus-derived glucocorticoids on α_{1B}-AR expression in thymus and thymocytes

A, representative photomicrographs illustrating α_{1B}-AR-positive thymocytes (arrows) and thymic non-lymphoid cells (arrowheads) in thymi from adrenalectomized (Adx) and sham-Adx rats. Original magnification ×400. Abbreviations: C, cortex; and M, medulla. Dashed black lines indicate cortico-medullary borders. The histograms indicate numerical density (Nv) of thymocytes, thymic non-lymphoid cells and all thymic cells expressing α_{1B}-AR (*a*), thymic α_{1B}-AR mRNA expression (*b*), the percentage of α_{1B}-AR-positive thymocytes (*c*), the α_{1B}-AR mean fluorescence intensity (MFI) on α_{1B}-AR+ thymocytes (*d*) and the α_{1B}-AR mRNA level (*e*) in thymocyte suspensions from Adx and sham-Adx rats (*B*) and KET- and PEG-treated Adx and orchidectomized rats (*C*). Results are expressed as means ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$.

cortico-medullary junction, display autofluorescence due to a significant lipofuscin content (Milićević *et al*. 1986), the lack of significant difference in the intensity of cellular autofluorescence between control thymic sections (cryosections which were coverslipped only with non-fluorescent immersion oil) from Adx and control rats (data not shown) suggests that adrenalectomy did not influence the CA content in thymic stromal cells. This finding could be associated with the data indicating that adrenalectomy has a differential effect on GC synthesis in thymocytes and TECs (Qiao *et al.* 2009; Taves *et al.* 2011). Thus, adrenalectomy, acting via ACTH-dependent mechanisms, decreases and increases GC synthesis in thymocytes and TECs, respectively (Qiao *et al.* 2009; Taves *et al.* 2011). It may therefore be assumed that the increased local production of GCs by TECs was sufficient to prevent adrenalectomy-induced changes in the expression of the CA biosynthetic enzyme in thymic non-lymphoid cells, and consequently in their NA synthesis and content. This also implies a differential glucocorticoid-dependent regulation of NA content in thymic lymphoid and non-lymphoid cells.

In addition, we showed that adrenalectomy downregulated β_2-AR mRNA expression in thymic tissue and, in thymocytes in particular. The diminished β_2-AR MFI on β_2-AR-positive thymocytes indicated a decrease in the receptor surface density in Adx rats. To corroborate these findings, there are data suggesting that GCs increase β_2-AR expression in thymocytes and lymphocytes, as well as in some other cell types (Jazayeri & Meyer, 1988; Abraham *et al.* 2003). Considering that NA augments α_{1B}-AR expression via a β-AR-mediated mechanism (Kavelaars, 2002), the diminished thymocyte surface β_2-AR expression, coupled with the decrease in thymic NA level and thymocyte NA content, could explain the diminished α_{1B}-AR surface density and α_{1B}-AR mRNA level in thymocytes from Adx rats. Considering the decrease in the thymic α_{1B}-AR mRNA expression in Adx rats in conjunction with the substantial increase in Nv of α_{1B}-AR-ir cells, the reduced amount of α_{1B}-AR mRNA per thymocyte with unaltered Nv of α_{1B}-AR-ir thymocytes and the markedly reduced Nv of α_{1B}-AR-ir thymocytes compared with α_{1B}-AR-ir non-lymphoid cells, a diminished expression of α_{1B}-AR mRNA in non-lymphoid cells from Adx rats may also be supposed. The subsets of TECs, macrophages and dendritic cells have been shown to express α_{1B}-ARs (Leposavić *et al.* 2011).

Effect of ketoconazole inhibition of thymic steroid synthesis and action on thymic CA-containing nerve fibres and cells and AR expression in Adx and orchidectomized male Dark Agouti rats

This set of experiments showed that KET-induced withdrawal of thymic GCs in the absence of systemic GCs increased the thymic expression of NA biosynthetic enzymes and NA concentration. This could be associated with the rise in the density of CA-containing nerve fibres and the rise in the fluorescence intensity of large autofluorescent cells, which is most likely to reflect an increase in their CA content. Given that thymic NA concentration was increased in KET-treated rats compared with control animals, it seems obvious that in these animals the increase in nerve fibre density and non-lymphoid cell NA content was more pronounced than the decrease in the thymocyte NA content. The differential effect of KET on CA content in thymocytes and thymic non-lymphoid cells was consistent with the findings from the first set of experiments. Furthermore, considering the data from both sets of experiments it may be assumed that thymic GCs diminish CA (NA) synthesis in TECs, which is different from their effect in thymocytes. In favour of this option are data showing that GCs influence the expression of enzymes involved in CA biosynthesis and degradation in a tissue- and cell-type-specific manner (Hiwatashi *et al.* 2002; Kano *et al.* 2005; Lindley *et al.* 2005; Kvetnansky *et al.* 2009).

The expression of β_2-AR mRNA measured in thymic tissue and in thymocytes was not affected by inhibition of local GC synthesis. However, the expression of α_{1B}-AR at both mRNA and protein levels per thymocyte and, most probably, α_{1B}-AR mRNA in thymic non-lymphoid cells, was upregulated. In light of our previous data indicating that chronic blockade of α_1-ARs increases thymic weight and cellularity (Leposavić *et al.* 2011), the increased α_{1B}-AR expression on thymic cells and the elevated thymic NA level in KET-treated rats were fully consistent with the decline in thymic weight and thymocyte number in these rats. The increase in α_{1B}-AR expression following removal of the local GC synthesis in rats deprived of adrenal GCs could be linked with the data showing that the gene for α_{1B}-AR contains a GC response element within its promoter region (Gao & Kunos, 1993) and that adrenalectomy increases α_{1B}-adrenoceptor mRNA levels in the rat hypothalamic paraventricular nucleus (Day *et al.* 1999). Alternatively, it may be ascribed to augmented stimulation of β-ARs in thymic settings enriched with NA (Kavelaars, 2002).

Conclusion

In conclusion, the study provided novel evidence for the following: (i) a modulatory role of GCs in the expression of genes encoding enzymes involved in CA synthesis and metabolism and AR synthesis; (ii) thymic cell-type-specific GC regulation of the mRNA expression of CA synthetic and degrading enzymes; and (iii) local GC synthesis in the thymus. It also indicates a putative role of local GCs in modulation of thymic CA synthesis

and bioavailability and AR expression. Therefore, CAs may be viewed as mediators in GC-mediated tuning of thymopoiesis and, consequently, the T-cell compartment, suggesting that pharmacological modulation of CA action may modulate GC-dependent effects. The data on the differential effects of β-AR blockade in Adx and non-Adx rats support this concept. This may be particularly important when homeostasis is disturbed by various stressors or in the state of adrenal insufficiency.

References

Abraham G, Schusser GF & Ungemach FR (2003). Dexamethasone-induced increase in lymphocyte beta-adrenergic receptor density and cAMP formation in vivo. *Pharmacology* **67**, 1–5.

Aloe L (1989). Adrenalectomy decreases nerve growth factor in young adult rat hippocampus. *Proc Natl Acad Sci U S A* **86**, 5636–5640.

Ashwell JD, King LB & Vacchio MS (1996). Cross-talk between the T cell antigen receptor and the glucocorticoid receptor regulates thymocyte development. *Stem Cells* **14**, 490–500.

Biguet NF, Rittenhouse AR, Mallet J & Zigmond RE (1989). Preganglionic nerve stimulation increases mRNA levels for tyrosine hydroxylase in the rat superior cervical ganglion. *Neurosci Lett* **104**, 189–194.

Boldizsar F, Talaber G, Szabo M, Bartis D, Palinkas L, Nemeth P & Berki T (2010). Emerging pathways of non-genomic glucocorticoid (GC) signalling in T cells. *Immunobiology* **215**, 521–526.

Chrousos GP & Gold PW (1992). The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *JAMA* **267**, 1244–1252.

Chung H, Choi YI, Ko MG & Seong RH (2002). Rescuing developing thymocytes from death by neglect. *J Biochem Mol Biol* **35**, 7–18.

Cima I, Corazza N, Dick B, Fuhrer A, Herren S, Jakob S, Ayuni E, Mueller C & Brunner T (2004). Intestinal epithelial cells synthesize glucocorticoids and regulate T cell activation. *J Exp Med* **200**, 1635–1646.

Davies AO & Lefkowitz RJ (1980). Corticosteroid-induced differential regulation of β-adrenergic receptors in circulating human polymorphonuclear leukocytes and mononuclear leukocytes. *J Clin Endocrinol Metab* **51**, 599–605.

Day HE, Campeau S, Watson SJ Jr & Akil H (1999). Expression of α_{1b} adrenoceptor mRNA in corticotropin-releasing hormone-containing cells of the rat hypothalamus and its regulation by corticosterone. *J Neurosci* **19**, 10098–10106.

Deuschle M, Lecei O, Stalla GK, Landgraf R, Hamann B, Lederbogen F, Uhr M, Luppa P, Maras A, Colla M & Heuser I (2003). Steroid synthesis inhibition with ketoconazole and its effect upon the regulation of the hypothalamus–pituitary–adrenal system in healthy humans. *Neuropsychopharmacology* **28**, 379–383.

Elenkov IJ, Wilder RL, Chrousos GP & Vizi ES (2000). The sympathetic nerve– an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev* **52**, 595–638.

Felten DL, Felten SY, Carlson SL, Olschowka JA & Livnat S (1985). Noradrenergic and peptidergic innervation of lymphoid tissue. *J Immunol* **135**, 755s–765s.

Gao B & Kunos G (1993). Isolation and characterization of the gene encoding the rat alpha 1B adrenergic receptor. *Gene* **131**, 243–247.

Hiwatashi Y, Kurahashi Y, Hatada R, Ueno S, Honma T, Yanagihara N, Yanase H, Iwanaga T, Ohizumi Y & Yamakuni T (2002). Glucocorticoid inhibits expression of V-1, a catecholamine biosynthesis regulatory protein, in cultured adrenal medullary cells. *FEBS Lett* **528**, 166–170.

Jazayeri A & Meyer WJ 3rd (1988). Glucocorticoid modulation of β-adrenergic receptors of cultured rat arterial smooth muscle cells. *Hypertension* **12**, 393–398.

Kano Y, Otsuka F, Takeda M, Suzuki J, Inagaki K, Miyoshi T, Miyamoto M, Otani H, Ogura T & Makino H (2005). Regulatory roles of bone morphogenetic proteins and glucocorticoids in catecholamine production by rat pheochromocytoma cells. *Endocrinology* **146**, 5332–5340.

Karapetrovic B, Micic M & Leposavic G (1995). Stereological analysis of the sexually mature rat thymus after orchidectomy. *Indian J Med Res* **102**, 42–48.

Kavelaars A (2002). Regulated expression of α-1 adrenergic receptors in the immune system. *Brain Behav Immun* **16**, 799–807.

Kvetnansky R, Sabban EL & Palkovits M (2009). Catecholaminergic systems in stress: structural and molecular genetic approaches. *Physiol Rev* **89**, 535–606.

Lee HW, Kim SM, Shim NR, Bae SK, Jung IG, Kwak JY, Kim BS, Kim JB, Moon JO, Chung JS & Yoon S (2007). Expression of nerve growth factor is upregulated in the rat thymic epithelial cells during thymus regeneration following acute thymic involution. *Regul Pept* **141**, 86–95.

Leposavić G, Mićić M, Ugresić N, Bogojević M & Isaković K (1992). Components of sympathetic innervation of the rat thymus during late fetal and postnatal development: histofluorescence and biochemical study. Sympathetic innervation of the rat thymus. *Thymus* **19**, 77–87.

Leposavić G, Pilipović I & Perišić M (2011). Age-associated remodeling of neural and nonneural thymic catecholaminergic network affects thymopoietic productivity. *Neuroimmunomodulation* **18**, 290–308.

Lindley SE, She X & Schatzberg AF (2005). Monoamine oxidase and catechol-*o*-methyltransferase enzyme activity and gene expression in response to sustained glucocorticoids. *Psychoneuroendocrinology* **30**, 785–790.

Loose DS, Stover EP & Feldman D (1983). Ketoconazole binds to glucocorticoid receptors and exhibits glucocorticoid antagonist activity in cultured cells. *J Clin Invest* **72**, 404–408.

Lucas CA & Thoenen H (1977). Selective induction by glucocorticoids of tyrosine hydroxylase in organ cultures of rat pheochromocytoma. *Neuroscience* **2**, 1095–1101.

McMahon A & Sabban EL (1992). Regulation of expression of dopamine beta-hydroxylase in PC12 cells by glucocorticoids and cyclic AMP analogues. *J Neurochem* **59**, 2040–2047.

Manoli I, Le H, Alesci S, McFann KK, Su YA, Kino T, Chrousos GP & Blackman MR (2005). Monoamine oxidase-A is a major target gene for glucocorticoids in human skeletal muscle cells. *FASEB J* **19**, 1359–1361.

Milićević NM, Milićević Z & Mujović S (1986) Histochemical characterization of the lipid content in the macrophages of the cortico-medullary zone of the rat thymus. *Anat Histol Embryol* **15**, 355–360.

Nagaiah K, MacDonnell P & Guroff G (1977). Induction of tyrosine hydroxlase synthesis in rat superior cervical ganglia in vitro by nerve growth factor and dexamethasone. *Biochem Biophys Res Commun* **75**, 832–837.

Ou XM, Chen K & Shih JC (2006). Glucocorticoid and androgen activation of monoamine oxidase A is regulated differently by R1 and Sp1. *J Biol Chem* **281**, 21512–21525.

Pilipović I, Kosec D, Radojević K, Perisić M, Pesic V, Stojić-Vukanić Z & Leposavi G (2010). Glucocorticoids, master modulators of the thymic catecholaminergic system? *Braz J Med Biol Res* **43**, 279–284.

Pilipović I, Vidić-Danković B, Perisić M, Radojević K, Colić M, Todorović V & Leposavić G (2008). Sexual dimorphism in the catecholamine-containing thymus microenvironment: a role for gonadal hormones. *J Neuroimmunol* **195**, 7–20.

Qiao S, Okret S & Jondal M (2009). Thymocyte-synthesized glucocorticoids play a role in thymocyte homeostasis and are down-regulated by adrenocorticotropic hormone. *Endocrinology* **150**, 4163–4169.

Radojević K, Kosec D, Perišić M, Pilipović I, Vidić-Danković B & Leposavić G (2011). Neonatal androgenization affects the efficiency of β-adrenoceptor-mediated modulation of thymopoiesis. *J Neuroimmunol* **239**, 68–79.

Rastogi RB & Singhal RL (1978). Evidence for the role of adrenocortical hormones in the regulation of noradrenaline and dopamine metabolism in certain brain areas. *Br J Pharmacol* **62**, 131–136.

Sabban EL (1997). Control of tyrosine hydroxylase gene expression in chromaffin and PC12 cells. *Semin Cell Dev Biol* **8**, 101–111.

Sacedón R, Vicente A, Varas A, Jiménez E, Muñoz JJ & Zapata AG (1999). Glucocorticoid-mediated regulation of thymic dendritic cell function. *Int Immunol* **11**, 1217–1224.

Stojić-Vukanić Z, Rauski A, Kosec D, Radojević K, Pilipović I & Leposavić G (2009). Dysregulation of T-cell development in adrenal glucocorticoid-deprived rats. *Exp Biol Med (Maywood)* **234**, 1067–1074.

Talaber G, Kvell K, Varecza Z, Boldizsar F, Parnell SM, Jenkinson EJ, Anderson G, Berki T & Pongracz JE (2011). Wnt-4 protects thymic epithelial cells against dexamethasone-induced senescence. *Rejuvenation Res* **14**, 241–248.

Taves MD, Gomez-Sanchez CE & Soma KK (2011). Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. *Am J Physiol Endocrinol Metab* **301**, E11–E24.

Tollefson L & Bulloch K (1990). Dual-label retrograde transport: CNS innervation of the mouse thymus distinct from other mediastinum viscera. *J Neurosci Res* **25**, 20–28.

Trotter RN, Stornetta RL, Guyenet PG & Roberts MR (2007). Transneuronal mapping of the CNS network controlling sympathetic outflow to the rat thymus. *Auton Neurosci* **131**, 9–20.

Weigent DA & Blalock JE (1997). Production of peptide hormones and neurotransmitters by the immune system. *Chem Immunol* **69**, 1–30.

Wiegers GJ, Knoflach M, Böck G, Niederegger H, Dietrich H, Falus A, Boyd R & Wick G (2001). CD4(+)CD8(+)TCR(low) thymocytes express low levels of glucocorticoid receptors while being sensitive to glucocorticoid-induced apoptosis. *Eur J Immunol* **31**, 2293–2301.

Acknowledgements

This work was supported by grant no. 175050 from the Ministry of Education and Science of the Republic of Serbia.

Supporting Information

The following supporting information is available in the online version of this article.

Figure S1. Influence of adrenal- and thymic-derived glucocorticoids on noradrenaline (NA) level in (a) thymi and (b) thymocyte suspensions of (Panel A) adrenalectomised (Adx) and sham-Adx rats and (panel B) Ketoconazole- (KET) and polyethylene glycol-administered (PEG) Adx and orchidectomised rats. Arrows indicate peaks corresponding to NA.

Figure S2. Influence of adrenal- and thymic-derived glucocorticoids on thymocyte expression of (a) tyrosine hydroxylase (TH), (b) β_2-adrenoceptor (AR) and (c) α_{1B}-AR in (Panel A) adrenalectomised (Adx) and sham-Adx rats and (panel B) Ketoconazole- (KET) and polyethylene glycol-administered (PEG) Adx and orchidectomised rats, as determined by flow cytometry analysis.