ELSEVIER

Journal of Neuroimmunology 158 (2005) 67–75

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Ontogenesis and functional aspects of oxytocin and vasopressin gene expression in the thymus network

I. Hansenne[a,1], G. Rasier[a,1], C. Péqueux[a], F. Brilot[a], Ch. Renard[a], C. Breton[b], R. Greimers[a], J.-J. Legros[a], V. Geenen[a,*], H.J. Martens[a]

[a]*Liege University Center of Immunology, Institute of Pathology CHU-B23 Neuroendocrine-Immunology and Embryology, B-4000, Liege-Sart Tilman, Belgium*
[b]*Neuroendocrinology of Development, Physiology Department, UPRES-EA 2701, Lille Science and Technology University, F-59655 Villeneuve d'Ascq cedex, France*

Received 23 April 2004; received in revised form 11 August 2004; accepted 12 August 2004

Abstract

Ontogenesis of oxytocin (*OT*) and vasopressin (*VP*) gene expression and function were investigated in murine thymus. *OT* and *VP* transcripts were detected in the thymus on embryonic days 13 and 15, respectively. Corresponding messenger RNAs were evidenced in thymic epithelial cells by in situ hybridization with a neurophysin probe. From all OT and VP receptors, only *OTR* was expressed by all T-cell subsets, while *V1bR* was found in double positive and single positive CD8 cells. In fetal thymic organ cultures, OTR antagonist d[D-Tyr(Et)^2, Thr^4]OVT increased early apoptosis of CD8 cells, while V1bR antagonist (Sanofi SSR149415) inhibited T-cell differentiation, and favored CD8 T-cell commitment.

Keywords: Thymus; Oxytocin; Vasopressin; Receptors; T-cell differentiation; Fetal thymic organ cultures

1. Introduction

The thymus exerts a fundamental role both in the establishment of T-cell self-tolerance by deletion of self-reactive T cell clones and generation of self-antigen specific $CD4^+CD25^+$ regulatory T cells, as well as in the development of major histocompatibility complex (MHC)-restricted competent and self-tolerant T cells (thymopoiesis) (Geenen et al., in press). An important factor leading to T-cell death or development is the density of the natural ligands (peptide/MHC complexes) expressed in the thymus and presented to pre-T cells (Ashton-Rickardt et al., 1994; Sebzda et al., 1999). Stochastic recombination of T-cell antigen receptor (TCR) chain genes generates a repertoire of TCR potentially reactive to a wide spectrum of self-antigens. Cells that express a TCR unable to recognize any self-peptide/MHC complexes die by neglect. T-cell clonal deletion results from high-affinity TCR ligation to self-peptide/MHC complexes at high density (negative selection). On the contrary, TCR recognition of self-peptide/MHC complexes at low density, or a high density of low affinity interactions, mediates T-cell positive selection.

Our understanding of thymic physiology highly progressed with the demonstration that thymic stromal cells transcribe a repertoire of neuroendocrine-related and other peripheral tissue antigen-encoding genes (Geenen et al., 1995; Kyewski et al., 2002). Thymic neuroendocrine precursors play a dual role in T-cell selection according to their processing either as self-antigens for T-cell negative selection, or as accessory ligands for the development of self-tolerant T cells (Martens et al., 1996a). The repertoire of thymic neuroendocrine precursors expressed is organized in such a way that one dominant member per family is expressed in the thymus. Thymic neuroendocrine self-antigens usually correspond to sequences highly conserved throughout evolution of one given family: oxytocin (OT) vs.

* Corresponding author. Tel.: +32 43 66 25 50; fax: +32 43 66 29 77.
E-mail address: vgeenen@ulg.ac.be (V. Geenen).
[1] Both authors contributed equally to this work.

0165-5728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2004.08.007

vasopressin (VP) for the family of related hormones, neurokinin A vs. substance P for tachykinins (Ericsson et al., 1990), and insulin-like growth factor 2 (IGF-2) vs. IGF-1 and proinsulin for the insulin family (Geenen et al., 1993; Martens et al., 1996a).

Hypothalamic magnocellular neurons are the major site of *OT* and *VP* expression (for a complete review, see Gainer and Wray, 1993). These genes encode 16- and 20-kDa precursors, respectively, that are processed during their axonal transport towards neurohypophysis into active nonapeptides OT and VP, and their specific carriers 10-kDa neurophysins. Though they only differ by two amino acids, the physiological actions of these two peptides are completely different. After binding to a specific receptor (OTR), OT triggers milk ejection and intervenes in the contractility of uterine myometrium during parturition. VP inhibits water diuresis through kidney VP type 2 receptors (V2R), contributes to the regulation of vascular tone through V1a receptors (V1aR), and stimulates secretion of adrenocorticotropin (ACTH) through V1b receptors (V1bR). As a source of hypothalamus-specific antigens, *OT* and *VP* are also expressed in TEC from different species, although with a dominance of OT at the peptide level. Thymic OT is not secreted but targeted to the outer surface of TEC plasma membrane where it is recognized by specific monoclonal antibodies (mAbs) (Martens et al., 1996b). Specific and functional receptors for these peptides are expressed by human thymocytes and a murine double negative (DN) $CD4^{-}CD8^{-}$ pre-T cell line RL12-NP (Martens et al., 1992). After binding to their cognate receptors, OT and VP act as mitogenic signals through activation of the phosphoinositide pathway in T cells (Elands et al., 1990; Martens et al., 1992). In addition, a rapid phosphorylation of $p125^{FAK}$ in murine pre-T cells follows their treatment with OT and VP, in particular with OT (Martens et al., 1998). Thus, thymic OT constitutes an early activation signal for immature T cells and promotes focal adhesions to TEC during their early differentiation in the thymus. However, the precise nature of the receptors expressed by T-cell subsets remains undefined, and previous studies have suggested that more than one receptor could be implicated (Martens et al., 1992; Torres and Johnson, 1988). The identification of those receptors based on transduction mechanisms and biochemical events is further intricate by the fact that some of these receptors poorly discriminate their ligands. For example, OT drives ACTH release from rat pituitary after binding to V1bR (Schlosser et al., 1994).

The main objectives of this study were to compare the ontogenesis of *OT* and *VP* expression in murine brain and thymus, to determine the distribution of *OTR*, *V1aR*, *V1bR* and *V2R* in murine thymic T cell subsets, as well as to investigate the effects of the blockade of OT- and VP-mediated signaling upon T-cell differentiation and apoptosis.

2. Materials and methods

2.1. Cells and tissue collection

Balb/c and C57BL6/Ka mice were mated overnight. Plugged females were considered as positive and separated in the morning of the so-called embryonic day 0 (E0). Matched thymi and brains were collected from E13 to E20, and on post-natal day 2. Thymi were used for fetal thymic organ cultures (FTOC), as described by Plum et al. (1995), for total RNA extraction, or were embedded in OCT (Sakura, Torrance, CA) for in situ hybridization. The experimental procedures were carried out in accordance with the Ethical Committee on Animal Experimentation at the University of Liege.

FTOC were grown for 7 days in 1-ml medium (IMDM, penicillin 50 IU/ml, streptomycin 50 ng/ml, FCS 10%, L-Glutamine 2 mM, NEAA 1%, HEPES, 1%, Na Pyruvate 1%) (Cambrex) in basal conditions or in presence of d[D-$Tyr(Et)^2$, Thr^4]OVT (Atosiban), a specific OTR antagonist (1 μM) kindly provided by Maurice Manning (Medical College of Ohio, Toledo) (Manning et al., 1995), and SSR 149415, a specific V1b antagonist (1 μM) from Sanofi-Synthelabo (Montpellier, France) (Serradeil-Le Gal et al., 2002). Control cultures were performed in basal medium, or added with 0.002% DMSO. For the analysis of antagonist effects, medium was supplemented each day of the culture with a 20 μl of 50-fold concentrated tested antagonist solutions and with 0.002% DMSO for matched controls.

The type of receptor expressed by thymic T cell subpopulations was analyzed from thymic lobes surgically removed from 8-week-old female C57BL6/Ka mice. They were cut in small pieces, filtered and washed in DPBS buffer. After three successive incubations (15 min, 4 °C), thymocyte suspension was incubated with a mAb cocktail prepared for negative selection of CD4 and CD8 T cells (StemCell Technologies). DN cells were obtained by combination of procedures. DP cells were >90% in the crude suspension. After washing in DPBS buffer, an anti-biotin tetrameric Ab complex mix (StemCell Technologies) was added. The last incubation was performed with a magnetic colloid (StemCell Technologies). Separation was realized with a MACS column (Miltenyi Biotec). This pre-purification step (from 70% to 80% purity) allows a FACS sampling of highly purified DN, DP, $CD4^{+}$ and $CD8^{+}$ subpopulations (>95%).

2.2. RNA preparation, cDNA synthesis and PCR amplification

Total RNA from 4-week-old mice hypothalamus was used as positive control for *OT* and *VP* expression. RT-PCR was performed using Ready-to-Go beads (Amersham-Pharmacia Biotech, The Netherlands). Fifty nanograms of total RNA extracted with RNeasy Mini Kit (Qiagen), DNAse I-treated, from hypothalamus and fetal thymic lobes

were transcribed in cDNA 30 min at 42 °C with random hexamers, denatured at 94 °C for 5 min and amplified by 35 PCR cycles of 45 s at 94 °C; 45 s at 66 °C, 90 s at 72 °C and a final 10-min extension at 72 °C with 10 pmol of specific *OT* and *VP* primers (Table 1). To ensure mRNA quality and similar loading in each sample, β-actin mRNA was amplified in the same conditions, except that annealing temperature was 54 °C (Pleau et al., 1996). Alternatively, *OT* and *VP* nested-PCR was performed with 1:20 dilution of RT-products from 250 ng total RNA transcribed with AMV (Roche, Belgium), followed by 35 PCR cycles with *OT** and *VP** primers (Table 1, annealing temperature=62 and 64 °C, respectively) and a second step of 35-cycles PCR with *OT* and *VP* primers and 1:5000 dilution of first step products, both with 2 IU of FastStart Taq polymerase (Roche). RT-PCR products were subcloned in pCR2.1-TOPO plasmid (Invitrogen) and sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit protocol (Perkin Elmer) to ensure *OT* and *VP* identity.

OT and *VP* expression in the thymus during FTOC was investigated by RT-PCR followed by Southern blotting. The amplified RT-PCR products were separated by 2% gel agarose, transferred on membrane (GeneScreen-Plus, NEN, Belgium), and hybridized with the corresponding internal oligonucleotide probes. Oligonucleotides (50 ng) were labeled with [γ ^{32}P] dATP (5 μl, 4500 Ci/mmol) using T_4 kinase (7.9 IU) (Pharmacia), then purified on BIO-RAD Micro Bio-spin Column P-30 Tris, RNase-Free. Gene Screen Plus membranes were prehybridized (2 h, 60 °C) in SSC (5×), Na_3PO_4 (20 mM), Denhardt solution (50×), SDS (7%) and salmon sperm DNA (0.1 ng/ml). This was followed by hybridization (12 h, 60 °C) in prehybridization solution+dextran sulfate (20%) with labeled OT ($\approx 10^6$ cpm/ml) or VP ($\approx 10^6$ cpm/ml) probe. Membranes were washed in SSC (5×) (30 min, 60 °C) and in SSC (2×) (30 min, 60 °C). Autoradiographies were developed after a 2-day exposure at −70 °C.

For receptor analyses, positive controls were RNA from adult mice uterus (*OTR*), liver (*V1aR*), kidney (*V2R*) and anterior pituitary (*V1bR*). Milli-Q quality water was used as negative control. Total RNA from murine uterus, liver, kidney and anterior pituitary was extracted with TriPure Isolation Reagent (Boerhinger Mannheim). Total RNA from purified thymocytes subsets was extracted with RNeasy Mini Kit (Qiagen). All were treated (15 min, 37 °C) with DNase I. RNA was quantified with Ribogreen RNA Quantitation Kit (Molecular Probes). Primers were designed from murine *OTR*, *V1aR*, *V2R*, *V1bR,* sequences (Table 1) and selected in different exons in order to exclude genomic DNA from amplification. Total RNA (50 ng) of each sample was reverse-transcribed with random hexamers (30 min, 42 °C). After denaturation (5 min, 94 °C), the samples were submitted to PCR with 35 cycles (for *OTR*, *V1aR* and *V2R*), or with 37 cycles (for *V1bR*). For each cycle, denaturation was 94 °C for 1 min (*OTR*, *V1aR*, *V2R* and *V1bR*),

Table 1
Sequences of oligonucleotides used for RT-PCR

	Forward primer	Reverse primer	Internal probe
OT*	TTGCTGCCTGCTTGGCTTAC [1]	CGCGCTAAAGGTATTCCCAG [3]	
OT	ACCTCGGCCTGCTACATCCAGAA [1]	ACTGGCAGGGCGAAGGCAGGTA [2]	CCCCTGGGCGGCAAGAGGCTGTGCTGGACCTGGATATGC
VP*	TGCTCGCCAGGATGCTCAACAC [1]	TAGACCCGGGGCTTGGCAGAA [3]	
VP	GGCATCTGCTGCAGCGACGAGA [2/3]	TAGACCCGGGGCTTGGCAGAA [3]	CACAGCTGGACGGCCCTGCTCGGGCGCTGCTGCTAAGGCT
OTR	CTGGAACGTCAATGCGCCCAAAGAAG [3]	CATGCCGAGGATGGTTGAGAACAGCTC [4]	GAGACGAGCATTAGCAAGAAAAGCAACTCCTCCACC
V1a R	TGGTCACGCCTTGTGTCAGCAGCGTGA [1]	GATTTAGGTGAATCCTTCCACGTCCCA [2]	GAAATTGGTATCCCAGACTGACCACATCTGGACGATGAAG
V1b R	CGGGTCAGCAGCATCAGTACCATCTCC [1]	GGAACGTGGCAACAGGTGGCTGTTGA [2]	AAGCAATGTAGGCCAGCACAATGACAAAGGTCATCTTC
V2 R	CCCTAGGCATTGCTGCCTGCC [2]	GAAGCTGGCTGTGGCACAGGACTCATCTTG [3]	CACCAGACTGGCATGTATCTCCCGGAAGATAAGAACC
β-Actin	TAAAGACCTCTATGCCAACACAGT	CACGATGGAGGGGCCGGACTCATC	

Exons where primers localized are given in brackets.

hybridization was 1 min at 56 °C (*OTR*, *V1aR* and *V2R*) and 64 °C (*V1bR*), elongation was 1 min (*OTR*, *V1aR*, *V2R*, *V1bR*) at 72 °C and a final 10-min extension at 72 °C. β-actin mRNA was amplified in the same conditions, excepted that annealing temperature was 54 °C. RT-PCR products were analyzed on 2% agarose gel with ethidium bromide. RT-PCR products were subcloned in pCR2.1-TOPO plasmid (Invitrogen) and sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit protocol (Perkin Elmer). Southern blotting was performed to ensure the specificity of the products. Receptor RT-PCR products were transferred on Hybond-XL membrane (Amersham Pharmacia Biotech). Oligonucleotide probes were synthesized in the internal sequence of the amplified products (Table 1) and labeled as described. Hybond XL membranes were prehybridized (2 h at 65 °C) in Denhardt solution (5×), SSC (5×) and SDS (0.5%) followed by hybridization (12 h, 65 °C) in the same solution with labeled probe ($\approx 10^6$ cpm/ml). Membranes were successively washed in a SSC solution (2×) and SDS (0.1%) (5 min), SSC (1×) and SDS (0.1%) (15 min), SSC (0.1×) and SDS (0.1%) (10 min). cDNA was visualized with PhosphorImager after exposition (2 h) or by autoradiography (−70 °C, 2-day exposition).

2.3. In situ hybridization

The probe derived from the complete coding sequence of human preprovasopressin (exon 2) cloned in the pGEM-3Z plasmid and linearized with *Eco*RI and *Bam*HI (Hara et al., 1990; Rehbein et al., 1986). One microgram of sense and antisense matrix riboprobes was synthesized, and 8-μm tissue sections were fixed and stained as described (Kecha et al., 2000).

2.4. Flow cytometry

After 7-day FTOC, cell suspensions were prepared and 5×10^5 cells were stained for 20 min at 4 °C. Quadruple staining was achieved with anti-CD4 coupled to phycoerythrin (PE) (Biosciences Pharmingen, clone GK 1.5), and anti-CD8 coupled to fluorescein isothiocyanate (FITC) Ab (Biosciences Pharmingen, clone 53–6.7), annexin V coupled to APC (Biosciences Pharmingen); propidium iodide (PI, Sigma) or 7-AAD (Biosciences Pharmingen) was added prior to lecture. For each sample, events were gated according to forward and side scatter profiles. Flow cytometry was performed using a FACVantage Plus Cell sorter (BD-Pharmingen) with a blue argon laser (488 nm) and with CellQuest software. Fluorescence was determined on 10,000 cells and stored in list mode data files.

2.5. Statistics

Results were expressed as mean±standard deviation (m±S.D.) of values after treatment vs. basal conditions. Differences between groups were estimated by nonparametric ANOVA Kruskal–Wallis test and analyzed by multicomparative Dunn's test.

3. Results

3.1. Ontogenesis of OT and VP transcription in Balb/c brain and thymus

We have extracted total RNA from brain and thymi of same mother's fetuses in order to compare *OT* and *VP* expression at the same age. By RT-PCR, *OT* transcripts were detected in the thymus and in the brain from E13 to post-natal day 2, while *VP* transcription was detected in the brain on E14, started in the thymus on E14, but was clearly observed on E15. A faint PCR product was detected on E13 with *OT* primers in one out of five brains and in five out of five thymus. To check specificity of the transcripts, a nested *OT* PCR was performed and revealed the presence of transcripts in the brain on E13. Nested PCR allowed detection of *VP* transcripts in the thymus and brain on E14 (Fig. 1). *OTR* transcripts were detected from E13 thymus and brain to post-natal day 2 with a decrease in the thymus and an increase in the brain after E17. Although PCR conditions were not quantitative, these data nevertheless indicates that *OT* transcription in the thymus slightly precedes or coincides the one observed in the central nervous system (CNS).

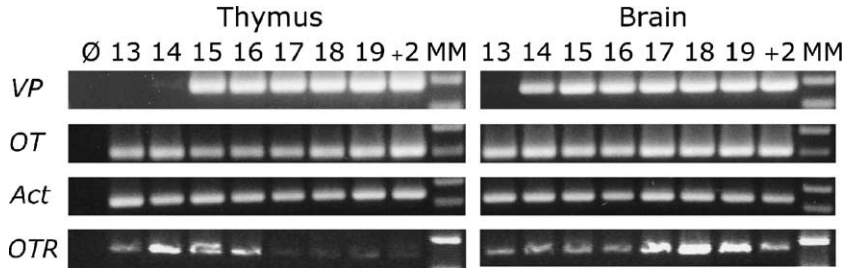

Fig. 1. RT-PCR amplification of *OT*, *VP* and *OTR* mRNA in murine thymi from E13–19 to post-natal day +2. RT-PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. The predicted sizes for the amplification bands using primer specific for *OT* are 211 bp, 188 bp for *VP* primers, 273 bp for *OTR* primers and 250 bp for *Act* (β-actin) primers. Thymic total RNA extracts are presented on the left panel and brain total RNA extracts on the right panel. MM=molecular markers EZ load, Ø=negative control (H_2O).

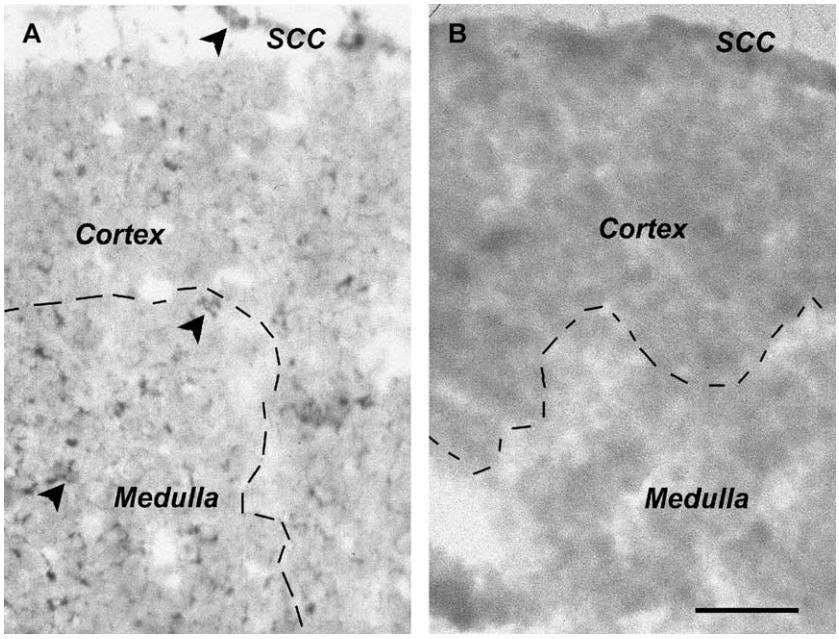

Fig. 2. RNA in situ hybridization of E18 murine thymus with neurophysin probe. Slices (magnification 200×) were labeled with neurophysin antisense (A) and sense (B) digoxigenin-labeled probe. Most of labeled cells are located in the subcapsular cortex (SCC) and in the medulla (arrows); a few positive cells are also stained in the cortex, whereas methyl-green reveals a majority of T-cell nuclei in the cortex. Dashed lines delineate boundary between cortex and medulla in a thymic lobule. Scale bar is 25 μm.

3.2. In situ hybridization

In the fetal murine thymus, specific transcripts were evidenced on E18 while no staining was detected with the sense probe used as a negative control (Fig. 2). The use of the neurophysin domain (exon 2) coding sequence allowed to stain and to localize both *OT* and *VP* transcripts. A staining of reticular cells was observed in the section. A staining of a thin layer in the subcapsular cortex also emerged from the background. The pattern of gene transcription in the thymus was restricted to the epithelial reticular network.

3.3. OT and VP receptor expression by thymic T-cell subsets

RT-PCR with *V1aR* and *V2R* primers revealed the presence of 409- and 437-bp products in liver and kidney, respectively, but failed to amplify any product in thymic T cells (Fig. 3). Hybridization with specific probes confirmed the absence of *V1aR* and *V2R* transcripts in all T cell samples (data not shown). Note that *V2R* primers allow amplification of genomic DNA at 593-bp in all samples and a *V2R* short transcript (±300 bp) in positive control. RT-PCR with *OTR* specific primers revealed the presence of a 273-bp product in the mouse uterus as positive control. A same sized RT-PCR product was detected in all thymic T cell subsets. Southern blotting of the PCR product and hybridization with a specific probe confirmed the presence of *OTR* transcripts in thymocytes (data shown). Sequencing of the 237-bp T-cell product confirmed homology with murine *OTR* sequence. RT-PCR with *V1bR* primers identified a 254-bp product in mouse anterior pituitary as positive control. A same sized product was similarly detected in thymic DP and $CD8^+$T cells, but never in DN nor in $CD4^+$. Southern blotting and hybridization with a specific probe confirmed *V1bR* expression in DP and $CD8^+$ thymic T cells (data not shown).

3.4. Fetal thymic organ cultures (FTOC)

RNA from thymic lobes was extracted at different days of FTOC. Hybridization was performed after RT-PCR amplification and southern blotting with specific *OT* and *VP* probes. Both *OT* and *VP* transcriptions were maintained

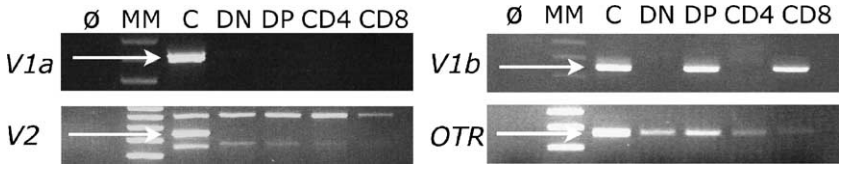

Fig. 3. OT and VP receptor (*OTR*, *V1aR*, *V1bR* and *V2R*) expression in T-cell subsets. RT-PCR of total RNA from murine T cell subsets. Ø: negative control; MM: molecular mass marker; C: positive control; DN: double negative $CD4^-CD8^-$ cells, DP: double positive $CD4^+CD8^+$ cells, CD4: single positive $CD4^+$ cells, CD8: single positive $CD8^+$ cells. Arrows indicate the RNA amplified products for each receptor.

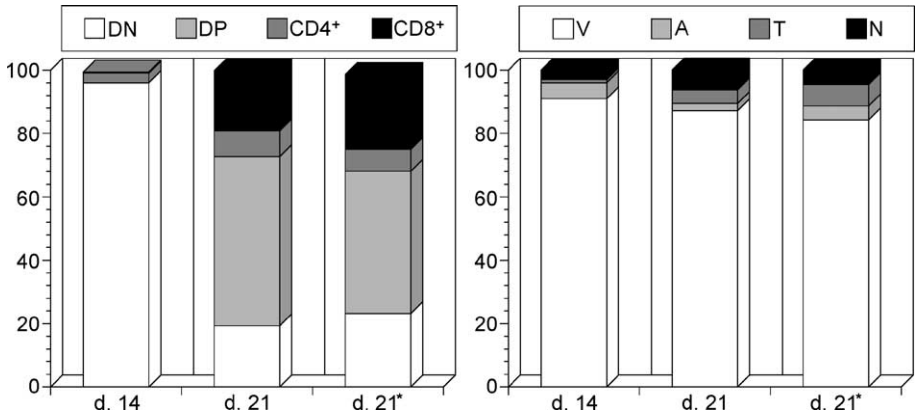

Fig. 4. FTOC control conditions. DN: double negative $CD4^{-}CD8^{-}$ cells, DP: double positive $CD4^{+}CD8^{+}$ cells, $CD4^{+}$: single positive $CD4^{+}$ cells, $CD8^{+}$: single positive $CD8^{+}$ cells, V: viable cells, A: early apoptotic cells, T: terminal, late apoptotic cell, N: necrotic cells. Mean of all experiments is represented. D14 T-cell subpopulations at the day of thymic sampling, n=8; d.21 T-cell subpopulations at the end of the culture in medium, n=14; d.21*: T-cell subpopulations at the end of the culture in medium with control DMSO addition, n=16. Results are expressed as mean of the population percentage. Statistical significance of differences was tested with nonparametric ANOVA Kruskal–Wallis followed by Dunn's multi-comparative post-test.

during the time length (7 days) of FTOC (data not shown). When FTOC were initiated, thymocytes were essentially DN (>95%) with a small population of $CD4^{low}$ cells (±5%) (Fig. 4). Very little apoptotic cells were numbered. After 7-day culture, DN cells differentiated into four T-cell subsets: DN (19.3%±3.00, m±S.D.); DP (53.4%±4.72); $CD4^{+}$ (8.1%±1.93) and $CD8^{+}$ (18.9%±2.85). Living cells were 82.2%±3.84, early apoptotic cells were 2.1%±2.06 and late apoptotic cells were 4.4%±1.73. Vehicle control (DMSO/medium, 1:50,000) only induced a nonsignificant increase in the percentage of DN (23.17±2.02) and $CD8^{+}$ (23.5±1.84) cells, as well as early and late apoptotic cells (4.34±1.65 and 6.8±1.82, respectively) and decreased DP cells (44.9±3.98).

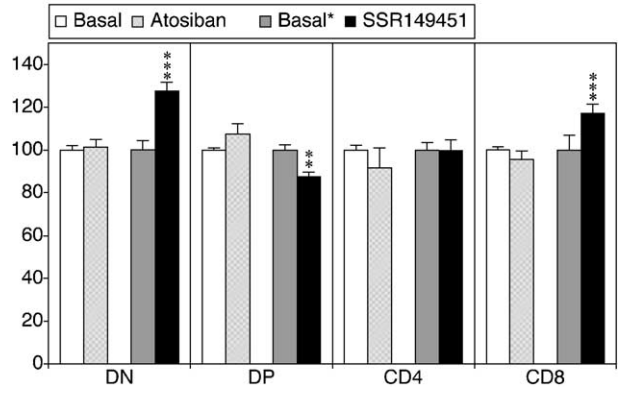

Fig. 5. Blockade of OTR and V1bR signaling during T-cell differentiation in FTOC. Flow cytometry analyses show the percentage of T-lymphocyte subpopulations stained either with anti-CD4, anti-CD8, Annexin-V and 7-AAD, recovered from FTOC exposed to OTR antagonist or V1bR antagonist for 1 week. Hundred percent are control conditions. DN: $CD4^{-}CD8^{-}$ cells; DP: $CD4^{+}CD8^{+}$ cells; $CD4^{+}$: single positive $CD4^{+}$ cells; $CD8^{+}$: single positive $CD8^{+}$ cells. Each column represents five experiments in triplicate. Results are expressed as mean±standard error of percentage vs. reference values. Statistical significance was tested with nonparametric ANOVA Kruskal–Wallis followed by Dunn's multi-comparative post-test. **p<0.01, ***p<0.001; Dunn's test.

The effects of specific receptor antagonists on T-cell differentiation (Fig. 5) were investigated in FTOC. The OTR antagonist Atosiban exerted a slight effect that, however, was not significant. On the contrary, the V1bR antagonist SSR149451 significantly increased DN and $CD8^{+}$ populations, and decreased DP cells. Significant effects on cell viability were a decrease in early apoptosis of the DN cells with the V1bR antagonist and an increase in CD8+early apoptosis with the OTR antagonist (Table 2). No significant effects were observed on total, DP and $CD4^{+}$ cells viability parameters, and no difference appeared between medium alone and controls (DMSO 1:50,000) (data not shown).

4. Discussion

Ontogenesis of *OT* and *VP* transcription in the murine thymus has not been investigated so far. In the present study, *OT* transcripts were detected by RT-PCR in the murine fetal thymus as early as on E13, while *VP* transcription started on

Table 2
Blockade of OTR and V1bR signaling on T-cell viability in FTOC

	Basal*	Atosiban	SSR149451
DN/V	70.6±7.76	68.5±10.76	69.3±4.55
DN/A	3.5±1.19	6.6±3.80	3.0±2.63*
DN/T	8.6±3.13	12.7±8.09	8.9±4.19
DN/N	17.3±8.49	12.3±2.83	18.7±5.52
CD8/V	87.5±6.02	88.2±4.75	88.4±2.81
CD8/A	1.9±0.86	3.4±3.09*	1.6±1.06
CD8/T	4.8±1.97	5.5±2.27	4.7±2.00
CD8/N	5.9±4.84	2.9±1.24	5.3±2.68

V: viable cells; A: early apoptotic cells; T: terminal, late apoptotic cells; N: Necrotic cells. DN: $CD4^{-}CD8^{-}$ cells; $CD8^{+}$: single positive $CD8^{+}$. Results are expressed as m±S.D. of total population percentage. N=18. Statistical significance was tested with nonparametric ANOVA Kruskal–Wallis followed by Dunn's multi-comparative post-test; Dunn's test.

* p<0.05.

E14 and was clearly detected on E15. Before these days, thymic lobes could not be identified. By comparison, *OT* transcription in the brain appeared on E13, while *VP* expression was detected on E14. It must be pointed out however that, only in 1 out of 5 experiments, *OT* transcription could be detected in the brain on E13 without nested PCR procedures. This unique result might be linked to the imprecision of the plug date in relation to the exact time of egg fertilization and embryonic development. The expression of *OT* and *VP* in CNS has already been investigated during murine ontogenesis. *VP* transcripts were detected by in situ hybridization on E16 in the supraoptic nucleus (SON), but were observable only on E21 (time of birth) in the hypothalamic suprachiasmatic nucleus (SCN) (Reppert and Uhl, 1987). A previous study has shown that the OT precursor is detected in CNS as soon as on E16, confirming the delay between fully processed VP and OT (Whitnall et al., 1985). Thus, based on our observations and the available literature, *OT* transcription in the thymus precedes or more rarely coincides with that in the CNS.

Whereas preliminary assays with *OT* and *VP* oligonucleotide probes failed to define a clear staining of thymic sections, in situ hybridization with a neurophysin probe (chosen in the conserved exon 2 of *OT* and *VP*) revealed that fetal thymic reticular cells express *OT* and/or *VP* transcripts. The cell feature, morphology, and overall distribution correspond to TEC. This is in accordance with our previous immunocytochemical studies showing that OT and VP are identified in human and murine TEC, including thymic nurse cells (TNC) (Geenen et al., 1988; Martens et al., 1996b; Robert et al., 1991). *OT* and *VP* transcription in the thymus is therefore coupled to mRNA translation and precursor processing. A specific aspect of this peptide axis in the thymus appears to be a peptide maturation that is more precocious than in the hypothalamo-neurohypophysial system during ontogenesis. In the rat CNS, OT prohormone was not detected before E16, while the mature OT nonapeptide appeared on E20–22 (Whitnall et al., 1985). This difference in the delay of OT appearance in the thymus and in the brain suggests a difference in the processing between hypothalamic neurons and TEC. Such discrepancy does not concern the late steps of OT processing since peptidyl-glycine-α-amidating monooxygenase (PAM), the last enzyme in the processing of neurohypophysial peptides (Bradbury et al., 1982), is present in its active form in rat thymic TEC (Martínez et al., 1998). It may be related to the difference in the precursor processing in association with neurohormone secretion in the hypothalamo-neurohypophysial axis, and in association with self-antigen presentation in the thymus during induction of central T-cell self-tolerance. Supporting this hypothesis, it is very significant that fully processed OT as the neurohypophysial self-antigen appears in the central tolerogenic organ before or at least simultaneously with the one in CNS.

We previously showed that T-cell lines with an immature $CD4^{-}CD8^{-}$ phenotype express functional receptors for OT and VP peptides (Martens et al., 1992). Among all types of receptors (*V1aR*, *V1bR*, *V2R* and *OTR*), only *OTR* transcripts were clearly evidenced in all thymus T-cell subsets, while *V1bR* was detected in DP and $CD8^{+}$ cells. Previous binding studies have also suggested *V1bR* expression by immature T cells (Elands et al., 1990; Martens et al., 1992; Torres and Johnson, 1988). Interestingly, Northern blot analyses have detected the presence of *V1bR* transcripts in rat thymus extracts (Lolait et al., 1995), and recent data evidence *OTR* expression in intralesional Kaposi's sarcoma $CD4^{+}$ and $CD8^{+}$ T cells (Cassoni et al., 2002). This last study suspected up-regulation of *OTR* expression in the Kaposi's sarcoma environment, but we show here that it is detected in all naive thymic T-cell subsets. We failed to detect any *V1aR* transcripts in the T-cell subsets. This is in accordance with a recent study using $V1aR^{-/-}$ mice (Hu et al., 2003) that shows the absence of effect of *V1aR* deletion upon thymic T-cell differentiation, while it affects B-cell late differentiation through modulation of B cell receptor. Taken together, our data substantiate an active intrathymic signaling mediated through OT/VP and OTR/V1b receptors. In the thymus, OT is targeted to the outer surface of human TEC plasma membrane from where it behaves either as a self-antigen, or as a ligand for OTR expressed by immature T cells. This latter interaction mediates mitogenic effects in freshly isolated human thymocytes, and induces phosphoinositide hydrolysis in RL12-NP cells. In addition, OT- and VP-induced phosphorylation of focal adhesion kinases in pre-T cells could promote the establishment of immune 'synapses' between very immature pre-T cells and thymic stromal cells (Martens and Geenen, 2000). Such morphological units are of crucial importance for the implementation of the T-cell differentiation program.

The other objective of this study was to investigate the functional importance of the thymic OT/VP-mediated signaling on parameters of T-cell differentiation and survival. FTOC is an appropriate model to study in vitro T-cell differentiation since FTOC conserve spatial interactions between differentiating T cells and thymic stromal cells (Jenkinson and Anderson, 1994). In this model, immature thymic T cells proliferate and differentiate whereas isolated thymocytes quickly die in vitro. *OT* and *VP* transcription in murine FTOC was confirmed by RT-PCR followed by Southern blotting with specific *OT* and *VP* oligonucleotides. The expression of both genes was maintained during the 7-day culture. After 7-day FTOC, the V1bR antagonist SSR 149451 decreased the early differentiation from DN to DP stage, while it favored $CD8^{+}$ commitment. SSR 149451 induced only a little decrease in DN T cell early apoptosis. It will be interesting to investigate the $CD4^{+}/CD8^{+}$ balance in the recent $V1bR^{-/-}$ mice (Tanoue et al., 2004) so to see whether an increase appears in the percentage of thymic $CD8^{+}$ T cells. The OTR antagonist increased apoptosis of $CD8^{+}$ T cells in this experiment, in accordance with the OT-

mediated mitogenic properties evidenced in a previous study (Martens et al., 1992).

A number of studies are currently revealing the importance for the establishment of central T-cell self-tolerance resulting from the intrathymic expression of genes encoding neuroendocrine and peripheral tissue-specific antigens. Our findings show that the neuroendocrine precursors encoded by those genes also control T-cell differentiation after binding to neuroendocrine receptors expressed by pre-T cells. This supports the model of a dual role for thymic neuroendocrine precursors according to their behavior either as the source of self-antigens in T-cell negative selection, or as accessory signals for the development/survival of self-tolerant T cells (Martens et al., 1996a). With regard to *OT* and *VP* transcription in the thymus, our studies until now provide evidence that thymic OT, besides its role as the tolerogenic self-antigen of this family, can also bind to OTR expressed by all thymic T-cell subsets and phosphorylate focal adhesion kinases thereby promoting the formation of immune 'synapses' between TEC and early immature T-cells. Furthermore, thymic OT and VP can bind to V1bR on DP and $CD8^+$ thymic T cells, thereby facilitating the DN–DP transition during T-cell differentiation, and regulating the final balance between mature $CD4^+$ and $CD8^+$ thymic T lymphocytes.

Acknowledgements

Our gratitude is due to Maurice Manning and to Claudine Serradeil-Le Gal for their generous gifts of specific OT and VP receptor antagonists. These studies were supported by National Fund of Scientific Research (Belgium), by the Fondation Leon Fredericq (Liege Medical School), by the Belgian Federation against Cancer, by the Fondation Vaugrenier (Switzerland), and by the EU FP6 Integrated Project Euro-Thymaide (LSHB-CT-2003-503410).

References

Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Van Kaer, L., Pircher, H.P., Zinkernagel, R.M., Tonegawa, S., 1994. Evidence for a differential avidity model of T cell selection in the thymus. Cell 76, 651–663.

Bradbury, A.F., Finnie, M.D., Smyth, D.G., 1982. Mechanism of C-terminal amide formation by pituitary enzymes. Nature 298, 686–688.

Cassoni, P., Sapino, A., Deaglio, S., Bussolati, B., Volante, M., Munaron, L., Albini, A., Torrisi, A., Bussolati, G., 2002. Oxytocin is a growth factor for Kaposi's sarcoma cells: evidence of endocrine–immunological cross-talk. Cancer Res. 62, 2406–2413.

Elands, J., Resink, A., De Kloet, E.R., 1990. Neurohypophyseal hormone receptors in the rat thymus, spleen, and lymphocytes. Endocrinology 126, 2703–2710.

Ericsson, A., Geenen, V., Robert, F., Legros, J.J., Vrindts-Gevaert, Y., Franchimont, P., Brene, S., Persson, H., 1990. Expression of preprotachykinin-A and neuropeptide-Y messenger RNA in the thymus. Mol. Endocrinol. 4, 1211–1218.

Gainer, H., Wray, S., 1993. Cellular and molecular biology of oxytocin and vasopressin. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction. Raven Press, New York, pp. 1099–1130.

Geenen, V., Defresne, M.P., Robert, F., Legros, J.J., Franchimont, P., Boniver, J., 1988. The neurohormonal thymic microenvironment: immunocytochemical evidence that thymic nurse cells are neuroendocrine cells. Neuroendocrinology 47, 365–368.

Geenen, V., Achour, I., Robert, F., Vandersmissen, E., Sodoyez, J.C., Defresne, M.P., Boniver, J., Lefebvre, P.J., Franchimont, P., 1993. Evidence that insulin-like growth factor 2 (IGF2) is the dominant thymic peptide of the insulin superfamily. Thymus 21, 115–127.

Geenen, V., Goxe, B., Martens, H., Vandersmissen, E., Vanneste, Y., Achour, I., Kecha, O., Lefebvre, P.J., 1995. Cryptocrine signaling in the thymus network and T cell education to neuroendocrine self-antigens. J. Mol. Med. 73, 449–455.

Geenen, V., Brilot, F., Hansenne, I., Renard, C., Martens, H., 2004. Thymus and T cells. In: Adelman, G., Smith, B.H. (Eds.), Encyclopedia of Neuroscience, 3rd ed. on CD-ROM Elsevier, New York, ISBN: 0-444-51432-5.

Hara, Y., Battey, J., Gainer, H., 1990. Structure of mouse vasopressin and oxytocin genes. Brain Res. Mol. Brain Res. 8, 319–324.

Hu, S.B., Zhao, Z.S., Yhap, C., Grinberg, A., Huang, S.P., Westphal, H., Gold, P., 2003. Vasopressin receptor 1a-mediated negative regulation of B cell receptor signaling. J. Neuroimmunol. 135, 72–81.

Jenkinson, E.J., Anderson, G., 1994. Fetal thymic organ cultures. Curr. Opin. Immunol. 6, 293–297.

Kecha, O., Brilot, F., Martens, H., Franchimont, N., Renard, C., Geimers, R., Defresne, M.P., Winkler, R., Geenen, V., 2000. Involvement of insulin-like growth factors in early T cell development: a study using fetal thymic organ cultures. Endocrinology 141, 1209–1217.

Kyewski, B., Derbinski, J., Gotter, J., Klein, L., 2002. Promiscuous gene expression and central T-cell tolerance: more than meets the eye. Trends Immunol. 23, 364–371.

Lolait, S.J., O'Carroll, A.M., Mahan, L.C., Felder, C.C., Button, D.C., Young III, W.S., Mezey, E., Brownstein, M.J., 1995. Extrapituitary expression of the rat V1b vasopressin receptor gene. Proc. Natl. Acad. Sci. U. S. A. 92, 6783–6787.

Manning, M., Miteva, K., Pancheva, S., Stoev, S., Wo, N.C., Chan, W.Y., 1995. Design and synthesis of highly selective in vitro and in vivo uterine receptor antagonists of oxytocin: comparisons with Atosiban. Int. J. Pept. Protein Res. 46, 244–252.

Martens, H.J., Geenen, V., 2000. Focal adhesion kinases: interest in immunoendocrinology, developmental biology, and cancer. Endocrine 13, 233–242.

Martens, H., Robert, F., Legros, J.J., Geenen, V., Franchimont, P., 1992. Expression of functional neurohypophysial peptide receptors by murine immature and cytotoxic T cell lines. Prog. NeuroEndocrinImmunol. 5, 31–39.

Martens, H., Goxe, B., Geenen, V., 1996a. The thymic repertoire of neuroendocrine self-antigens: physiological implications in T-cell life and death. Immunol. Today 17, 312–317.

Martens, H., Malgrange, B., Robert, F., Charlet, C., De Groote, D., Heymann, D., Godard, A., Soulillou, J.P., Moonen, G., Geenen, V., 1996b. Cytokine production by human thymic epithelial cells: control by the immune recognition of the neurohypophysial self-antigen. Regul. Pept. 67, 39–45.

Martens, H., Kecha, O., Charlet-Renard, C., Defresne, M.P., Geenen, V., 1998. Neurohypophysial peptides stimulate the phosphorylation of pre-T cell focal adhesion kinases. Neuroendocrinology 67, 282–289.

Martínez, A., Farr, A., Vos, M.D., Cuttitta, F., Treston, A.M., 1998. Peptide-amidating enzymes are expressed in the stellate epithelial cells of the thymic medulla. J. Histochem. Cytochem. 46, 661–668.

Pleau, J.M., Esling, A., Van Acker, C., Dardenne, M., 1996. Glutamic acid decarboxylase (GAD 67) gene expression in the pancreas and brain of the nonobese diabetic mouse. Biochem. Biophys. Res. Commun. 224, 747–753.

Plum, J., De Smedt, M., Leclercq, G., Vandekerckhove, B., 1995. Influence of TGF-beta on murine thymocyte development in fetal thymus organ culture. J. Immunol. 154, 5789–5798.

Rehbein, M., Hillers, M., Mohr, E., Ivell, R., Morley, S., Schmale, H., Richter, D., 1986. The neurohypophyseal hormones vasopressin and oxytocin. Precursor structure, synthesis and regulation. Biol. Chem. Hoppe-Seyler 367, 695–704.

Reppert, S.M., Uhl, G.R., 1987. Vasopressin messenger ribonucleic acid in supraoptic and suprachiasmatic nuclei: appearance and circadian regulation during development. Endocrinology 120, 2483–2487.

Robert, F., Geenen, V., Schoenen, J., Burgeon, E., De Groote, D., Defresne, M.P., Legros, J.J., Franchimont, P., 1991. Colocalization of immunoreactive oxytocin, vasopressin and interleukin-1 in human thymic epithelial neuroendocrine cells. Brain Behav. Immun. 5, 102–115.

Schlosser, S.F., Almeida, O.F., Patchev, V.K., Yassouridis, A., Elands, J., 1994. Oxytocin-stimulated release of adrenocorticotropin from the rat pituitary is mediated by arginine vasopressin receptors of the V1b type. Endocrinology 135, 2058–2063.

Sebzda, E., Mariathasan, S., Ohteki, T., Jones, R., Bachmann, M.F., Ohashi, P.S., 1999. Selection of the T cell repertoire. Annu. Rev. Immunol. 17, 829–874.

Serradeil-Le Gal, C., Wagnon, J., Simiand, J., Griebel, G., Lacour, C., Guillon, G., Barberis, C., Brossard, G., Soubrie, P., Nisato, D., Pascal, M., Pruss, R., Scatton, B., Maffrand, J.P., Le Fur, G., 2002. Characterization of (2*S*,4*R*)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidine carboxamide (SSR149415), a selective and orally active vasopressin V1b receptor antagonist. J. Pharmacol. Exp. Ther. 300, 1122–1130.

Tanoue, A., Ito, S., Honda, K., Oshikawa, S., Kitagawa, Y., Koshimizu, T.A., Mori, T., Tsujimoto, G., 2004. The vasopressin V1b receptor critically regulates hypothalamic–pituitary–adrenal axis activity under both stress and resting conditions. J. Clin. Invest. 113, 302–309.

Torres, B.A., Johnson, H.M., 1988. Arginine vasopressin (AVP) replacement of helper cell requirement in IFN-gamma production. Evidence for a novel AVP receptor on mouse lymphocytes. J. Immunol. 140, 2179–2183.

Whitnall, M.H., Key, S., Ben-Barak, Y., Ozato, K., Gainer, H., 1985. Neurophysin in the hypothalamo-neurohypophysial system: II. Immunocytochemical studies of the ontogeny of oxytocinergic and vasopressinergic neurons. J. Neurosci. 5, 98–109.