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Neuropeptides and thymic hormones in the *Xenopus* thymus

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1. ABSTRACT

T-cell development is characterised by a complex series of events in the thymus, which results in the development of self-restricted immunocompetent lymphocytes. We have previously reported the expression of neuropeptides in the thymus of various species, highlighting the evolutionary importance of neuroendocrine interactions in thymocyte development. Despite the many physiological and functional similarities in their immune systems, no study has addressed the importance of neuropeptides and thymic hormones in T-cell development in *Xenopus*. Immunohistochemical analysis revealed that the neuropeptides substance P, neuropeptide Y, somatostatin, calcitonin gene related peptide, and vasoactive intestinal polypeptide and the thymic hormones thymosin alpha1, thymosin beta4, and thymopoietin are found in the *Xenopus* thymus. This was further corroborated by RT-PCR. Furthermore, double staining revealed that neuropeptides and thymic hormones are coexpressed within the epithelial cell component of the thymus. These results show that neuropeptides and thymic hormones are expressed in the thymus of *Xenopus*, and suggest that they are likely to play a role in T-cell development.

2. INTRODUCTION

Despite its immunological importance, for many centuries the thymus remained an obscure and enigmatic organ with unknown functions. Only around 40 years ago has this organ been shown to be responsible for T cell development (1). T cell development is characterised by a complex series of events in the thymus which results in the development of self-restricted immunocompetent T cells with both effector and regulatory activities. A variety of thymic hormones, steroids and cytokines as well as extracellular matrix (ECM) components are known to be produced by thymic epithelial cells (TEC) that influence migration, differentiation, apoptosis and maturation of thymocytes (2-4). However, the scale of molecules and signalling events involved in T cell development is not yet fully characterised.

Despite the evolutionary distance between mammals and amphibians, it is becoming clear that the processes that regulate thymus physiology and T cell development have been conserved throughout evolution. Indeed, thymectomy of *Xenopus* at larval stages results in a deficiency in T cells which is irreversible through adult stages (5-7), leading to the animal becoming

immunocompromised (8). Much less is known about the pattern of development of mature T cell subsets in amphibians than mammals. However, it seems clear that immature thymocytes pass through a CD4/CD8 double positive state (9). Furthermore, amphibians have T cell receptors and CD3 molecules homologous to their mammalian counterparts (10), and also have cell surface receptors including MHC class I and class II molecules (11), CD5 (12) and CD8 co-receptors (13). The conservation of molecules during T cell development also extends to the recently characterised CTX protein that was first discovered in *Xenopus* and identifies cortical thymocytes. ChT1, the chicken homologue for CTX, has been shown to be required for T cell differentiation and development (14) and gene homologues for CTX, have since been identified in mice and humans (15).

3. CROSS-TALK BETWEEN THE NEUROENDOCRINE AND THE IMMUNE SYSTEM

Increasing evidence suggests cross-talk between the neuroendocrine and the immune system, with shared ligands and receptors used as a common chemical language within and between the two systems (16, 17). This bi-directional interaction is likely to serve as an important homeostatic mechanism. Both primary and secondary mammalian lymphoid organs are highly innervated, and the sympathetic nerve fibres are in direct contact with lymphocytes as well as other cell types, thus providing the anatomical link between the two systems (18). Similarly, such interactions also appear to occur in birds, reptiles, amphibians (see article by Kinney and Cohen in this issue) and teleosts (19-21). Hormones enter lymphoid organs via blood circulation, and neuropeptides released from the terminals of nerve fibres innervating the lymphoid tissues act as transmitters of signals from the neuroendocrine to the immune system, thus modulating the function and state of lymphocytes and their precursors, macrophages, and dendritic cells that carry these specific receptors (2, 22). For example, growth hormone (GH) has been shown to enhance interferon (IFN)-gamma secretion by antigen presenting cells, as well as to direct homing of recent thymic emigrants to lymph nodes (23). Furthermore, there are reports that have suggested that neuropeptides play a role in cytokine production, migration and immunomodulation in various immune cells (24-26). In addition, there is increasing evidence indicating that both hormones and neuropeptides are produced by several cell types within lymphoid organs (22). Indeed, we have made the observation that several neuropeptides, namely substance P (SP), neuropeptide Y (NPY), somatostatin (SOM), calcitonin gene related peptide (CGRP), and vasoactive intestinal polypeptide (VIP) are endogenously produced and expressed in the thymus of different species (20, 27, 28). Furthermore, cells within the thymus express neuropeptides receptors and we have shown that such substances are able to modulate thymocyte development in murine and avian fetal thymic organ culture. (27, 28). Thus, these observations highlight the evolutionary significance of neuropeptide expression and activity within the thymus, which suggests that they may play a role in T cell development.

3.1. Neuropeptides

Neuropeptides, such as substance P (SP), neuropeptide Y (NPY), somatostatin (SOM), calcitonin gene related peptide (CGRP), and vasoactive intestinal polypeptide (VIP) are small acting proteins that regulate many physiological activities within the body. They are located within the central and peripheral nervous system and exert their action by binding to specific G protein-coupled receptors. Increasing evidence suggest that neuropeptides are also endogenously produced within lymphoid tissue and can modulate many cells of the immune system.

3.1.1. Somatostatin (SOM)

SOM is a peptide hormone that is widely distributed throughout the body and was originally isolated from mammalian hypothalamus and characterised as a physiological inhibitor of GH secretion (29). The peptide is composed of either 14 or 28 amino acids, with the 14-aa being the most predominant isoform. The diverse activity induced by SOM occurs due to its effect on neurotransmission, secretion and proliferation. These effects of SOM are exerted through five receptors that belong to the 7 transmembrane G-protein-coupled receptor family (29). Numerous studies have indicated that SOM modulates the proliferation of lymphoid tissues. For instance, it has been shown to influence antibody secretion, to inhibit IgA production, and downregulate IL-8, IL-1beta and IFN-gamma secretion (22, 30, 31).

3.1.2. Vasoactive intestinal polypeptide (VIP)

VIP is another neuropeptide that is believed to have immunoregulatory properties. Besides of its function as a neuromodulator in the central nervous system, this 28 amino acid polypeptide also has various potent effects on smooth muscle, epithelial cells, and endothelial cells. VIP exerts its effect via binding to either of two receptors that are also members of the 7 transmembrane G-protein-coupled receptor family (32). A number of reports have implicated VIP in immune regulation. For instance, it has been observed that VIP inhibits the proliferation of Concanavalin A (ConA)-stimulated T cells as well as phorbal myristate acetate-or calcium ionophore-stimulated splenocytes and CD4+ and CD8+ T cells (33-36). Overall this peptide is generally recognised as a potent anti-inflammatory mediator (37).

3.1.3. Substance P (SP)

Containing 11 amino acids SP is a small neuropeptide that belongs to the tachykinin family. There are three specific receptors for SP, namely neurokinin 1 receptor (NK1 R), NK2 R and NK3 R, although SP preferentially binds to NK1 R. SP is widely distributed in the central and peripheral nervous system and is involved in the transmission of sensory information such as pain and stress, as well as in the stimulation of smooth muscle cells, vasodilatation and glandular secretion (38). There are also data to suggest that SP modulates several aspects of immune regulation, particularly those of inflammatory responses. SP enhances macrophage and mast cell activation and degranulation (39-41), and upregulates tumour necrosis factor 1 (TNF-1), IL-1beta, IL-2, and IL-6

in macrophages, NK and T cells (42, 43), as well as skewing Th1/Th2 cytokine responses (44) and enhancing Ab production (45, 46). In addition, SP modulates adhesion and chemotaxis of T cells through upregulation of LFA-1 (47, 48), and it modulates proliferation of a variety of immune cells including T cells and thymocytes (49, 50). Thus, the cummulative evidence suggests that SP is a potent pro-inflammatory neuropeptide.

3.1.4. Neuropeptide Y

NPY is a 36 amino acid peptide that belongs to the neuropeptide Y family, of which pancreatic polypeptide is also a member. Originally isolated from the porcine brain and intestine, NPY is present in all vertebrate classes (51). Mainly synthesised in the central and peripheral nervous systems, NPY acts on G-protein coupled receptors, particularly through NPY Y1 R and NPY Y2 R, playing a major role as a neurotransmitter and neuromodulator, but also regulating behavioural effects such as food intake, anxiety, learning, and memory (52, 53). Studies have also suggested that NPY acts as an immunomodulator of immune responses. NPY has been found to upregulate IFN-gamma and inhibit IL-4 production in murine T cells (54), as well as induce adhesion to fibronectin in sites of inflammation (47). NPY has also been implicated in skewing the Th1/Th2 cytokine profile (47), influencing NK cell activity in tissue in an age-dependent fashion (55), and influencing cell migration of monocytes and NK cells *in vivo* (56, 57). Furthermore, evidence suggests that NPY modulates the activity of macrophages (58) and T cells (55), as well as inhibiting Ab production (59) and thymocyte proliferation (60).

3.1.5. Calcitonin gene related peptide (CGRP)

CGRP is a 37 amino acid peptide generated by alternative splicing of the calcitonin gene, which is predominantly synthesised and stored in sensory neurons. CGRP mediates its activities through either of two specific receptors. These receptors belong to the G-protein-coupled receptor superfamily and consist of at least three proteins: calcitonin receptor-like receptor, CGRP receptor component protein, and the receptor activity modifying protein (RAMP). Upon interaction with CGRP, the receptors induce intracellular cyclic adenosine monophosphate formation (cAMP) (61). CGRP acts primarily as a modulator of vasodilatation, metabolism, and secretion in a number of different cells of the neuronal, cardiovascular, respiratory, and gastrointestinal systems (62-64), but evidence suggests that this neuropeptide also influences cells of the immune system. CGRP stimulates eosinophil migration (65), as well as T cell adhesion to fibronectin in inflammatory sites (47). CGRP also inhibits IL-2 and IFN-gamma production, and impairs T cell proliferation (54, 66, 67). Furthermore, CGRP administration impairs macrophage hydrogen peroxide secretion and their ability to activate T cells (68). A similar inhibitory effect of CGRP is also observed on the activation of dendritic cells (69), including inhibition of antigen presentation (70). Several reports have also shown that CGRP enhances thymocyte and T cell apoptosis (71-73). The vast majority of experimental evidence therefore suggests that CGRP has potential immunosuppressive and anti-inflammatory activities which are currently being explored in a variety of pharmacological treatments (63).

4. THYMIC HORMONES

Thymic hormones are so called because they were first isolated in fractions from crude extracts of calf thymus (74-76). The original aim was to identify the hormones that underlie T cell maturation, and those discovered are a group of small proteins that appear to be highly conserved. Of all the peptides isolated, three were identified as potent stimulators of the immune system and subsequently characterised and named thymosin alpha1, thymosin beta4, and thymopoietin (TMPO). It is now recognised that these peptides are not restricted to the thymus. Furthermore, their status as true hormones are in question (77, 78). Nevertheless, it is becoming increasingly apparent that these peptides are able to carry out a multitude of functions and may prove to have therapeutic properties.

4.1. Thymosin alpha1

Thymosin alpha1 is able to affect a number of different cell types. Presently, thymosin alpha1 is being successfully used to enhance the immune responses in patients with AIDS, cancer and hepatitis B and C. This is thought to be in part due to the ability of thymosin alpha1 to activate T helper cells by enhancing dendritic cell antigen presentation (79), possibly via Toll-like receptor signalling (80). Additionally, thymosin alpha1 has been shown to increase major histocompatibility complex Class I expression at a transcriptional level by Rat FRTL-5 Thyroid Cells (81), which could augment presentation of cancer antigens to CD8 cytotoxic T cells. The effects of thymosin alpha1 on T cells have not been as extensively investigated, although treatment of human lymphocytes with thymosin alpha1 caused an upregulation of IL-2 receptor expression, improving T cell sensitivity to proliferative stimuli (82). Even less is known about the role of thymosin alpha1 in the thymus. Studies have shown thymosin alpha1 is able to protect murine thymocytes against dexamethasone-induced apoptosis (83), perhaps by the induction of cAMP and protein kinase C dependent pathways (84). Moreover, there are data to suggest that thymosin alpha1 may also influence the developmental pathway of thymocytes. For example, in cyclophosphamide-induced immunodeficient mice, thymosin alpha1 injections sped up the recovery of the thymocyte population, almost exclusively due to an increase in the number of double positive thymocytes (85).

4.2. Thymosin beta4

The immunological properties of thymosin beta4 have been overshadowed by the discovery that it is an actin-sequestering peptide, able to regulate actin polymerization in many cells (78). Furthermore, thymosin beta4 has recently been implicated in the migration and survival of cardiac cells (86) and is essential for coronary vessel development in mice (87). However, earlier studies showed that thymosin beta4 is rapidly upregulated in response to ConA stimulation in rat thymocytes (88). Moreover, there is a specific splice variant of thymosin

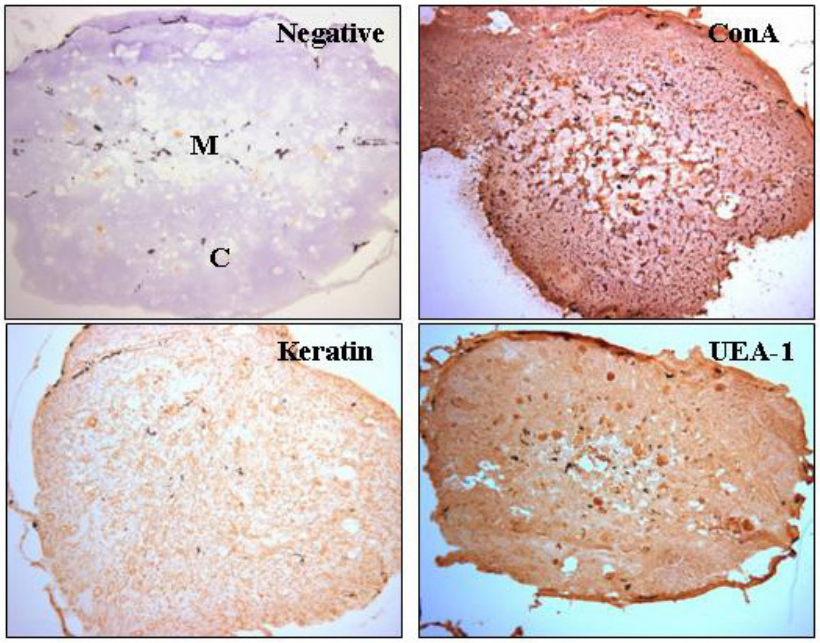

Figure 1. Architecture of the *Xenopus* thymus. Immunohistochemistry of frozen adult *Xenopus* (four month old) thymic sections with stromal cell markers defining the architecture of the thymus (20, 28). Unlike other species, the *Xenopus* thymus has a peculiar cortex (C) and medulla (M). Keratin expression revealed a reticular network of TEC as in other species. Cystic bodies are marked by arrows. Interestingly, receptors for ConA were detected throughout the thymus, representing TEC and scattered thymocytes. A similar result was found when staining with UEA-1 on stromal cells. No immunoreactivity was detected when sections were incubated with normal rabbit and goat sera Abs (negative control). All sections (including negative control) were counterstained with Mayer's haematoxylin solution. Data presented is representative of at least three individual experiments. Magnification x100.

beta4 that is expressed only in lymphoid tissue, believed to have anti-inflammatory effects (89). Furthermore, thymosin beta4 may influence thymopoiesis as it is able to induce the expression of terminal deoxynucelotidyl transferase, an enzyme involved in TCR gene rearrangement (90). Incidental evidence also implicates thymosin beta4 in thymus organogenesis as thymosin beta4 knockdown mice exhibit thymic defects (87).

4.3. Thymopoietin (TMPO)

The biology of TMPO is still confused, primarily because it is a splice variant of the lamin-associated polypeptide which is a component of the nuclear envelope (77). Nevertheless, this 49 amino acid peptide is detectable in human serum (91), and is thought to regulate T cell differentiation and function (92) because administration to athymic mice increases the total number of T cells.

5. LOCALISATION OF NEUROPEPTIDES AND THYMIC HORMONES IN THE XENOPUS THYMUS

Despite the increasing amount of data showing the presence of neuropeptides and thymic hormones in the thymus (2, 93), the expression of these molecules in the *Xenopus* remains to be fully elucidated. Below we provide evidence indicating that the neuropeptides CGRP, NPY, SOM, SP and VIP together with the thymic hormones thymosin alpha1, thymosin beta4 and TMPO are expressed in the *Xenopus* thymus. We believe that the expression of these molecules in the *Xenopus* thymus, which is similar with other phylogenetically distant species (20), suggests a conserved functional role of neuroendocrine interactions in T cell development across evolution.

5.1. Organisation of the *Xenopus* thymus

Like mammals, amphibians have two thymic lobes, although their anatomical location is somewhat altered. Thymic lobes in *Xenopus* are found between the maxillary jaw and the front legs/arms. Each individual spherical thymic lobe is surrounded by a thick layer of connective tissue, and trabeculae penetrate in from the capsule through to the cortex (Figure 1). In mammals and other higher vertebrate species, medullary and cortical areas are easily distinguishable (20), however in the *Xenopus* this distinction becomes more challenging. Stromal cells formed an interlacing meshwork giving a sponge-like thymic structure, as previously described for the amphibian thymus (94, 95). In the mammalian thymus the binding activity of the lectin Ulex Europeaus Agglutinin 1 (UEA-1) is restricted to the medullary epithelial cells (23). Interestingly, when the *Xenopus* thymus is analysed for UEA-1 binding potential, the entire thymic structure is found to possess lectin binding activity (Figure 1) suggesting that MHC class II^+ TEC are associated with developing thymocytes throughout the entire organ, unlike mammalian species. In addition, the expression pattern of ConA suggests that the receptor for this lectin is also likely to be highly expressed by cells of

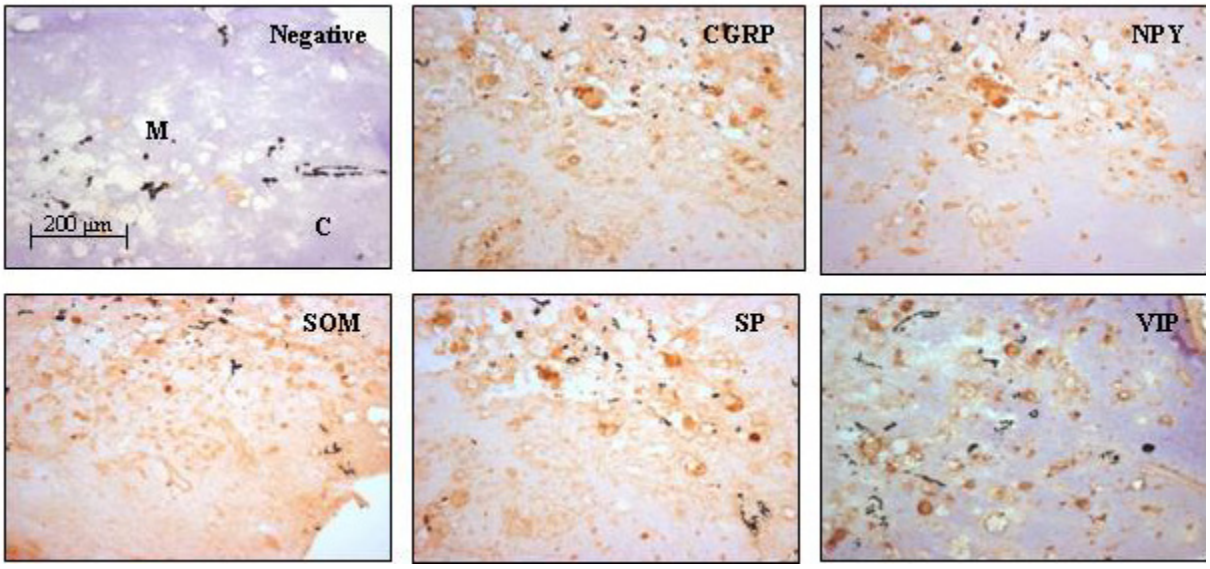

Figure 2. Neuropeptides are expressed in the *Xenopus* thymus. Immunohistochemistry of frozen adult *Xenopus* (four month old) thymic sections with anti-CGRP, anti-NPY, anti-SOM, anti-SP and anti-VIP Abs (20, 28). Immunoreactive cells for CGRP, SOM and SP give a reticular staining pattern which can be observed throughout the medulla (M), and the cortex (C). NPY is expressed in the subcapsular region and medulla, while VIP expression seems to be restricted to cystic bodies. No immunoreactivity was detected when sections were incubated with normal rabbit and goat sera (negative). All sections (including negative control) were counterstained with Mayer's haematoxylin solution. Data presented is representative of at least three individual experiments. Magnification x200.

the thymic microenvironment, in particular TEC, as previously described in the mouse (96) and in the chicken (97, 98). This staining was specific, as background staining was not detected in the negative control. However, the negative control did present high levels of endogenous peroxidase, activity which suggests the presence of high numbers of macrophage clusters associated with thymic nurse cells as previously described in amphibians (94, 99). When looking at keratin expression, the *Xenopus* thymus was found to display a typical network of TEC although more globular in organisation (particularly in the medulla). Cysts were also observed in the medulla, as previously described (95, 100).

5.2. Analysis of the expression of neuropeptides and thymic hormones in the *Xenopus* thymus

We had previously reported that neuropeptides are expressed in the thymus of other phylogenetically distant species (20, 27), highlighting that the expression of such proteins are an evolutionary conserved feature of the thymus and also suggesting that they may play a role in T cell development. Here, by using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) we show that the neuropeptides CGRP, NPY, SOM, SP and VIP, as well as the thymic hormones thymosin alpha1, thymosin beta4 and TMPO are expressed in the thymus of the amphibian. Immunohistochemical analysis revealed positive cells for all of the neuropeptides in the *Xenopus* thymus. Indeed, the neuropeptides GCRP, NPY, SOM and SP could be found throughout cortical and medullary areas, including associated with cysts. In contrast, VIP expression appeared to be restricted to medullary cystic bodies (Figure 2).

It has long been recognised that the thymic epithelium is vital for the successful production of immunocompetent T cells (3, 4, 101). It has been previously reported that in the mouse, neuropeptides are expressed within the thymic stromal compartment (20, 27). However, whether neuropeptides and thymic hormones are also expressed within the epithelial compartment of the thymus, particularly in non-mammalian species remained to be fully answered. This was determined using double fluorescence microscopy with *Xenopus* thymic sections. For identification of TEC component of the thymus, the anti-pancytokeratin mAb clone C-11 and wide spectrum polyclonal anti-keratin was used to help identify epithelial cells from the *Xenopus*. All neuropeptides were found to be co-localised, in varying degrees, with keratin positive TEC; especially associated with cyst-like structures that make up the amphibian thymic microenvironment (Figure 3). Likewise, thymosin alpha1 and thymosin beta4 were highly expressed and co-localised with thymic epithelium (Fig.4). Although not strongly expressed, TMPO staining also revealed that this thymic hormone co-localises with the thymic epithelium (Figure 4). Interestingly, both neuropeptides and thymic hormones seem to be expressed by keratin-negative cells, which might indicate that they are expressed by other stromal cells and developing thymocytes.

5.3. Endogenous production of neuropeptides and thymic hormones in the *Xenopus* thymus

To further investigate the expression of neuropeptides and thymic hormones, RT-PCR was performed in the *Xenopus* thymus (Figure 5). mRNA encoding for prepro-NPY, prepro-SOM and prepro-VIP

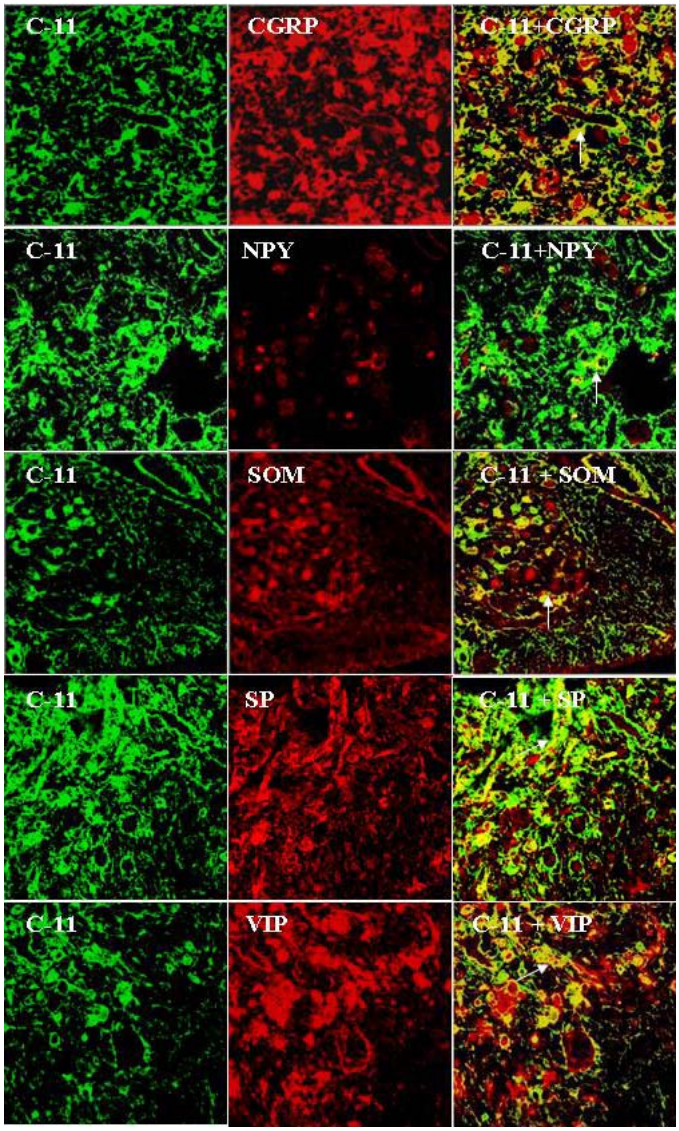

Figure 3. Neuropeptides are expressed by TEC in the *Xenopus* thymus. Double staining was performed on frozen adult *Xenopus* (four month old) thymic sections (20, 28). Tissues were sequentially stained for anti-CGRP, anti-NPY, anti-SOM, anti-SP and anti-VIP in red (centre column) and the anti-pancytokeratin (C-11) mAb, which detected the vast majority of the epithelial components of the thymus of *Xenopus* in green (left column). Co-expression (C-11 + Neuropeptide Ab) can be visualised when overlaying the neuropeptide and TEC pictures (right column; as marked with an arrow), although other keratin-negative cells appear to be positive for these neuropeptides. No immunofluorescence was detected when staining with normal rabbit and goat sera. Data presented is representative of at least three individual experiments. Magnification x10, optical zoom 2.7.

was found in the *Xenopus* thymus where amplicons were of the expected size and sequence (results not shown). Likewise, amplicons of the expected size were found for the thymic hormones thymosin alpha1, thymosin beta4 and TMPO (Figure 5). The intrathymic production of neuropeptides and thymic hormones in the thymus of the *Xenopus* and other species (20, 27), and their co-localisation to the thymic epithelial cell compartment seems to suggest that both neuropeptides and thymic hormones are a conserved feature of the thymus and suggests that they might play a functional role in thymus physiology and T cell development.

6. DISCUSSION AND PERSPECTIVES

Antigen receptors, such as B cell immunoglobulin and the TCR expressed on T cells, rearrange their genes following RAG expression. Immunoglobulin gene recombination is thought to have first evolved nearly 450 million years ago, during the so called “Big-Bang” of immunology (102, 103). Therefore, teleost fish, amphibians, birds and mammals all share similar types of blood cells, including T and B cells, and use common molecular mechanisms that regulate the development of immune cells (11, 104-106). Indeed,

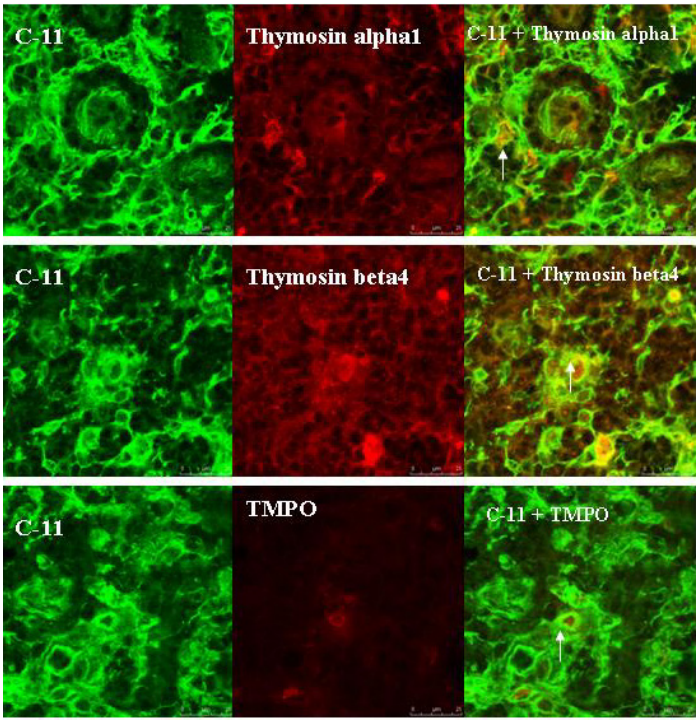

Figure 4. Thymic hormones are expressed by TEC in the *Xenopus* thymus. Double staining for the thymic hormones thymosin alpha1, thymosin beta4 and TMPO. *Xenopus* thymic sections were sequentially stained for thymosin alpha1, thymosin beta4 or TMPO in red (centre column) and the thymic epithelial cell marker anti-pancytokeratin (C-11) in green (centre column). Superimposing the two stains revealed high levels of co-localisation of both thymosin alpha1 and thymosin alpha1 (right column; as marked with an arrow), although other cells of the *Xenopus* thymus appear to express these thymic hormones. In contrast, TMPO is present at much lower levels than the other thymic hormones but likewise appears to co-localise with TEC marker keratin (as marked with an arrow). No immunofluorescence was detected when staining with normal rabbit and goat sera. Data presented is representative of at least three individual experiments. Magnification x20, optical zoom 7.

evidence is accumulating demonstrating that the specific immune system in amphibians and fish is very similar to that of higher vertebrates, as sequence homologues of all major genes involved in specific immune responses such as MHC class I and MHC class II, TCR, and immunoglobulin have been identified and characterised (10, 11, 13, 107-111). Likewise, the involvement of the thymus in the development of T cells has also been conserved throughout evolution, and mechanisms of thymic development, thymocyte development, and thymic physiology appear to be conserved among other phylogenetically distant species, including the amphibians (9, 11, 105, 106, 112).

The intricate thymic stromal cell compartment and three-dimensional organisation of the mammalian thymus allow T cell precursors to interact with particular subtypes of cells required for specific stages of T cell development (3, 4). The function of the thymus in all jawed vertebrate species is that of assisting T cell development. As a result, the thymic microenvironment has also been shown to be an important feature of the thymus of *Xenopus* (95, 100). For example, the mammalian thymus involutes with age and, as a result, the thymic architecture is severely disrupted (113, 114). A similar ageing process has long been recorded in amphibians (115). In addition, in several conditions where thymus architecture is absent or disrupted, including DiGeorge syndrome in humans and nude mutation in mice, the affected individual produces B cells but few if any T cells (4). Again, a similar process occurs when thymectomy is performed in *Xenopus* larval stages, as there is a reduction in alloimmunoreactivity (6) and loss of helper activity necessary for Ab production (5), which ultimately results in features of immunodeficiency (7, 8).

Comparative studies have been useful in identifying novel molecules, such as the *Xenopus* CTX protein, whose homologues are likely to be involved in mammalian T cell development (116). ChT1, the chicken homologue for CTX, has been shown to be required for T cell differentiation and development (14), and gene homologues for CTX have since been identified in mice and humans (15). However, to date little is known about the thymic architecture of the thymus of *Xenopus*, despite the many similarities in thymic function and T cell development. The amphibian thymus was found to have cortical and medullary areas similar to mammals, as previously described (94, 95). In addition, when the thymus was stained for keratin, this marker revealed a typical TEC organisation although more globular in structure. However, UEA-1 and ConA binding studies revealed that their binding activity is throughout the cortex and medulla,

Table 1. *Xenopus* PCR primer sequence and annealing temperatures used for neuropeptide and thymic hormone detection

Gene of Interest	Forward Primer ***5' – 3'***	**Reverse Primer** 5' – 3'	**Annealing Temp.**	***Acession Number***
***Xenopus* EF-alpha**	TGCCAATTGTTGACATGATCCC	TACTATTAAACTCTGATGGCC	61°C	NM_203970
***Xenopus* NPY**	ATGCAGGGAAACATGAGGTTG	CACATGGGAGGGTCTTCAAAC	61°C	S55577
***Xenopus* SOM**	ATGCAGTCCTGCCGTGTGCGC	TCCTGCTTTGCGTTCCCTGGG	61°C	AY316318
***Xenopus* VIP**	GGAAATCGGTTACCATTTGAG	TCTTTACAGCCATTTGCTTCC	61°C	BC043792
***Xenopus* Prothymosin alpha1**	GACCACCAAGGACTTAAAAGC	CCTCATCATCTTCTGCTGCTC	61°C	NM_001087373
***Xenopus* Thymosin beta4**	CCAGATATGGCTGAGATTGAG	CCACCAGAATCAAAGCCAGCC	61°C	NM_001090852
***Xenopus* TMPO**	CTGCAAGACCCGTCGGTAC	TTGGGCCTGGTTTCATTCC	61°C	BC061384

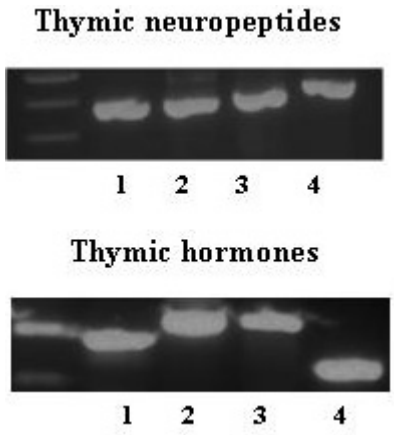

Figure 5. Messenger RNA transcripts for neuropeptides and thymic hormones are detected in *Xenopus* thymus. In the *Xenopus* thymus mRNA was present for NPY (lane 2 – 290bp), SOM (lane 3 – 309bp) and VIP (lane 4 – 340bp). Similarly, RT-PCR revealed mRNA transcripts for thymosin beta4 (lane 2 – 345bp), TMPO (lane 3 – 334bp) and Prothymosin alpha, the precursor of thymosin alpha1 (lane 4 – 224bp), were expressed in the *Xenopus* thymus. EF-alpha was used as a positive control (lanes 1 – 276bp). DNA marker is a 100bp ladder. Amplicon identities were confirmed by nucleotide sequencing (data not shown). Data presented is a representative of at least three independent experiments (see Table 1 for primers and annealing conditions).

unlike the mammals where UEA-1 and ConA binding is most, if not exclusively, restricted to the medullary area. ConA is a lectin specific for D-*mannose*, which can bind to late stage thymocytes in the medulla. However, it has also been shown to detect TEC of different species (96-98), which could explain the presence of some positively stained cortical cells in the *Xenopus* thymus. In the *Xenopus* thymus, the presence of cysts was also recorded. These morphological structures have been reported before (95) and have been suggested to act as sites for the deposition of dead cellular material (117). Indeed, when *Xenopus* thymus is irradiated there is an increase in thymocyte cell death accompanied by an increase in cystic activity (95). Therefore, *Xenopus* cysts might act as mammalian Hassall's corpuscles, by being involved in the clearance of apoptotic thymocytes. This suggests that the TEC component in the amphibian is structurally different, although appearing to have the same physiological and functional properties as in other higher vertebrates.

The expression of Foxn1, RAG and Ikaros family members has also been well-documented in a variety of higher and lower vertebrate species (118-123), which further demonstrates that the basic mechanisms that regulate T cell development are phylogenetically conserved. We have previously reported the expression of neuropeptides in the thymus of different species (20). Here, we extend the analysis of neuropeptides and thymic hormones in the thymus of *Xenopus*. We found that the neuropeptides CGRP, NPY, SOM, SP and VIP, as well as the thymic hormones thymosin alpha1, thymosin beta4 and TMPO were all expressed in the thymus. This potentially identifies these molecules as key mediators of thymus physiology and thymocyte development. Indeed, neuropeptides and thymic hormones are known to influence the development, migration and proliferation in several species (2). For example, in a model of *in vitro* murine T cell development, SOM was found to increase thymic cellularity, as well as influencing the development and migration of thymocytes (27).Additionally, SOM and CGRP were found to inhibit chicken T cell development while SP seemed to stimulate differentiation (28). Therefore, neuropeptides and thymic hormones appear to have been evolutionary conserved in the thymus as they regulate T cell development. Studies using pituitary-deficient mice have shown that these animals have a number of immune deficiencies, including reduced thymus size and impaired cellular and humoral immune responses (124-126). These deficiencies can however be rescued in developing mice using growth hormone and thyroid hormone treatment (127, 128). These studies demonstrated the importance of neuropeptides and hormones in the immune system and the thymus gland. Similarly, the *Xenopus* thymus also seems to be under the influence of neuropeptides and hormones produced by the pituitary gland. Rollins-Smith *et al.,* have shown that when pituitary tissue is removed from young tadpoles, it renders adult animals with smaller thymus, spleen and lymph nodes (129). In addition, there is a reduction in thymocyte and T cell number in the thymus and spleen and a higher proportion of T cells is arrested in the G_0/G_1 phase of cell cycle when compared to controls (129).

At least in mammals, TEC are known to be responsible for providing many of the signals that dictate T cell development. When using a keratin marker, we showed that neuropeptides and thymic hormones are expressed by TEC. Furthermore, it was also found that neuropeptides and thymic hormones are co-localised with cyst like structures which are reminiscent to Hassall's corpuscles. We and others have reported the expression of neuropeptides and hormones in Hassall's corpuscles in various species (20,

28, 130). It appears then that this co-localisation is conserved throughout lower vertebrate species and may indicate that this expression could be involved in the clearance of apoptotic thymocytes. This finding is of great importance as it further highlights the importance of these molecules in the thymic microenvironment. However, not all neuropeptide and thymic hormone expression was found to be in TEC. This expression is likely to be from other stromal cells and developing thymocytes themselves, indicating that these molecules act via autocrine and paracrine circuits. To corroborate these finding, further studies involving RT-PCR analysis for neuropeptide and thymic hormones expression on purified subset of *Xenopus* TEC and thymocytes should be performed (28).

In conclusion, we show that the *Xenopus* thymus is likely to possess neuroendocrine activity, as judged by the expression of various neuropeptides and thymic hormones. Given the evolutionary conserved intrathymic expression of neuropeptides these findings suggest that they may play an important role in T-cell development and provide further evidence of cross talk between the immune and neuroendocrine systems. Moreover, it places *Xenopus* on a list of candidate animal models to study the effects of neuropeptides and hormones in T cell development. The disclosure of neuropeptide mode of action could provide important strategies for the treatment of immune disorders such as various immunodeficiencies, autoimmunity and thymic dysfunctions.

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Abbreviations: Ab: antibody, CGRP: calcitonin-gene related peptide, ConA: Concanavalin A, cAMP: cyclic adenosine monophosphate formation, ECM: extracellular matrix, GH: growth hormone, IFN: interferon, MHC: major histocompatibility complex, mAb: monoclonal antibody, NK1 R: neurokinin 1 receptor, NPY: neuropeptide Y, NPY Y1 R, neuropeptide Y Y1 receptor, PBS: phosphate buffered saline, RT-PCR: reverse transcription polymerase chain reaction, SOM: somatostatin, SP: substance P, TEC: thymic epithelial cell, TMPO: thymopoietin, UEA-1: Ulex Europeaus Agglutinin 1, VIP: vasointestinal polypeptide, VIP R1, vasointestinal polypeptide receptor 1.

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