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REVIEW

Neuroendocrineimmunology (NEI) at the turn of the century: towards a molecular understanding of basic mechanisms and implications for reproductive physiopathology

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The interactions between the nervous, endocrine and immune systems require a complex communication network. The central nervous system (CNS) affects the immune system through endocrine, paracrine and neuronal mechanisms. Evidence that this bidirectional communication plays a vital role in the regulation of physiological homeostatic mechanisms while a disfunction of the neuroendocrineimmune balance favors the susceptibility to a number of diseases is derived largely by animal models but also by an increasing number of clinical studies in different fields, including endocrinology, reproductive physiology, pediatrics, oncology, neurology and psychiatry. An increasing number of endocrine hormones, neurotransmitters and neuropeptides are expressed in immune tissues and cells and are actively involved in the physiological regulation of immunity. Conversely, the endocrine and nervous systems harbor receptors for a wide variety of immunologically-derived substances, suggesting potential regulatory feedback loops between the three major integrative bodily systems. Major implications for the reproductive endocrinology field are that psychoneuroendocrine processes may alter fertility via immunomodulation, and that events that occur as part of immune responses influence the neuroendocrine axes, which in turn counter-regulate immune function. In the present article, some features of reproductive-immune interactions will be described, and the neuroendocrineimmune dialogue via the chief reproductive hormone, luteinizing hormone-releasing hormone (LHRH), will be summarized as prototype of intersystem crosstalk. A particular emphasis will be given to the cytokine-LHRH interrelationships both at central (i.e. especially with the astroglial compartment) and peripheral levels. The surprisingly similar communication network systems used by the gonads and the thymus will be summarized, and the sexually-driven dimorphisms dictating female versus male reproductive and immunological capacities reviewed. Evidence that neural, endocrine and immune systems work together as a single unit are emphasized in animal models and human pathologies where interruption of NEI feedback loops results in long lasting pathological consequences for the nervous, endocrine and immune functions.

Keywords: nervous system; endocrine system; immune system; luteinizing hormone-releasing hormone (LHRH); LHRH receptors; thymus

Introduction

At nearly two decades from its 'birth' (Pierpaoli & Sorkin, 1967), neuroendocrineimmunology (NEI) which is no longer a 'teenager', having acquired a certain degree of maturity, leaves the phenomenological approach to embrace a molecular dimension. The turning point in this emerging discipline certainly represents the acquisition of the 'molecularity' in looking at the different aspects of this intersystem crosstalk. Recent research accumulated in the last ten years strongly supports the notion that neuroendocrine peptides are 'endogenous' to the immune system and as such are used both for physiological regulation of immune cell activity as well as for bidirectional communication between the neuroendocrine and immune systems (Blalock, 1984; Blalock & Smith, 1985, Blalock, 1987; 1990; 1992; 1994; Dunn, 1990; Pierpaoli & Maestroni, 1988; Pierpaoli & Spector, 1988; 1991; 1994; Spector, 1990a,b; MacCann *et al.*, 1990; 1993; 1994; Yankovic, 1994; Pierpaoli, 1994; Maier *et al.*, 1994). Conversely, neuroendocrine cells are able to express a variety of immune products such as the cytokines and other growth factors of immunological origin, together with their cognate receptors (Farrar *et al.*, 1987; Takao & De Souza, 1993; Blalock, 1994). The field of lymphokines and Cytokines is continuously expanding with an increasing number of new lymphokines and monokines. A hallmark of the soluble mediators in the immune system is to be multifunctional, and then, it will not surprise that these pluripotent molecules also have many systemic effects (Smith, 1988; 1992; Dunn, 1990; Besedowsky *et al.*, 1981; 1985). It would appear that within the immune and neuroendocrine systems, the normal functioning of these cytokines is often dependent upon multiple (neuroendocrine/immune) signals generated by the tissue-specific microenvironment that will dictate the appropriate cellular response. The production and expression of a wide variety of peptide hormones and neurotransmitters by the cells of the immune system is surprisingly increased. From corticotropin (ACTH) (Smith and Blalock, 1981; Harbor *et al.*, 1985; 1987; Lolait *et al.*, 1985) the first neuroendocrine hormone to be described in the immune system, to suppressin (LeBoeuf, 1994), a negative regulatory molecule in the neuroendocrine and immune systems, the production and regulation of these peptide hormones seems remarkably like that observed in neuroendocrine cells.

Although a number of noteworthy differences appear to regulate the peptide hormones of immunological origin, in view of their capacity to regulate immune functions and to convey informations to the neuroendocrine system, they represent the sensory function of the immune system (Blalock, 1984).

Importantly enough the cells and organs of the immune system are richly innervated by the sympathetic and parasympathetic branches of the autonomous nervous system, and a number of reviews have appeared on this subject (Ackerman *et al.*, 1991; Belinger *et al.*, 1989; 1990; Bulloch & Moore, 1981; Bulloch & Pomerantz, 1984; Bulloch, 1987; Felten *et al.*, 1985; Felten & Felten, 1992; Kendall *et al.*, 1994; Clarke & Kendall, 1994). The extensive neuro-

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immune anatomical connection between nervous and immune cells is emphasized by the close contacts of nerves with macrophages and lymphocytes. Not only the classical neurotransmitter noradrenaline, but a number of neuropeptides including vasoactive intestinal peptide (VIP), somatostatin, substance P (SP), calcitonin gene related peptide (CCRP), neuropeptide Y (NPY) and opioid peptides have been identified in nerves in spleen, lymph nodes, thymus and/or other lymphoid tissues (see Felten *et al.*, 1992; Geenen *et al.*, 1986; 1987; 1994; Clarke & Kendall, 1994). The presence of receptors for catecholamines, glucocorticoids (Marchetti *et al.*, 1990d,e; Morale *et al.*, 1992a,b; Marchetti *et al.*, 1994a; Peiffer *et al.*, 1994; Morale *et al.*, 1995) and peptides on these cells (see Blalock, 1992; 1994) coupled with functional evidence that these neural signals can modulate immune responses (Morale *et al.*, 1992a,b) and are subjected to hormonal control (Marchetti *et al.*, 1990d,e; 1994a; Morale *et al.*, 1995), bring this class of molecules at the very edge of the NEI field and the study of alterations in this circuitry is currently under scrutiny to characterize peripheral markers of altered central nervous system (CNS) activity (Morale *et al.*, 1992b).

The biochemical and molecular mechanisms responsible for NEI crosstalk have been deeply examined in this last decade and the fact that cells of the immune system not only produce most if not all the neuroendocrine hormones but also exhibit and express their receptors certainly adds a further biochemical and molecular dimension in this bidirectional signaling system (Carr & Blalock, 1989; Carr *et al.*, 1989a,b). The field has been variously reviewed by different investigators (see Carr & Blalock, 1990; Carr, 1992), and a variety of receptor systems including arginine vasopressin, corticotropin, β-endorphin, growth hormone, nerve growth factor, opioid, prolactin, somatostatin, SP, thyrotropin, VIP, CCRP and other hypothalamic peptide receptors, (see Blalock, 1994). The integrated way by which neuroendocrine hormones can modify immunity and specify the receptors and post-receptor signaling pathways used for this crosstalk has been disclosed in recent years, and appears surprisingly similar to that used by the endocrine cell (see Roszman & Brooks, 1992; Marchetti *et al.*, 1994a).

A recent area of particular interest in neuroendocrine-immune relationships within the CNS is the one of immunological reactions in the brain (see Beneviste, 1992; McGeer & McGeer, 1994; Hefti, 1994). There is increasing evidence that soluble factors from lymphoid/mononuclear cells are able to modulate the growth and function of cells found in the CNS. Specifically macroglia and microglia cells. Furthermore glial cells can secrete immunoregulatory molecules that influence immune cells, as well as the glial cells themselves (Beneviste, 1992; McGeer & McGeer, 1994). On the other hand, glia cells harbor receptors for a number of neuroendocrine peptide hormones, neurotransmitter molecules as well as growth factors and neurotrophic factors (see Murphy & Pearce, 1987; Hefti, 1994). Thus, the potential exists for bidirectional communication not only between lymphoid cells and glial cells, but also between neuronal cells and glial cells (see Marchetti *et al.*, 1995b,c; Gallo *et al.*, 1995a-c).

In the present article some features of reproductive-immune interactions will be described with a particular attention to one principal system, i.e., the luteinizing hormone-releasing hormone (LHRH) receptor-signaling system, acting as 'primum movens' of the cascading actions and interactions concerned with reproduction. The advances in reproductive-immunology will be summarized by following the achievements in LHRH-immune field, from the biochemical to the molecular characterization of a paracrine intralymphocytic LHRH system involved in physiological control immune physiology, and at the same time serving as channel for bidirectional communication with the nervous-reproductive axis. The teleological assumption that the chief hormone governing the fertility capacity of a living organism 'should' directly signal the master gland of the immune system, i.e. the thymus (see Marchetti *et al.*, 1988b, 1989a-c), is now corroborated by a number of biochemical, molecular as well as clinical evidences, all supporting the concept that this neuropeptide, may, indeed, behave as immunological response modifier. The exquisite functional interplay between the LHRH system and the immune 'world' is reflected at the CNS level, where a bidirectional functional interaction between the differentiating LHRH neuron and the maturing astroglial cell has been for the first time demonstrated (Gallo *et al.*, 1993; 1994; 1995a-c). Of importance, the link between reproductive and immunological functions is underlined when the study of the molecular mechanisms involved in sexually-driven immunological functions disclose the involvement of three major 'target' genes participating in estrogen action at the thymus gland level. The neuroendocrineimmune consequences of disruption of NEI communications are emphasized in animal models as well as in human pathologies.

1985-1995: the era of neuroendocrinology of reproduction switches to neuroendocrineimmunology

The purification, sequencing, and synthesis (Amoss *et al.*, 1971; Schally *et al.*, 1971; Matsuo *et al.*, 1971) of the decapeptide, LHRH, has certainly represented a major transition point in the study of the neuroendocrinology of reproduction. Indeed, the hypothalamic coordination of a series of control mechanisms subserving the reproductive capacity of mammals by LHRH represents an extraordinarily complex relationship in time and function among a number of structures anatomically distant. A number of events including changes in the activity of the trophic structures in the brain and pituitary that control the gonadotropins; the maturation of an ovum and ovulation, the associated changes in the sex steroid background that periodically prepare the endometrium for implantation of an embryo, are dynamically orchestrated by the chief reproductive hormone, LHRH, interacting with specific receptors located in the neuroendocrine-reproductive axis (Clayton & Catt, 1981; Naor *et al.*, 1981; Marchetti and Labrie, 1982; Marchetti *et al.*, 1982; 1983a,b; Conn *et al.*, 1984; Conn, 1986; Naor, 1990; 1995; Conn & Crowley, 1991; Krsmanovic *et al.*, 1993; Yankovick & Conn, 1994; Stojikovic *et al.*, 1994).

On the other hand, specific mechanisms responsible for protecting the mammalian embryo against the potentially hostile immunological maternal environment are known to occur, and appear to vary according to different stages of reproduction, i.e. during the menstrual cycle, and from fertilization to implantation and to full development of the fetus (Hodgen & Itskovitz, 1988; Gill, 1988; Sargent, 1993; Ussa *et al.*, 1994; Lahita, 1994). They are also unique since they vary from species to species and result from exceptional genetic and immunoligical processes (Gill, 1988). At a century from Calzolari (1898) first observation of a functional link between the thymus gland and the gonads, we are now facing the molecular evidence of the presence of LHRH messenger ribonucleic acid (mRNA) transcripts which are identical to hypothalamic LHRH within the primary organ of the immune system (Maier *et al.*, 1992; Wilson *et al.*, 1995). Such finding will not surprise if one recalls that survival of a living organism depends, besides upon environmental conditions and the availability of nutrients, upon a. the ability to prevent invasion by other organisms and b. the presence of a perfectly operative reproductive axis for the successful completion of reproduction and species perpetuation. Then, the recognized interconnections linking the three major integrative bodily systems are likely to play a major role especially in the control of fertility, to dictate the required homeostatic

adjustments during each phase and at every level of a reproductive process.

LHRH is the central and peripheral orchestrator of reproductive events

Even though originally recognized as a hypothalamic peptide acting on gonadotrophs, LHRH has been demonstrated to have extrapituitary direct effects on both ovary and testis in rats (Hsueth & Jones, 1981; Hsueh & Schaeffer, 1985; Labrie *et al.*, 1982a,b; Tremblay *et al.*, 1985; Bramley *et al.*, 1985; 1986). LHRH and LHRH-like peptides are found in extrapituitary tissues including the ovary, placenta, mammary gland, testis, prostate, pancreatic islets and thymus gland and spleen (Khodr & Siler Khodr, 1978; Koch & Baram, 1977; Seppala *et al.*, 1970; Belisle *et al.*, 1984; Miller *et al.*, 1985; Ireland *et al.*, 1988; Behrman *et al.*, 1989; Emanuele *et al.*, 1990; Azad *et al.*, 1991). Although isolation of LHRH or LHRH-like peptide has not been accomplished in all these tissues, low levels of mRNA for LHRH have been described by the use of reverse transcription polymerase chain reaction (Oikawa *et al.*, 1991; Goubau *et al.*, 1992; Clayton *et al.*, 1992; Maier *et al.*, 1992; Wilson *et al.*, 1995). LHRH has been shown to act through similar specific receptors in extrapituitary tissues, but with different tissue-and specie-specific affinities (Eidne *et al.*, 1987; Lamberts *et al.*, 1982; Labrie *et al.*, 1982a,b; Marchetti *et al.*, 1987b,c; Pahua *et al.*, 1989; Fekete *et al.*, 1989; Qayum *et al.*, 1992; Krsmanovic *et al.*, 1983; Whitelaw *et al.*, 1995). Evidence for the implication of the decapeptide in all the reproductive phases concerned with reproduction came from the isolation of LHRH-like material within the mammary gland, milk and mammary tumors coupled to the presence of LHRH receptors and action of LHRH-A in breast tumors and carcinoma cell lines such as MCF-7, NMDA-M-231 and ZR-75 (see Eidne *et al.*, 1987; Krsmanovic *et al.*, 1993).

Interestingly enough, in a number of peripheral organs including ovary, testis, prostate and mammary gland, the LHRH system coexists with a wide number of peptides, hormones, and neurotransmitter substances, that in some instances may mimic the complexity of interactions reached at the hypothalamic level (see Marchetti *et al.*, 1985b; 1986; 1987a-c; Marchetti *et al.*, 1990f). In the ovary, direct neural pathways control different aspects of ovarian function from the development of compensatory ovarian hypertrophy to the production of sex steroid, and maturation of ovarian activity (see Gerendai *et al.*, 1978; 1979; Kawakami *et al.*, 1981; Aguado & Ojeda, 1984; Marchetti *et al.*, 1985b; 1986; 1987; Marchetti & Cioni, 1988; Lara *et al.*, 1990; Barria & Lara, 1991). In particular, the direct sympathetic innervation has been shown to directly modulate the maturation of the intraovarian LHRH receptor (LHRH-R) and β_2 adrenergic receptor (β_2AR) systems during the onset of puberty (Marchetti *et al.*, 1987a) as well as during the establishment of ovarian failure secondary to the aging process (Marchetti *et al.*, 1986; 1987c). The functional importance of the direct neural input to the ovary was recently supported (Lara *et al.*, 1993; Barria *et al.*, 1993) by the demonstration that an activation of sympathetic neurons innervating the ovary precedes the development of polycystic ovary syndrome.

Interestingly enough, receptors for β_2-adrenergic (β_2AR) agonists and for LHRH, are compartmentalized during the various phases of the rat estrous cycle, with a maximal distribution in corpora lutea and granulosa cells for β_2AR and LHRH-R, respectively (Figure 1). Using *in situ* hybridization it was recently demonstrated the presence of LHRH receptor mRNA expression in the granulosa cells of most follicles (principally in granulosa cells from follicles at all stages of development and the corpus luteum, Whitelaw *et al.*, 1995). Such findings are in accordance with previous data obtained using conventional radioreceptor techniques (Seguin

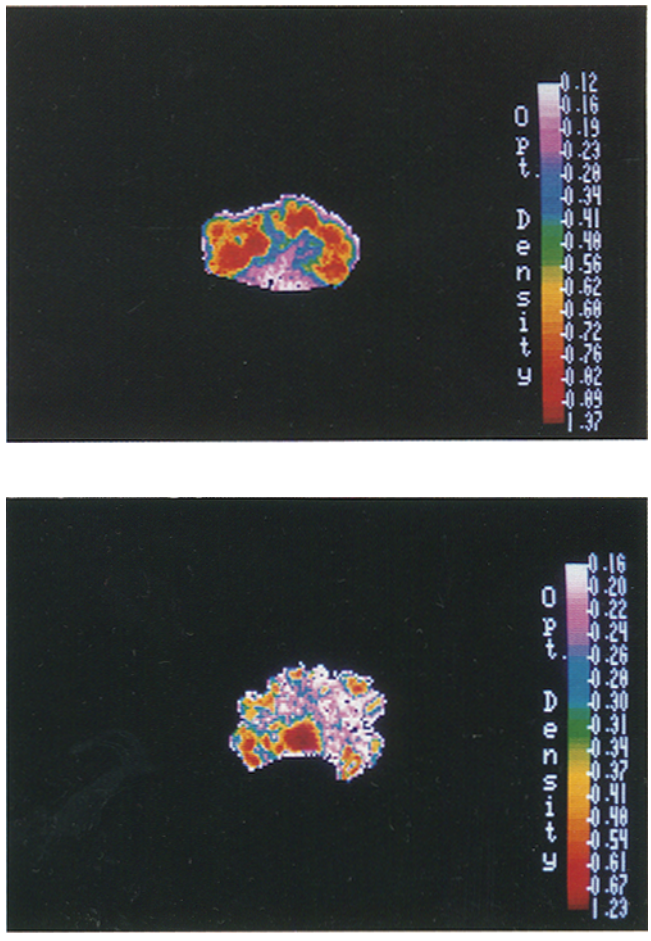


Figure 1 Radioautographic localization of LHRH receptors and β_2 adrenergic receptors in the rat ovary at estrus. A loats image analysis system was used to analyze the differences in receptor localization. As observed in the right upper corner of each picture, cold colors including blue, violet and rose, indicate the absence or a low density of receptor, while green, yellow, orange and red colors represent progressively the increase in receptor density. Note the compartmentalization of LHRH (upper panel) and β_2AR (bottom panel) receptors, with a high density of β_2AR within the corpus luteum of estrus, while the LHRH-R surround that area

et al., 1982; Labrie *et al.*, 1982a,b). A potential crosstalk between the intraovarian LHRH and adrenergic systems at the ovarian level is further corroborated in hypophysectomized rat models, where removal of the sympathetic input to the ovary results in a complete loss of LHRH-R. (Figure 2). The majority of the direct gonadal actions of the LHRH-like peptide are inhibitory. In particular, in the ovary, these receptors block many of the trophic actions of gonadotropins including follicle growth and differentiation, and because treatment with LHRH or its agonist analogues antagonizes the gonadotropin stimulation of follicle development, LHRH has been suggested to induce atresia in the ovary (Birnbauer *et al.*, 1985). Recent studies have demonstrated that apoptotic cell death (Hughes & Gorospe, 1991) is associated with follicular atresia in chicken, porcine and rodent ovaries, and Billig and coworkers (1994) have recently shown that LHRH-A directly induce apoptosis in the ovaries of hypophysectomized estrogen-treated rats, via a mechanism involving ovarian LHRH receptors and in specific ovarian compartment, i.e. the granulosa cells. The question, then, arises as to whether the decapeptide might have a direct balancing negative function, and whether it may serve as channel for bidirectional communication with the intragonadal immune system.

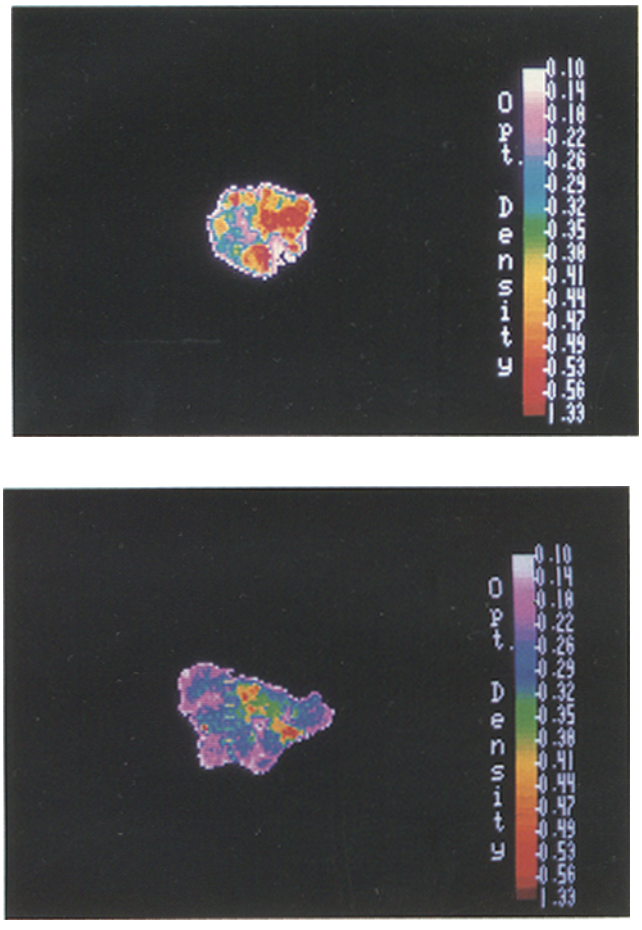


Figure 2 Effect of unilateral denervation in the distribution of LHRH receptors within the ovaries of hypophysectomized rats. Radioautographic localization of LHRH receptors within the intact (**A**) and the denervated (**B**) ovary of an immature hypophysectomized rat. Complete disconnection of the ovary from the superior ovarian nerve (SON) and ovarian plexus (OP) was carried out under light ether anesthesia in immature hypophysectomized female rats. Ovarian denervation was carried out unilaterally, with the contralateral ovary serving as control (see Marchetti *et al.*, 1987). Note the profound loss of LHRH receptors within the deafferented (**B**) gland

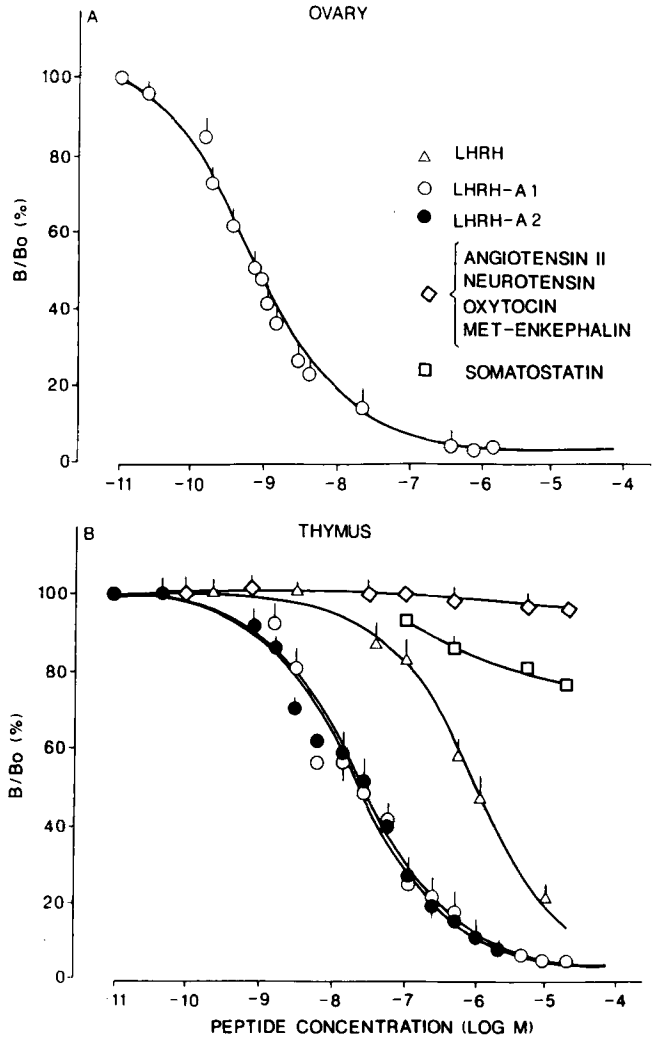


Figure 3 Computer-modelled curves of competition for [^{125}I]-Buserelin binding to rat thymic and ovarian membrane preparations by LHRH-agonists (LHRH-A) and different peptides. ○ LHRH-A 1: [D-Ser(TBU^6), Des-Gly^{10}]LHRH-N-ethylamide; ● LHRH-A2: [D-Tryp^6]Des-Gly^{10}-LHRH-N-ethylamide; △ LHRH; ◇ the indicated peptides. The curves were drawn after substraction of a non-specific (non-displaceable) binding remaining after addition of unlabelled buserelin (2×10^5 M^{-1}). ED_{50} values were calculated as described previously (from Marchetti *et al.*, 1989b)

Biochemical and molecular identification of LHRH and LHRH receptors in immune organs and cells: a further homeostatic level of integration

The first step in the action of the hypothalamic decapeptide is its binding to cognate receptors located in the plasma membrane of a number of central and peripheral structures. Specific LHRH binding sites are present in mouse blood lymphocytes (Marchetti *et al.*, 1988b), in rat mast cells (Sundaram *et al.*, 1988), thymocyte (Marchetti *et al.*, 1989a-c; Figure 3), and splenocyte (Costa *et al.*, 1990) cultures, as well as in cultured porcine lymphocytes (Standaert *et al.*, 1992). In addition, LHRH mRNA and/or LHRH-like molecules have been identified in rat thymus, in thymocyte and splenocyte cultures and in human peripheral T lymphocytes (Emanuele *et al.*, 1990; Azad *et al.*, 1991; 1993; Maier *et al.*, 1992). Moreover, by synthesizing and sequencing the rat thymus LHRH thymocyte and hypothalamic LHRH are shown to be identical (Figure 4), with the sequence data obtained 5′ and 3′ to the open reading frame being also identical to hypothalamic LHRH mRNA (Maier *et al.*, 1992). Not only LHRH mRNA is expressed in the primary immune organ, but it also appears to be developmentally regulated (Maier *et al.*, 1992). Of interest, using the rat immature T cell line Nb2, Wilson *et al.* (1995) have recently reported that the LHRH gene is regulated by PRL, at various times during the cell-cycle. Moreover, an alternatively spliced LHRH mRNA exists in Nb2 cells and may produce a new truncated gonadotropin-associated peptide (GAP) (Wilson *et al.*, 1995). Finally, the SH gene found on the opposite strand of the LHRH gene, is expressed in lymphocytes at the same time and in the same manner as the LHRH gene (Wilson *et al.*, 1995).

With the cloning and sequencing of the LHRH receptor (Tsutsumi *et al.*, 1992; Reinhart *et al.*, 1992; Perrin *et al.*, 1993; Kakar *et al.*, 1993), mRNA transcripts coding for the LHRH receptor were demonstrated to be present in immunologically competent cells (Wilson *et al.*, 1995). Using reverse transcription polymerase chain reaction (RT-PCR) and RNA from Nb2 cells the LHRH-R PCR fragment was sequenced and cloned and the DNA sequence shown to be identical to that previously described (Eidne *et al.*, 1992). In analogy to what observed at the anterior pituitary level for the LHRH-R protein (Marchetti & Labrie, 1982), in Nb2 cells the LHRH receptor mRNA was shown to drastically decrease immediately after PRL administration, suggesting

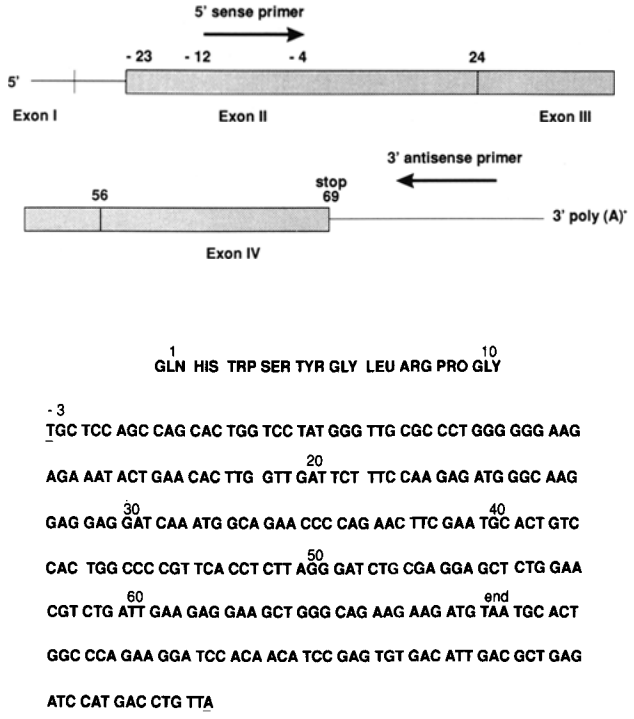


Figure 4 Rat hypothalamic pre-pro LHRH mRNA map (**A**) and sequence of the cloned LHRH PCR product (**B**). (**A**). The open reading frame (box) of the LHRH gene spans three exons. The primers used in PCR are represented as arrows and amplify a 332 basepair product from the mature LHRH mRNA. Aminoacid positions are numbered according to Adelman *et al.* (1986). (**B**). The nucleotide sequence of the cloned LHRH PCR product from thymus derived RNA is identical to the rat hypothalamic LHRH. The lack of introns indicate the cDNA was synthesized from mature LHRH mRNA, not contaminating genomic DNA. The animoacids are numbered and the sequence of the mature LHRH decapeptide is underlined (from Maier *et al.*, 1992)

possible paracrine regulation between the two hormones within immune cells (Wilson *et al.*, 1995).

LHRH as immunological response modifier

The idea that LHRH may be considered as an immunological response modifier with a specific tropism for the immune cells is derived by an increasing number of findings supporting a direct non-steroid-mediated effect of the hypothalamic decapeptide. Considerable evidence has been provided that LHRH and its agonist and antagonist analogs are able to influence immune functions, either with or without the intermediacy of the pituitary gland and/or the gonads. In adult rats, LHRH and its analogs have been shown to increase the absolute and relative thymic weight (Greensteen *et al.*, 1987; Marchetti *et al.*, 1989b,c; Ataya *et al.*, 1989; Blacker *et al.*, 1991) and to influence thymus weight, thymic morphology and thymocyte proliferative activity of hypophysectomized male rats (Marchetti *et al.*, 1989b). The ability of LHRH and its agonists to influence thymocyte proliferative potential was also demonstrated both in '*ex vivo*' as well as *in vitro* models. More importantly, LHRH agonist (LHRH-A) treatment of aging rats results in a significant increase in thymus weight and restoration of the morphological appearance of the gland (Greensteen *et al.*, 1987; Marchetti *et al.*, 1989c), and to reverse the aging-induced impairment of thymocyte proliferative capacity and IL-2 receptor expression in response to policlonal T-dependent mitogens (Marchetti *et al.*, 1989c; Marchetti *et al.*, 1990b,d; 1991). The biochemical mechanism of action of LHRH after the binding of the neuropeptide to its receptor present in the immune cell, appears to be the mobilization of polyphosphoinositide hydrolysis, translocation of the protein kinase C (PKC) and up-regulation of the interleukin 2 (IL-2) receptor expression (Batticane *et al.*, 1991; Figure 5). The physiological significance of the LHRH system within the rodent thymus has been also investigated (Marchetti *et al.*, 1990a; Morale *et al.*, 1991). Treatment of neonatal rats with a very potent LHRH-antagonist resulted in irreversible alterations in a series of immune parameters including thymus morphology, cell-mediated and humoral immune responses (Marchetti *et al.*, 1990a; 1991; Morale *et al.*, 1991). Conversely, the chronic treatment of nude (immunodeficient) mice with LHRH during the neonatal period resulted in both the restoration of reproductive and immune capacities (Marchetti, Morale & Pierpaoli, unpublished observations). Sequential changes in lymphocyte subpopulations during long-term treatment with LHRH-A in post-puberal rats were also described by Rao *et al.* (1993). Changes of intrathymic LHRH mRNA are observed as a function of the stage of the estrous cycle and pregnancy (see next sections), clearly underlying a physiological role of the hypothalamic decapeptide during the known changes of thymus-dependent immune functions observed in those conditions. Moreover, the fact that an LHRH-antisense (Marchetti, Maier, LeBoeuf & Blalock, unpublished observations) is able to counteract the mitogen-induced lymphocyte proliferation, clearly underlines a physiological paracrine/autocrine role of intralymphocytic LHRH in the control of lymphocyte responsiveness.

Of special interest, Glass (1991) reported a highly conserved sequence near the N-terminus of all human (HIV) and simian (SIV) immunodeficiency virus gag polyproteins that appears to be a precursor for a viral mimic of the amidated C-terminus of human LHRH. The gag polyproteins are known to be myristylated and processing of the amidation site would yield myristylated 23-residues peptides whose C-terminal sequence mimic LHRH and presumably behaves as a ligand for the LHRH receptor (Glass, 1991). The discovery of conserved LHRH-precursor-related sequences in HIV and SIV gag polyproteins and in the p-17 core proteins derived from them, would suggest that entry of the viral genomic RNA into hosts cells through the LHRH receptor may occur (Glass, 1991). This proposed mechanism of entry of HIV genomic RNA into host cells, if it proves to be correct, would suggest several new approaches to prevention and treatment of acquired immunodeficiency syndrome (AIDS) (Glass, 1991).

Other informations came from the study of Jacobson *et al.* (1994), demonstrating the ability of LHRH-A to modulate the expression of murine lupus in a gonadal steroid-independent fashion, since castrated (SWR X NZB)F1 mice treated with LHRH antagonist displayed decreased total IgG and anti-DNA antibody concentrations, delayed renal disease, and significant prolongation survival (Jacobson *et al.*, 1994). LHRH agonist might act on the immune system directly, by a direct effect on B- or T-lymphocytes. Alternatively the effects might be indirect, through a reduction in gonadotropins or alterations in cytokine production by immune cells. The fact that LHRH analogs are able to modulate murine lupus independently of effects on sex hormone production is of special interest, since they raise the possibility that hormones other than gonadal steroids might contribute to the well known gender differences in expression of autoimmune diseases.

Cytokine-LHRH interactions

The commonality of signaling systems used by immune and neuroendocrine cells is also exemplified by the powerful interaction between the cytokines and the LHRH system, both at central and peripheral levels. In particular, interleukin 1 (IL-1), one of the key mediators of immunological

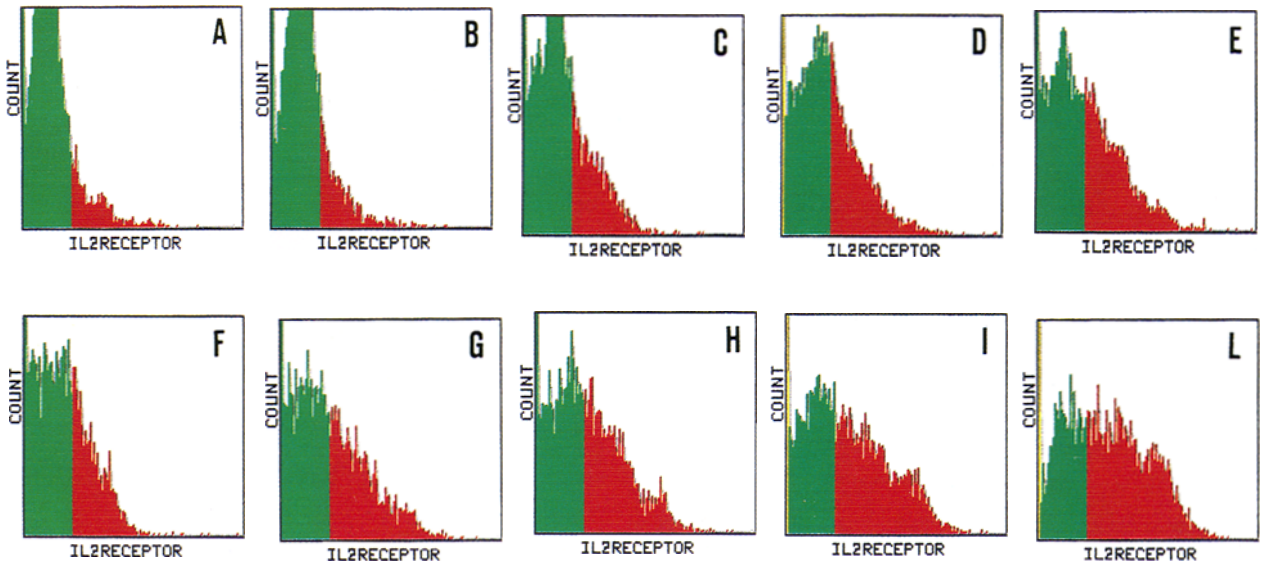


Figure 5 Flow cytometric analysis of thymocytes from proestrus rats. Cell cultures were treated and stained as described (see Batticane *et al.*, 1991). Samples passed on a flow cytometer (Facscan, Becton & Dickinson), gated to exclude non viable cells. (**A**) cell incubated with the medium alone, (**B–E**) cells incubated with concanavalin A (0.3, 0.6, 1.25, and 2.5 μg/ml); (**F**) cells incubated with LHRH (10^{-7}M) alone, (**G–L**) cells activated with 0.6 μg/ml Con-A and increasing (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}M) doses of LHRH. The area containing IL-2 receptor positive cells is outlined by the triangular red box (from Batticane *et al.*, 1991)

responses to stress, infection and antigenic challenge (see Dinarello, 1988; Takao & De Souza, 1993), has been shown to powerfully interfere with the hypothalamic-hypophyseal-gonadal axis (HHGA). At the CNS level, when administrated in an acute fashion. IL-1 has been shown to decrease plasma LH levels, a phenomenon attributed to the inhibition of hypothalamic secretion of LHRH and LHRH gene expression (Rivier & Vale, 1989; Kalra *et al.*, 1990; Rivier, 1990; Rivest *et al.*, 1993). That IL-1 represents an extremely potent factor inhibiting the activity of the HHGA is supported by different evidences. Interleukin-1α inhibits pulsatile release of LH, via a direct action on the LHRH neurons by suppressing the release of prostaglandin E_2 (PGE_2) from the medio-basal hypothalamus (MBH) (Rettori *et al.*, 1991; McCann *et al.*, 1994). Moreover, inhibition of the physiological or experimentally induced afternoon proestrus LH surge follows IL-1 administration (Rivest *et al.*, 1993), together with expression early c-fos gene which occurs within the LHRH cell nuclei during this same period of the cycle (Rivest *et al.*, 1993). The intermediacy of nitric oxide in IL-1-α control of LH *in vivo* and *in vitro* has been recently established (Rettori *et al.*, 1994; McCann *et al.*, 1994). In addition, it was demonstrated that when HHGA is chronically exposed to icv infusion of IL-1β a complete disruption of the estrous cycle, decreased biosynthesis/release of hypothalamic LHRH and gonadotropins was accompanied by a block in luteolisis of newly formed corpora lutea (CL) (Rivier & Erickson, 1993). It would, then, appear that according to the stage of the estrous cycle, the peptidergic and aminergic background, a number of potential interactions between the cytokines and the central LHRH system, may be envisaged.

LHRH-astroglial interactions

Interleukin-1 has been shown to be present in the cerebrospinal fluid, IL-1 mRNA is detected in normal brain and IL-1β-like immunoreactivity in both hypothalamic and extrahypothalamic sites in human brain have been identified (see Takao & De Souza, 1993). A major compartment of cytokine production is, however, represented by astroglial and microglial cells (see Fontana *et al.*, 1982; Beneviste, 1992; McGeer & McGeer, 1994). The capacity of astroglial cells to acquire major histocompatibility complex (MHC) antigens and function as antigen presenting cells within the CNS is well recognized, thus influencing immune reactions by their production of various agents that signal the immune system. Of major interest are the implications of these functions, cytokine secretion and antigen presentation, by glial cells, with respect to intracerebral immune responses, demyelination and inflammation in neurological diseases that have an immunological component (see Beneviste, 1992). Astroglia in culture can be induced to express MHC glycoproteins of class I and II by stimulation with gamma-interferon (γ-IFN), or tumor necrosis factor (see McGeer & McGeer, 1994). Astrocyte, for example, in culture, can be induced to secrete a variety of cytokines and growth factors including colony stimulating factor 1, which markedly stimulates the proliferation of macrophages (see McGeer & McGeer, 1994). The eicosanoids produced by astrocytes (for review see Murphy *et al.*, 1992) may also influence immune regulation (Figure 6). In turn, the interleukin family of growth factors alter powerfully astroglia cell physiology.

On the other hand, since astroglia is involved in the processes of neuronal-guided migration during embryology, another important facet of neuron-glia interactions involves the phenomena of axon guidance and target recognition, achieved by highly specific chemical mechanisms using diffusible trophic factors, cell surface and extracellular matrix molecules which allow tropism and cell-cell interactions (Hatten, 1993; Wang *et al.*, 1994). Indeed, the LHRH neuronal cell system appears to be unique among all neuropeptide expressing genes in the CNS, to make a migration pathway from the epithelium of the medial olfactory pit into the developing basal forebrain (Schwanzel-Fukuda *et al.*, 1987; 1992a,b). Failure of LHRH neuronal migration as in Kallmann's syndrome results in a suppression of the pituitary-gonadal axis (see Schwanzel-Fukuda *et al.*, 1992a,b). That glial elements contributed to LHRH axonal targeting was suggested by the early experiments of Kozlowski & Coates (1985) demonstrating the existence of ependymal tunnels and their association with LHRH axons. More recently, relationships of glia with LHRH axonal outgrowth have been described by Silverman and coworkers (1991).

Recent evidence suggest that astroglial cells represent a possible target and source of signals for the LHRH neuronal

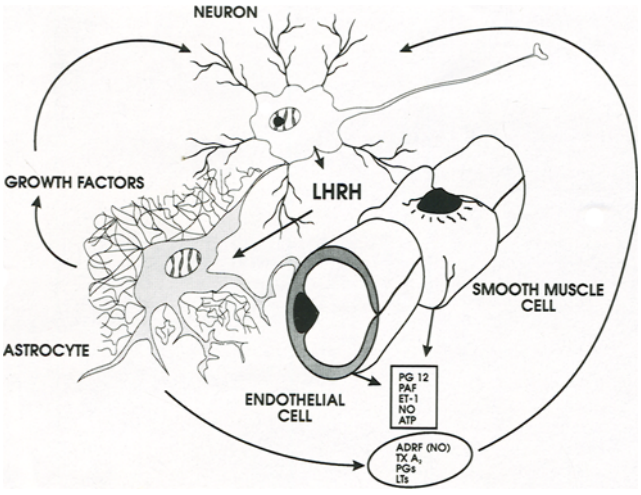


Figure 6 Dynamic interaction between the astroglial cell compartment, the endothelial and the neuronal cell. Upon selective stimulation astrocytes may release products able to alter the vascular endothelium. The expression of receptors on astrocytes, their ability to synthesize vasoactive products, and the close spatial relationships of these cells both with neurons and cell of the vasculature implicate astroglial cells in bi-directional signalling processes in the CNS. PG: prostaglandin, PAF: platelet activating factor, TXA_2: tromboxane, NO: nitric oxide, ATP: adenosin triphosphate, ADRF (NO): astrocyte-derived (vaso)-relaxing factor (nitric oxide). The potential interaction between the growths factors released by the astroglial compartment and LHRH released by neuron terminals is also illustrated

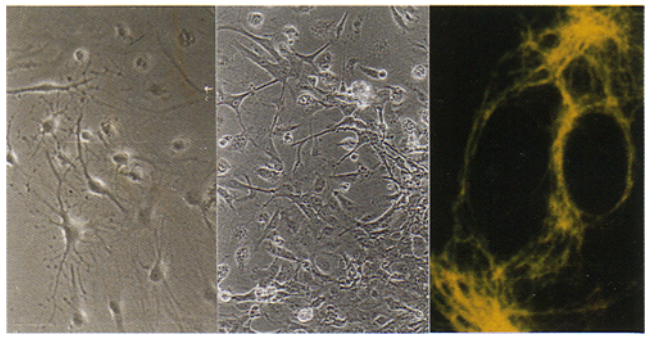


Figure 7 Photomicrograph of rat protoplasmatic and fibrous astrocytes. Primary rat astrocytes were prepared and isolated from cerebral hemispheres and cultured as described (Gallo *et al.*, 1995a) during maturation and differentiation *in vitro*. Cytoplasmatic staining performed on fixed cells with monoclonal antibody to glial-fibrillary protein, followed by incubation with FITC-conjugated goat-anti-mouse IgG (right corner panel); 35–40 days *in vitro* astrocytes (middle panel); 12 days *in vitro* astrocyte (left panel)

machinery (Olmos *et al.*, 1989; Garcia-Segura *et al.*, 1989; Torran-Aleman *et al.*, 1991; Langub *et al.*, 1992; Ma *et al.*, 1992; Junier *et al.*, 1993; Ojeda *et al.*, 1993; Duena *et al.*, 1994). Glial mechanisms are hypothesized to be involved in the marked changes in synaptic and interneuronal organization that occur in the supraoptic and paraventricular nuclei under various physiological conditions (see Gracia Segura *et al.*, 1989). Other studies (Ojeda *et al.*, 1990; Ma *et al.*, 1992; Junier *et al.*, 1993; Ojeda *et al.*, 1993) suggest a key role of neurotrophic factors, possibly of astroglial origin in the stimulation of LHRH release and induction of precocious puberty after lesions of the female rat hypothalamus. It seems then likely that besides offering a genetic pathway for neuronal migration, the glial network might provide other functional informations modulating neuronal maturation and differentiation.

As recalled, cytokines have recently shown to influence hypothalamic LHRH machinery (see also Marchetti *et al.*, 1995a-c). The ability of endotoxin to induce release of IL-6 from the medio-basal-hypothalamus (MBH) has been demonstrated by Spangelo and coworkers (1990). Moreover, hypothalamic LHRH neurons (Yamaguchi *et al.*, 1991) spontaneously secrete IL-6, and in turn exogenous IL-6 is able to stimulate LHRH release in a dose- and time-dependent fashion. A potential bidirectional communication between astroglial cells and LHRH neurons has been also disclosed (Gallo *et al.*, 1992; 1993; 1994; 1995a-c). Indeed, astroglia (Figure 7) may play a crucial regulatory function through the release of products able to alter LHRH neuronal morphology (see Figure 8), the LHRH intracellular secretory machinery and/or proliferation (Gallo *et al.*, 1995a). Interestingly, such modulation varies according to: the specific physiological conditions (i.e. stage of glia maturation and differentiation), the specific brain region examined, and the degree of neuronal differentiation. In fact, during its maturation and differentiation *in vitro* (8–40 days, DIV), astroglial cells in primary culture release factors able to markedly influence GT_{1-1} cell morphology and accelerate LHRH cell secretory potential, with a potency depending on both the 'age' of astroglia and the degree of GT_{1-1} neuron differentiation *in vitro* (Gallo *et al.*, 1995a). Regional differences in glial-derived factors that promote LHRH neuronal differentiation and secretion were observed, with hypothalamic astroglia being the most potent neurotrophic stimulus (Gallo *et al.*, 1995a, Figure 9). Such effects were specific for astroglia conditioned medium (CM), since oligodendrocyte conditioned medium (CM) was without effect (Gallo *et al.*, 1995a). In dynamic co-culture and mixed LHRH-astroglia system, it was further demonstrated the presence of a bidirectional informative network between the two cell types. In fact, astroglial cells can respond to GT_{1-1} neuronal signals resulting in both morphological and functional changes of the astroglia compartment. This mutual trophic and functional interaction is likely to occur via paracrine, 'intercrine' and/or autocrine mechanism(s). Although the ability of a thermostable astrocyte derived factor to influence in some instances LHRH release from the GT_{1-1} cell line has been recently reported (Melcangi *et al.*, 1995), our observations would support the contention that glial-derived, peptide growth factors are involved in LHRH-astroglia crosstalk (Gallo *et al.*, 1995a).

The extensive neurite outgrowth and establishment of cell-cell contacts between the glia and the LHRH neurons in mixed culture preparation (Figure 8), the fact that the presence of N-CAM-Ab in the GT_{1-1}-astroglial cell mixed cultures resulted in a dramatic disruption of GT_{1-1}-astroglia morphology and a 95% suppression of the stimulatory effect on both cell proliferation and LHRH release, clearly suggest that local adhesive mechanisms are importantly involved in the crosstalk between GT_{1-1} neurons and astroglial cells *in vitro* (Gallo *et al.*, 1995a-c).

These informations, coupled with the parallelism between the astrocyte and the macrophage, and in view of the powerful immunomodulatory properties of LHRH, add further support for a functional integration between the LHRH neuronal system and the immunological network of signals within the CNS (Figure 6). Then, besides the regulation of LHRH secretion at the level of LHRH cell bodies or terminals at the median eminence (ME), LHRH may locally be modulated by dynamic relationships among neuron terminals and glia (Figure 10). Accordingly, different interplays between products of the astroglial cell compartment (such as growth factors), the neuronal terminals and the hormonal background may be envisaged, according to the different developmental stages (astroglia differentiation), as well as the particular reproductive (i.e. specific sex steroid priming) conditions (Figures 6, 10).

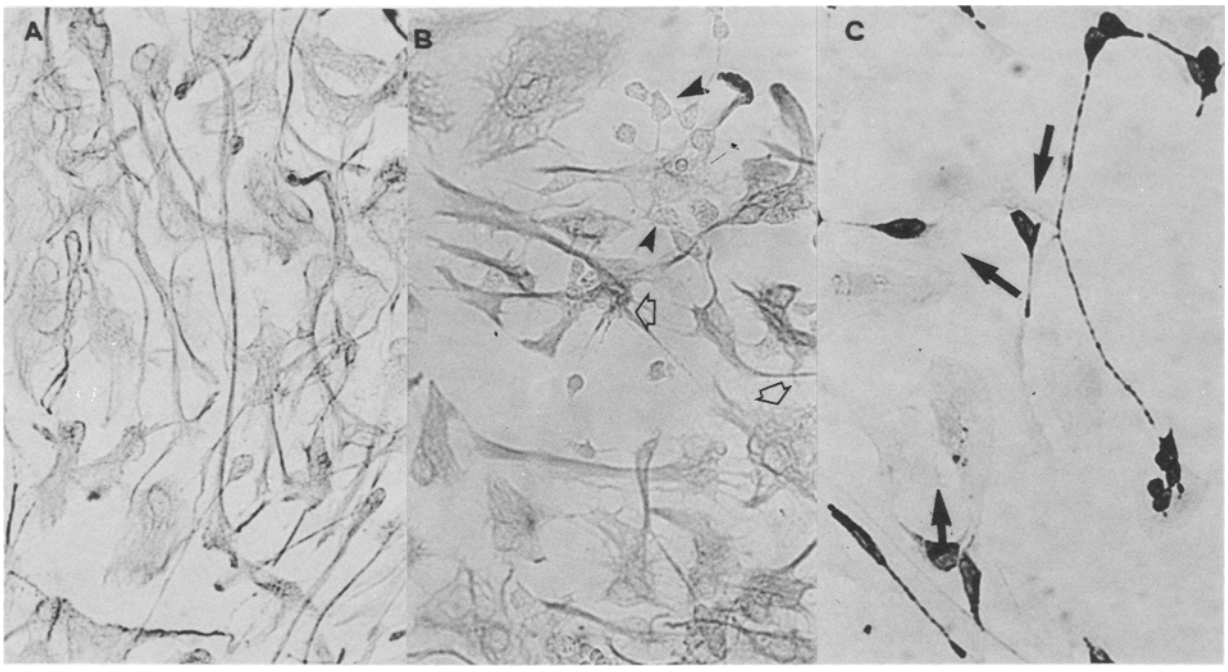


Figure 8 Immunohistochemistry of glial fibrillary acid protein (GFAP) and LHRH neurons. (**A**) Primary astroglial cell cultures (12 DIV) alone; (**B**) astroglial cells were cultured for 8 DIV and GT_{1-1} neurons were added on the top and grown for 4 DIV. Astroglia was labelled with anti-glial fibrillary acid protein (GFAP) antibody. Note that astroglia morphology changes from process-bearing (**A**) to polygonal and flat shapes (**B**), if 4 DIV GT_{1-1} cells were added in the preparation. In (**B**), glial cells (arrows) are stained, while GT_{1-1} cells (arrowheads) are not. (Magnification: × 300). (**C**) Immunocytochemistry of LHRH neurons grown in the presence of astroglial cells in a mixed culture preparation. GT_{1-1} neurons were fixed and labelled with anti-LHRH (LR-1) antibody. In LHRH-astroglia mixed culture GT_{1-1} neurons (arrowhead) extend neurite, and establish contacts with glial cells (arrows) already. Note the extensive neurite outgrowth and contacts of LHRH immunoreactive neurons (arrowheads) with astroglial cells (arrows) at 4 DIV (**D**). (Magnification: × 300)

Cytokine-ovarian interactions

Not only the central but also the peripheral administration of cytokines has potent neuroendocrine effects. In particular, the polypeptide hormone interleukin 1 (IL-1), secreted from monocytes and macrophages during inflammation and infection, directly inhibits the follicle stimulating hormone (FSH)-induced expression of luteinizing hormone (LH)/human chorionic gonadotrophin (hCG) receptors, suppresses progesterone secretion in cultured granulosa cells, and inhibits luteinization of porcine granulosa cells (Gottschall *et al.*, 1987; Fukuoka *et al.*, 1988). Supernate from mitogen-stimulated lymphocyte cultures have pronounced stimulatory and inhibitory effects on steroidogenesis by cultured granulosa cells (Gorospe & Kasson, 1989). The effects of interleukins 1, 2 and 3 on FSH-induced differentiation of rat granulosa cells (Kasson & Gorospe, 1989) was also reported. Communication between the intraovarian endocrine and the immune systems, was emphasized by Adashi in a series of articles (Hernandez & Hadashi, 1991; Kokia *et al.*, 1992; Hurwitz *et al.*, 1991; 1992; 1993). As the process of ovulation is evaluated from a perspective of an inflammatory process, as proposed by Espey, the role of cytokines falls within the domain of modulators of endocrine actions. Evidences supporting a role for IL-1β in ovulation is accumulating. Recently, Hurwitz *et al.* (1991; 1992) demonstrated gonadotropin-dependent increased rat ovarian thecal-interstitial cells IL-1β gene expression with positive up-regulation by IL-1 β in the preovulatory endocrine milieu. Kokia *et al.* (1992) have also shown the modulation of prostaglandin production in cocultured rat ovarian granulosa and theca interstitial cells exposed to IL-1β. Then, potential roles of bidirectional network between IL-1β and the endocrine system include the antigonadotropic effects on immature undifferentiated granu-

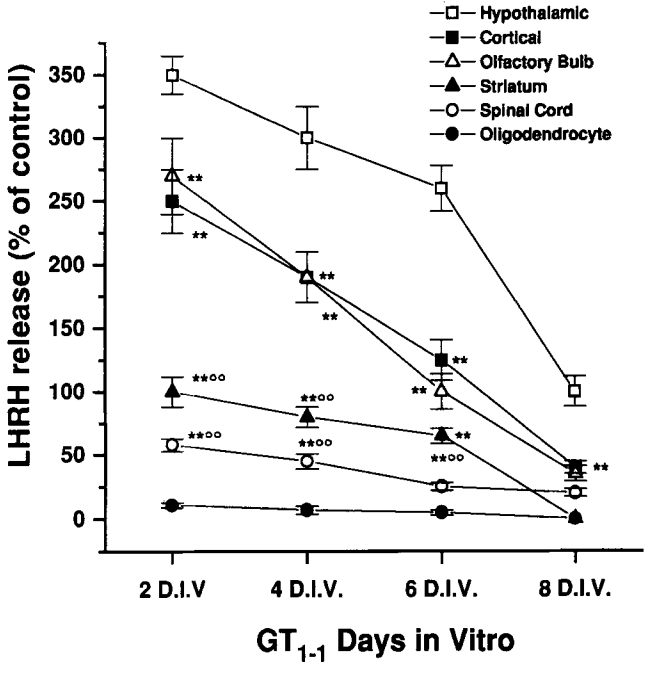


Figure 9 Regional differences of glial-derived factors that promote LHRH release from the GT_{1-1} neuronal cell line. Astroglial conditioned medium from the different regions was prepared as indicated (see Gallo *et al.*, 1995c) and 12 DIV ACMs or oligodendrocyte CM were tested during *in vitro* LHRH neuron differentiation (2–8 DIV). LHRH release in the medium is expressed as percentage (%) increase compared to LHRH released from GT_{1-1} neurons grown in DMEM (control). Results are the mean ± SEM of 2 different experimental manipulations. $^{**}P < 0.01$ *vs* hypothalamic glia; $^{\circ\circ}P < 0.01$ *vs* cortical and olfactory bulb glia

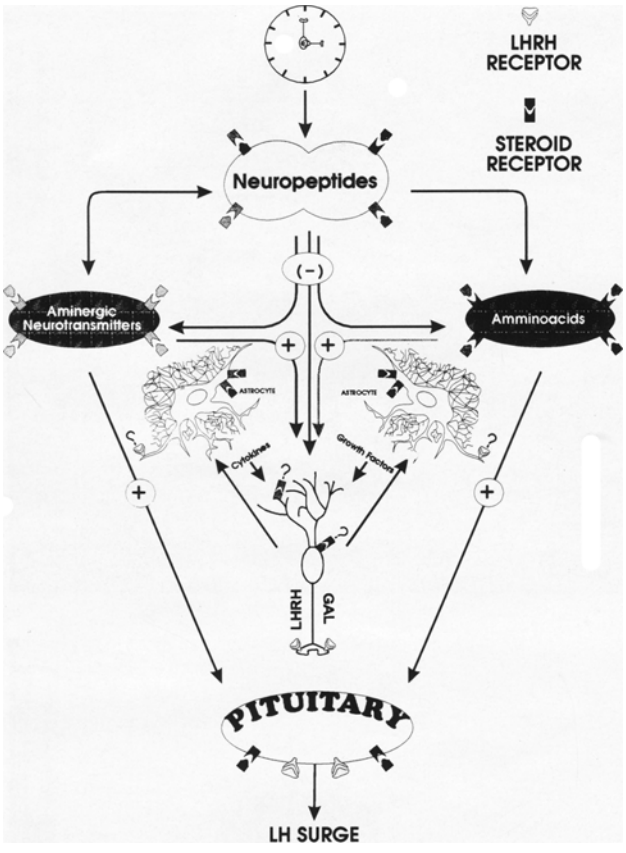


Figure 10 Schematic representation of hypothalamic peptidergic and aminergic signals together with integrating environmental factors, glial and hypophyseal-mediated mechanisms in the control of the episodic discharge of LHRH. The model includes the LHRH pulse generator, the neural elements (the clock) regulating directly the activity of this generator, and those elements involved in its indirect regulation via the negative feedback action of gonadal steroids. Around this unit are also pictured a number of other important factors modulating the phasic LHRH discharge leading to the preovulatory LH surge and ovulation (see Kalra, 1993). A modulatory influence is represented by the action of sex steroids impinging in this circuitry at both central and peripheral (hypophyseal level) via estrogen receptors, as well as by modifications in the number of pituitary LHRH receptors responsible for alterations in the sensitivity of the gonadotropes to LHRH (Marchetti *et al.*, 1982; 1983). Gonadal steroids may also influence astroglial cells (Langub *et al.*, 1992) to produce and release GFs impinging on the LHRH secretory machinery. The concomitant production of other peptides (i.e. galanine, GAL) together with LHRH and its influence in stimulating the proestrous LH surge is also illustrated

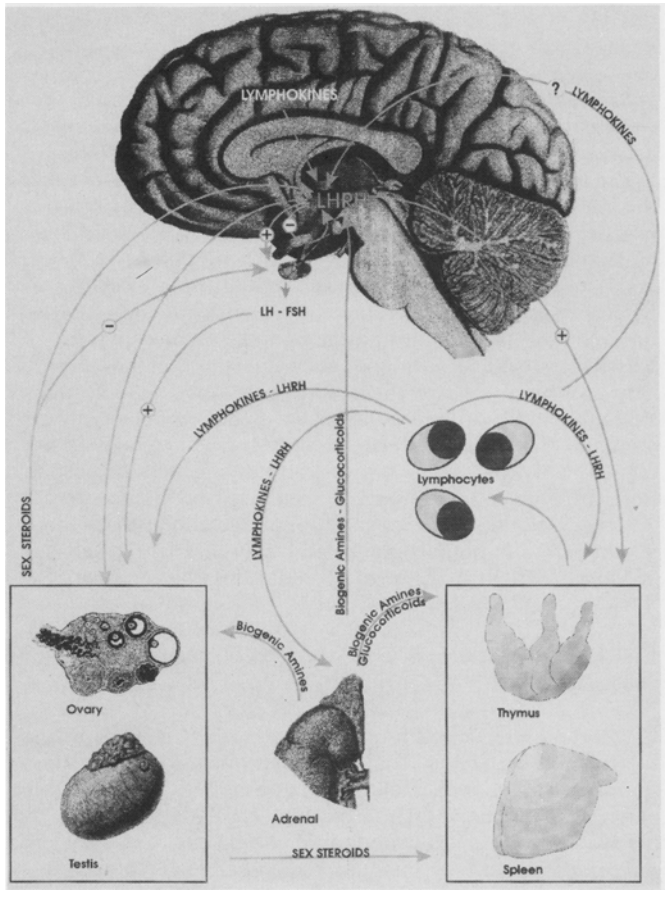


Figure 11 Schematic representation of the possible interactions between the hypothalamus-hypophyseal-gonadal axis and the thymus, with LHRH serving as a major channel of communication. Hypothalamic LHRH governs the release of the pituitary gonadotropins LH and FSH, responsible for gonadal production of the sex steroids. The gonadal hormones in turn, feed back informations to the thymus and hypothalamus. At the thymus level, sex steroids act on specific receptors present on the reticulo-epithelial matrix, and induce both up/down regulation of target genes involved in the control of T-cell response. On the other hand, the sex steroid background alters the production of thymic peptides (thymosins) and neuropeptides such as LHRH, with autocrine/paracrine regulatory influence within the thymic microenvironment. The direct neural pathways innervating immune and endocrine organs together the modulatory influence of glucocorticoids and catecholamines, are also indicated

losa and thecal cells, modulation of ovarian steroid and prostaglandin production, increased endothelial cell surface procoagulant and plasminogen activator inhibitor production, increased collagenase production, and vasodilatatory and angiogenic effect (Hurwitz *et al.*, 1991; 1992; 1993). The fascinating question still remains whether intraovarian LHRH may act as endocrine signal for the execution of a cascade of immunological mechanisms signaling the endocrine and immune cells.

LHRH coordinates the hypothalamo-hypophyseal-gonadal-thymic (HHGTA) axis

The fundamental importance of the thymus gland, as regulator of reproductive capacity is reflected by the fact that the physiological development of an operative hypothalamic-hypophyseal-gonadal axis (HHGA) necessitates the presence of an intact immune system and normal immune function, since immunosuppressed or incompetent animals show numerous reproductive disorders (see Besedowski and Sorkin, 1974; Pierpaoli & Besedowski, 1975; Michael *et al.*, 1980; Rebar *et al.*, 1980; 1981; Grossman, 1984). Moreover, inflammatory and infectious diseases often coincide with changes in reproductive functions including a decline in fertility, an increased incidence of spontaneous abortion, and full term birth of abnormal progeny (Farooki *et al.*, 1988). Conversely, hypogonadic patients with the Klinefelter's syndrome (Geswind & Behan, 1982) appear to have very high rates of lupus erythematosus, and hypogonadic hypogonadotropic patients (with a central deficiency of LHRH), present a number of immune abnormalities (Marchetti, Gallo & D'Agata, unpublished observations).

The primary communication between the immune and the reproductive systems is known to involve the thymus and its peptide secretion (see Marchetti, 1989; Kendall & Clarke, 1994; Kendall & Stebbinghs, 1994; Kendall *et al.*, 1994), it seems important to place the LHRH effects within the context of an hypothalamic-hypophyseal-gonadal-thymic axis

(HHGTA) (see Figure 11). Communication between the gonadal axis and the lymphoid organs has been proposed for over a century (Calzolari, 1898), and the studies of Grossman and coworkers (reviewed in Grossman, 1990) have even emphasized the existence of such reciprocal relationship between the HHGA and the brain-thymus-lymphoid axis. A schematic representation of the possible interactions between the HHGA and the thymus, with LHRH serving as a primary channel for communication is given. As observed, a bidirectional network carrying informations to both the immune and the neuroendocrine reproductive systems, via LHRH is depicted. Direct aminergic and peptidergic innervations of both the gonads and the immune organs (Figures 11, 12) are also illustrated, and underline the commonality of networks used by the neuroendocrine and immune cells. While hypophyseal and gonadal hormones feedback informations to the thymic cell providing a modulatory system and regulating thymic cell maturation and thymic peptide production, the thymus and its peptide secretion (the thymosins) can exert a modulation of gonadotropin secretion via a direct action at the hypothalamic LHRH neuronal level (Rebar *et al.*, 1981; Strich *et al.*, 1981; Kendall & Clarke, 1994).

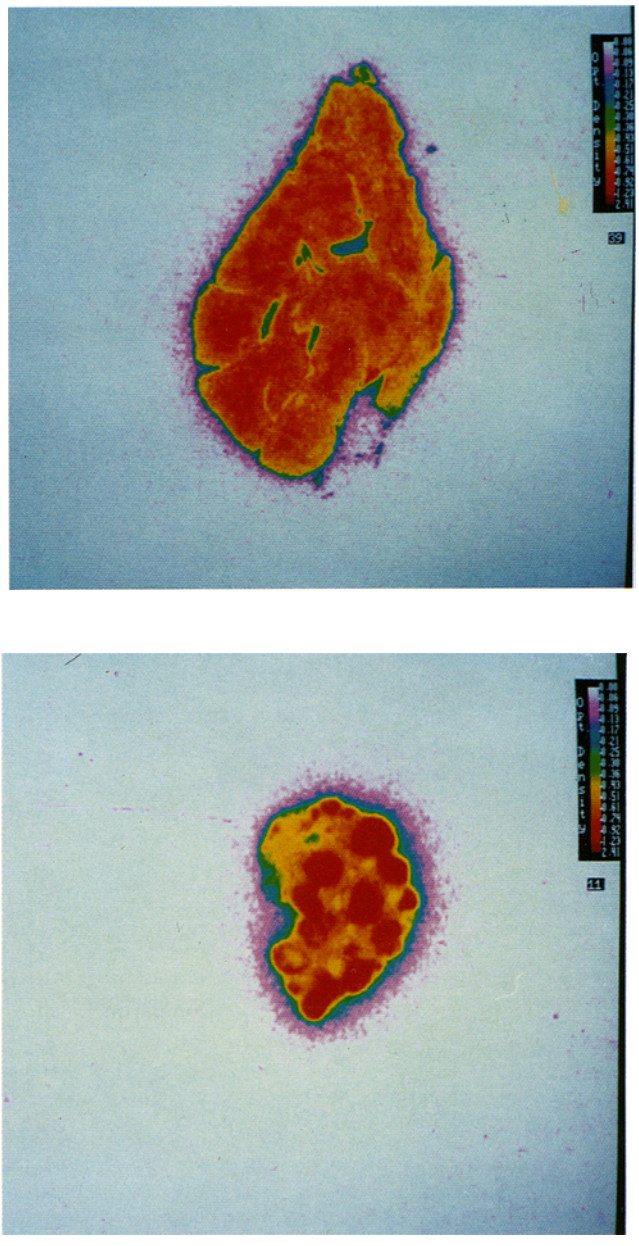


Figure 12 Autoradiographic localization of $\beta_2$adrenergic receptors (β_2AR) within the rat thymus and ovary during proestrus. Receptors are primarily localized in the medullar compartment of the thymus (upper panel). Note the high density of receptors in corpora lutea (bottom panel)

LHRH participates in sex steroid-induced immunological sex dimorphism

Given the presence and expression of LHRH mRNA within the thymus, its ability to alter thymocyte function a further step towards the understanding the physiological significance of its bidirectional function, was to clarify a possible participation of the neuropeptide in dictating the sex-dependent dimorphic pattern of immune responses. That steroid hormones mediate profound physiological and developmental effects in higher eukariotes by interacting with their intracellular receptors in target cells, is a well recognized phenomenon (Burnstein & Cidlowski, 1988). Immunological dimorphism might depend upon two fundamental influences of gonadal steroid hormones: the first one occurring during the perinatal period, when these hormones may permanently alter the developmental pattern of thymocyte selection and turnover and the establishment of the phenotypic (male *vs* female) T-cell repertoire, resulting in permanent effects in a particular subset of T-cells (see Marchetti *et al.*, 1995a; Morale *et al.*, 1995). For instance, the development of the medullary epithelium that occurs early in ontogeny may be irreversibly modulated by the sex steroid hormone milieu, giving rise to a population of progenitor cells (Grossman, 1990). The second influence of gonadal hormones may be exerted during adulthood, by maintaining the sex dimorphic immune function, through the production of adequate levels of circulating gonadal hormones. The sex-dependent regulation of immune responsiveness is a well known phenomenon. In fact, it is an accepted notion that both humoral and cell-mediated immune responses are more active in females than in males, except during pregnancy when this difference is abrogated (Butterworth *et al.*, 1967; Grossman, 1985; 1990; Sthoeger *et al.*, 1988; Kendal & Stebbings, 1994). The existence of a sexual dimorphism in the immune response, the marked alteration of immune function induced by gonadectomy and hormone replacement and the well documented immune alterations during pregnancy, strongly support the hypothesis that gonadal steroids regulate immune functions (see Grossman, 1990 & Marchetti *et al.*, 1995). Moreover when compared with males, females of many species including Human, demonstrate alterations in both T and B cell immune responses. These include higher Ig levels, increased antibody production after immunization, decreased susceptibility to a variety of infections, and the differences in graft rejection time. In particular, oestrogen have been shown to have an immunostimulatory effect on B cell differentiation and a suppressive effect on regulatory cell activity (see Grossman, 1990).

The benefit of immunological dimorphism is not clear at present, but it may relate to the ability of females to withstand the stress of reproduction, and thus increase the probability of species perpetuation (Rheins & Karp, 1985; Grossman, 1990). Sexual hormones have been indicated as the main factors responsible for the development, regulation and maintenance of the dimorphism, although other hormones including glucocorticoids (Peiffer *et al.*, 1984; Morale *et al.*, 1995) and neural influences (Marchetti *et al.*, 1990d,e; Morale *et al.*, 1992a,b; Clarke & Kendall, 1994; Marchetti *et al.*, 1995a), have been suggested to possibly play a role. The presence of receptors for estrogens and testosterone on the reticulo-epithelial matrix of the thymus (see Grossman, 1985) strongly argues in favor of a direct effect of these steroids at the thymus gland level, the molecular mechanism(s), however, involved in sexually-dimorphic responses are not completely understood.

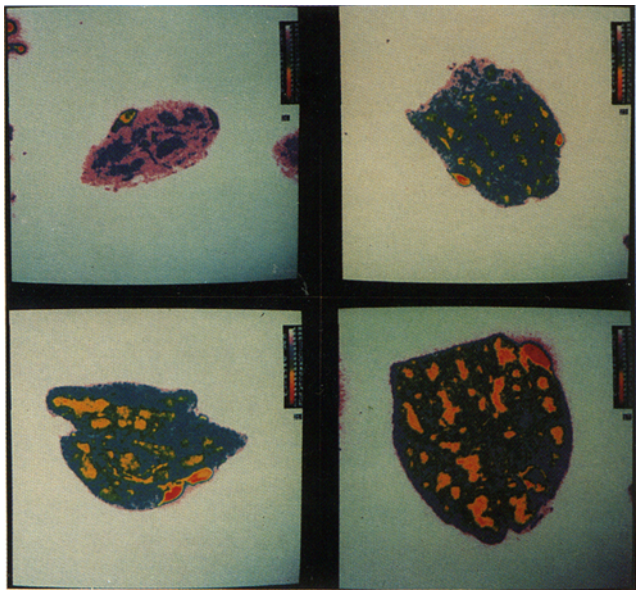
Molecular mechanism of sex steroid action within the thymus in the regulation of immune responsiveness: up- and down-modulation of target genes

LHRH gene expression

The intrathymic LHRH system activity varies according to the phases of the estrous cycle, but more importantly a sex dimorphic pattern of LHRH synthesis accompanies the sex-dimorphic immune response during ontogeny and cyclicity. Surprisingly enough, LHRH mRNA concentration exhibit clear-sex-dependent fluctuation during the rat estrous cycle, which are accompanied by a change in thymocyte sensitivity to LHRH with a maximal response to the natural decapeptide in proestrus (Marchetti *et al.*, 1995a). The dramatic decrease in both rodent thymocyte and human peripheral blood lymphocyte response to T-dependent mitogens after ovulation coupled to the diminished lymphocyte response to LHRH in that particular reproductive phase would indicate a possible participation of LHRH in that phenomenon and that sex steroid are capable of modulating that effect (Marchetti *et al.*, 1995a). Such a mechanism is then likely to participate in the reduction of immune responsiveness observed during pregnancy. Indeed, the ability of estrogens to directly alter LHRH gene expression has been demonstrated in the placenta (Radvick *et al.*, 1991).

β-adrenergic receptor gene expression

Recent studies, using *in vitro* autoradiography, demonstrated the presence of β_2-adrenergic receptors (β_2AR) in the rat thymus (Marchetti *et al.*, 1990d,e). In the thymus norepinephrine (NE) act via the β_2AR both as a paracrine hormone available to receptors on thymic cells, and as a localized transmitter in nerve terminals that directly contact cortical thymocytes, mast cells and eosinophils to modulate immune functions (Kendall *et al.*, 1994). The thymic β_2ARs are preferentially found in the medullar compartment of the gland and show a clear sexual dimorphism in receptor organization during sexual maturation (Figure 13). A cyclic variation receptor density accompanies the different phases of the oestrous cycle, with a significant increase in receptor density observed during the period of maximal oestrogenic stimulation (Marchetti *et al.*, 1990e) (Figure 14). The up-regulation of the thymic β_2AR induced by physiological changes in circulating sex steroid hormones is further supported by the sharp decrease in receptor density observed after castration, and by the dramatic stimulation of receptor levels accompanying the treatment of castrated rats with oestradiol (Marchetti *et al.*, 1990e, Figure 14). The expression of a β_2AR in the rat thymus was further confirmed by the presence in the thymic tissue of a mRNA species of 2.3 Kb which specifically hybridized with a cDNA encoding the gull coding sequence of the human β_2AR (Morale *et al.*, 1992; Marchetti *et al.*, 1994a). The β_2AR system observed in the thymic tissue preparations is functionally coupled to the adenylyl cyclase system, with a sensitivity characteristic of a β_2 subtype receptor (Morale *et al.*, 1992; Marchetti *et al.*, 1994a). Both a high affinity state of the receptor for isoproterenol and an isoproterenol-stimulated adenylyl cyclase activity could be detected in the thymus membrane preparations, with the guanine nucleotide converting all of the high-affinity-state receptors into low affinity state receptors (Marchetti *et al.*, 1994a). Moreover and of major interest sex steroids exert potent modulatory effects on the β_2AR-stimulated adenylyl cyclase activity, with direct consequences on thymic cell proliferative capacity (Marchetti *et al.*, 1994a). Parallel changes in both β_2AR density and β_2AR mRNA levels followed the hormonal changes associated with the rat estrous cycle, pregnancy or castration (Marchetti *et al.*, 1994a) (Figure 16). Such quantitative changes suggest a subtype specific hormonal regulation of the β_2AR population. It seems, then, possible that the hormonal priming of T-lymphocyte, through the amplification and/or potentiation of the intracellular inhibitory pathway (increased expression and coupling of the β_2AR) may participate in the dynamic regulation of the immune response (Marchetti *et al.*, 1994a).



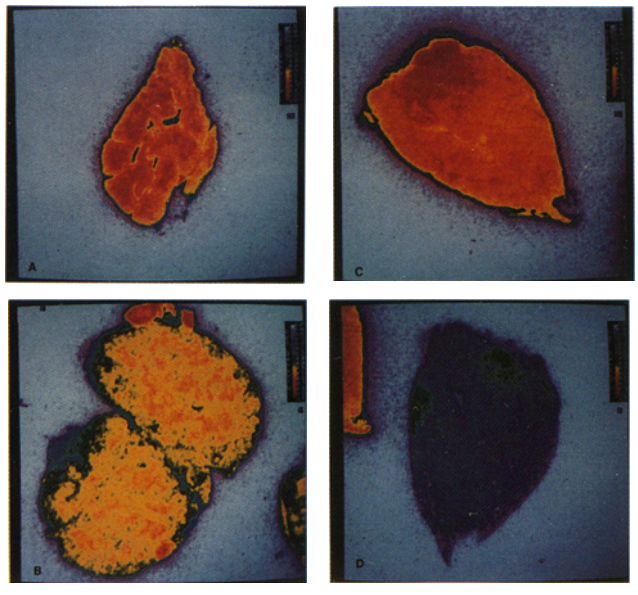


Figure 14 Autoradiographic distribution of β_2AR within the female rat thymus under different hormonal treatments. (**A**) Thymic section from a proestrous rat, note the high concentration of receptors distributed over the medullary islands; (**B**) Thymic section from a castrated female rat, note the loss of receptors and the enlargement of the gland; (**C**) Thymic section from a castrated rat treated with estradiol, note the important stimulation of receptor density accompanied by thymic atrophy; (**D**) Non-specific binding reaction after an excess of (−)propranolol (10^{-6}M). From Marchetti *et al.* (1990)

Type II glucocorticoid receptor (GR) gene expression

Glucocorticoids are crucial hormones in the control of immunity, being among the most potent anti-inflammatory,

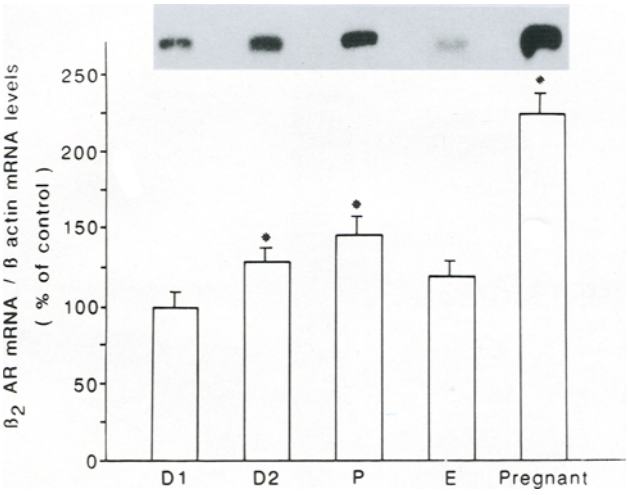


Figure 15 Hormonal regulation of β_2-adrenergic receptor mRNA content. Poly(A)$^+$ RNA samples from the different experimental groups were fractionated by electrophoresis on a 1.2% agarose-7% formaldehyde gel, blotted onto nylon filters and probed with ^{32}P-labelled beta-adrenergic receptor cDNA (2.5×10^6 d.p.m./ml) or actin cDNA (2.0×10^6 d.p.m./ml) (Marchetti *et al.*, 1994). The relative abundance of the β_2AR mRNAs are expressed as percentage of their values (of β_2AR mRNA/β-actin mRNA ratios) relative to diestrous 1, normalized to 100%. Results are the mean ± SEM of 4–6 independent determinations. $^*P < 0.01$ *vs* control, by ANOVA

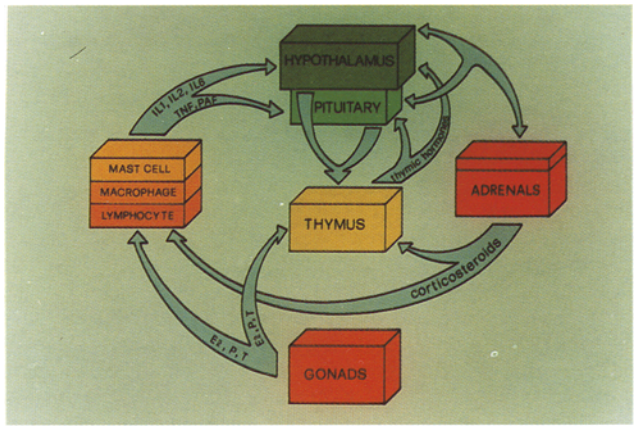


Figure 16 Schematic representation of the possible interactions between the hypothalamus-pituitary-adrenal (HPA) axis, the soluble products of stimulated immune cells, the thymus and gonadal hormones. The complex circuitry involves feedback effects at the central (hypothalamus), and peripheral sites (pituitary gland, adrenals, thymus, gonads, lymphocytes, mast cells and macrophages). Increased production of cytokines during immune system stimulation activates the HPA circuit, responsible for the subsequent shut-off of the immune response. On the other hand, the pre-existing sex steroid milieu may modulate glucocorticoid effects via an action on different immune compartments, possibly at the level of GR II receptor gene expression

anti-allergic and immuno-suppressive agents known, and they act in a very complex way, at various steps of the immune response (Van Rees *et al.*, 1991; Buckingham *et al.*, 1992; Marchetti *et al.*, 1994b; Peiffer *et al.*, 1994; Morale *et al.*, 1995) (Figure 16). The effects of corticosteroids on cells of the immune system, as in other corticosteroid responsive cells, are mediated through both soluble and nuclear glucocorticoid receptors (GR). Different experimental paradigms clearly demonstrated that thymic GR mRNA concentration is under the control of gonadal and adrenal hormones (Peiffer *et al.*, 1994). An up- and down-regulation of GR mRNA accompany the luteal and proestrus phases, respectively (Figure 17). The hormonal sensitivity of type II GR in the thymus was further substantiated by the sharp decrease in GR transcript during pregnancy, while the hormonal milieu of lactation lead to a normalization of GR mRNA concentration to levels measured in diestrous rats. In addition, the ability of corticosterone *in vitro* to influence a cell-mediated immune response in the thymus (i.e., the blastogenic transformation of thymocytes) seems to depend upon the sex steroid hormone milieu. The sexual dimorphism of immune system activity may be related to differential regulation of the HPA axis activity by estrogens since a sexual dimorphism has also been described in HPA axis regulation. In fact, higher circulating levels of corticosterone are found in females, and greater variations in plasma corticosterone both diurnally and in response to stress, have been described in female animals (Kitay, 1961). Transcortin concentrations of female rat are at least double than those found in males rat plasma (Critchlow *et al.*, 1963). Sex-related differences have also been found for glucocorticoid receptor affinity, binding capacity, nuclear translocation and gene expression in rat brain (Gala & Westphal, 1965; Turner & Weaver, 1985; Peiffer *et al.*, 1993). The link between the glucocorticoid tone and sex steroid modulation of immune functions is further supported by the finding that loss of GR function in transgenic animals expressing type II GR antisense RNA while resulting in higher plasma ACTH and corticosterone levels (Pépin *et al.*, 1992), abolishes the development of sex-dimorphism in immune response (see Morale *et al.*, 1995).

Figure 17 Alterations of glucocorticoid receptor (GR) mRNA transcript levels in female thymus gland during the rat estrous cycle. Total RNA was extracted from the thymus of adult cycling Sprague-Dawley female rats at different phases of the estrous cycle (diestrous 1, D1; diestrous 2, D2; proestrous, P; and estrus, E) and hybridized on Northern blots. Results are the mean ± SEM (6 animals/group), expressed in arbitrary units of the GR mRNA/β-actin mRNA ratio. ANOVA shows a significant decrease of GR transcript levels in proestrous and a marked increase at estrus when values are compared to those measured on the diestrous (D1 and D2) phases of the cycle. $^*P < 0.01$ *vs* D1 and D2 (by Dunkan-Kramer test). From Peiffer *et al.*, 1994

Moreover, the hyperactivity of the helper-inducer T-cell compartment, suggests a prominent role of this receptor system in the physiological counterregulation of immune response (see Peiffer *et al.*, 1994; Marchetti *et al.*, 1994b; Morale *et al.*, 1995).

Then, oestrogen may induce on the one hand, an increase in β_2AR and LHRH influence in the thymus via a stimulation of their mRNA concentrations, resulting in an increased sensitivity of the thymic cell to locally released and/or circulating catecholamines, as well as to endogenous LHRH, non excluding possible intersystem paracrine (i.e. catecholaminergic-LHRH-ergic) paracrine/autocrine/intercrine cross-talk. On the other hand, a sharp decrease of both GR

transcripts and thymocyte response to corticosterone follows E_2 treatment/exposure. Such oestrogen-induced up-or down-regulation of the β_2AR, LHRH and GR mRNA levels in the thymus may then result in a sophisticate hormonal control of lymphocyte sensitivity to endogenous hormones.

LHRH modulation of human immunological functions: the challenge

A further step in the study of the immunological properties of LHRH and its potent LHRH agonistic and antagonistic analogs was to verify the ability of these peptides to directly interact with human peripheral blood lymphocytes (PBMC) via an action on specific receptors. Human peripheral T cell subsets produce LHRH and its production is increased in T-cell activated with phytohemagglutinin (PHA) (Azad *et al.*, 1993). Stimulation of human peripheral blood lymphocytes with LHRH and its agonist significantly stimulates the incorporation of [^{3}H]thymidine (Marchetti, unpublished observations). To determine the ability of chronic treatments with the LHRH-A to interfere with human immune functions a cell-mediated immune response and the distribution of the different lymphocyte subsets in fertile women during the menstrual cycle, and in women undergoing a treatment with the potent agonist D-Tryp^{6}LHRH (Decapeptyl) for *in vitro* fertilization program, were assessed. A clear sex-dependent alteration of cell-mediated immune response characterized by a marked suppression of [^{3}H] thymidine incorporation during the periovulatory phase of the cycle, while in the luteal phase a significant increase in cell-mediated immune response was observed (Figure 18A). The chronic treatment with LHRH-A resulted in a biphasic effect, with a significant inhibition of immune response during the phase of maximal estrogenic production, while the desensitization of the hypophyseal-gonadal axis and inhibition of sex steroids production resulted in a marked increase in immune responsiveness (Marchetti *et al.*, 1995a) (Figure 18B).

Alterations in immunological functions are also studied in different gynecological endocrinopathies such as Kallmann's syndrome and endometriosis. In patients with hypogonadism of central origin, the PBMC cell-mediated immune response to polyclonal mitogens was dramatically reduced, while the pulsatile administration of LHRH resulted in a significant restoration of normal immune capacity (Marchetti, Gallo & D'Agata, unpublished observations). Such results are in line with the findings that patients suffering from Klinefelter syndrome have an especially high rate of immune disorders. Profound immune dysfunctions in patients with endometriosis both at systemic and local levels are known to occur. The analysis of the ability of the peripheral lymphocytes to respond when activated with the polyclonal phytomitogens Concanavalin-A, showed marked alterations of T-cell-mediated immune responses, as well as changes in the distribution of T-cell subset, B and NK cells before and during treatment with Decapeptyl. In experimental ovarian tumors (Marchetti, Ditrich, Gallo, Lomeo, Garozzo & Jagger, unpublished observations), Decapeptyl successfully reduced tumor growth, and this effect was accompanied by the re-establishment of a fully operative immune response. Such data, coupled to the profound immune suppression measured in experimental models of cancerogenesis (Gallo *et al.*, 1993), as well as in women with gynecological cancers (Marchetti *et al.*, 1995a) underline the potential use of LHRH-A in the treatment of different malignant endocrinopathies.

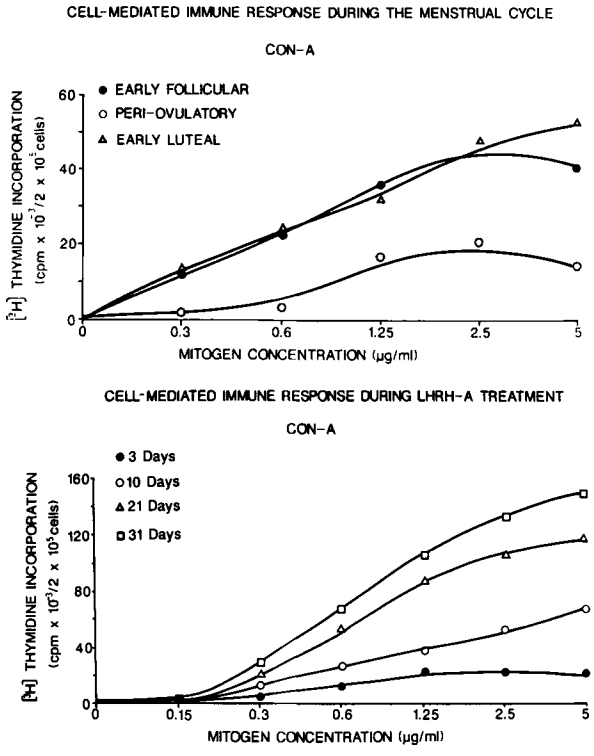


Figure 18 Cell-mediated immune response during the menstrual cycle and during a chronic treatment with LHRH-A. Individual peripheral blood monocyte proliferative (PBMC) activities were evaluated during the menstrual cycle. (**A**) [^{3}H]thymidine incorporation in activated PBMC during the late follicular, periovulatory and mid luteal phase of the patient before entering into the program of LHRH-A treatment for suppression of the axis for the *in vitro* fertilization protocol. Note the sharp suppression of the proliferative response in the ovulatory phase of the cycle. (**B**) [^{3}H]thymidine incorporation in activated PBMC from the same patient at different time intervals during treatment with Decapeptyl. Note that the phase corresponding to the stimulation of the HHGA by the LHRH-A is paralled by a marked suppression of the immune response, while the phase of down-regulation of the HHGA by the LHRH-A resulted in a sharp stimulation of cell-mediated immune response

Summary and conclusion

It seems apparent that the brain-pituitary-reproductive axis and the brain thymus-lymphoid axis are linked by an array of internal mechanisms of communication that use similar signals (neurotransmitters, peptides, growth factors, hormones) acting on similar recognition targets. Moreover, such communication networks form the basis and control each step and every level of reproductive physiology. This work has focused on the LHRH system, a primary central and peripheral clock of neuroendocrine functions. From the initiation of a sexually organized response, the detection of sexual odors and the induction of mating behaviour, extrahypothalamic and hypothalamic LHRH orchestrates the neuroendocrine modulation of gonadotropin secretion, while its expression within the ovary directly controls specific events such as follicular atresia. The presence of LHRH receptors in oocytes clearly anticipates a potential action of the decapeptide also during the process of fertilization and/or implantation. Within the thymus and other peripheral immune organs LHRH plays a unique role of immunomodulator, contributing to the sex-dependent changes in immune responsiveness during the estrous-menstrual cycle as well as pregnancy. The section of LHRH-immune network within the CNS emphasized the relationships between this neuropeptidergic secreting neuron and astroglia. It seems of interest to underline that to function normally neurons must migrate to specific structures, and this process relies on chemical communication between many different cells. In particular, neurons use glial fibers and special molecules on the surface of neurons (cell adhesion molecules) and glia cells that tell the neurons which path to follow. In Kallmann's syndrome, the LHRH neurons fail to migrate, leading to

hypogonadal hypogonadism. Unraveling astroglial-LHRH neuronal networks might then constitute an additional effort in prevention/treatment of migrating disorders.

The reciprocity of the neuroendocrine-immune signaling systems is further supported by the ability of sex steroids to modulate thymus dependent immune function via direct effects on specific target genes involved in the development of sex-dimorphism and sex dimorphic immune responses, including the down-regulation of immune response observed during pregnancy. Such cyclic changes in immune responsiveness could have a physiological implication, such as the decrease or suppression in cell-mediated immunity observed in the post-ovulatory phase of the cycle and pregnancy, respectively, might play a role during the implantation process and the establishment of pregnancy. In this context, the ability of corticosterone to directly inhibit both GR transcript levels as well as a cell-mediated immune response within the thymus, and the modulation of such inhibitory effect by the sex steroid hormone milieu, may offer an explanation and a molecular mechanism whereby stress may be deleterious for reproduction, also via immunomodulation. Due to the mutual influence between estrogenic milieu and immune competence, it would follow that the estrogenic status might influence the glucocorticoid-lymphokine interactions. Since estrogens can inhibit IL-6 production in IL-1-stimulated cells (Tabizadeh *et al.*, 1989) and this cytokine, in turn appears to enhance cortisol levels by activating the HPA axis (Beaumann *et al.*, 1987; Shegal, 1990; Spangelo & McLeod, 1990), the response to a given pro-inflammatory trigger, and the degree of susceptibility and the severity of inflammatory diseases may, thus, vary according to the sex steroid hormone milieu. On the other hand, hormonally-mediated alterations in immunity might also have a pathological implication in sexually related immune diseases. Sex steroid hormone milieu, might also have a role in controlling the stress response through immunomodulation. Such suggestions seem corroborated by the recent finding indicating that animals expressing a GR antisense RNA exhibit a number of immune abnormalities, including abolishment of immunological dimorphism and hyperactivity of the T-helper compartment, supporting a key role of the GR in the counterregulation of immune reactivity and their participation in estrogen-induced sex-dimorphic responses.

Even though not summarized in the present review, the neuroendocrine and immunomodulatory role of LHRH continues well during pregnancy (see Marchetti *et al.*, 1995a), as well as after parturition, since the presence of LHRH-like material within the mammary gland and milk participates in physiological modulation of hypophyseal, gonadal and immune functions of the pups (see Morale *et al.*, 1991). Such significant role played by the hypothalamic peptide in the modulation of immune responsiveness would indicate LHRH as the signal conveying informations to both neuroendocrine and immune cells, with the role of informing and then transducing the messages into appropriate biological responses.

From the recent cloning and sequencing of lymphocyte LHRH which appears to be identical to hypothalamic LHRH, the expression of LHRH receptor mRNA in lymphocyte which is identical to the pituitary counterpart, the transduction mechanisms involved and the steroidogenic sensitivity of the intralymphocyte LHRH system, it would appear that this peptide, besides its recognized neuroendocrine role might, indeed, act as an immunological response modifier in the brain pituitary-lymphoid-gonadal axis. The widespread therapeutical application of LHRH and is potent agonistic and antagonistic analogues in a large number of pathologies, in pediatric, gynecologic, urologic and oncologic medicine, underlines the potential clinical implications of the described experimental findings. The important positive effects of the LHRH-A therapy in post-menopausal breast cancer patients, the recently demonstrated direct effect of LHRH-A in murine lupus, and the powerful immunomodulatory role of LHRH-A in endometriosis and experimental ovarian cancer, certainly open a new chapter the one of NEI of reproduction, on the potential immune capabilities of hormones and anti-hormones, that are widely used in a variety of endocrinopathies. Further studies aimed to disclose at a biochemical and a molecular level the specific modulatory mechanisms involved in the control of the reproductive-immune axis, will give us new insights not only into the problems of fertility regulation but in more general issues concerned with the pathologic sequelae resulting from the malfunction of the endocrine-immune axis. More importantly, such studies will hopefully provide us with new tools that can be used to reverse, minimize or counteract some adverse/deleterious events associated with reproductive failure.

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