

# Immunological influences on reproductive neuroendocrinology

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One of the consequences of activation of the immune system, with its associated inflammatory responses and operation of the stress axes, is a generalised inhibition of reproductive function. This can be considered as part of the all-encompassing effects of an activated immune system, included in which is the 'immunological cost' arising from the nutritional demand required to maintain a competent, responsive immune system, and the pathological effects produced by severe immune responses. Elucidation of specific immune-neuroendocrine linkages has largely involved examination of corticosteroid-based mechanisms or use of bacterial endotoxin as a model stimulus and examination of effects on GnRH and LH pulsatility, on GnRH and LH surge processes and on pituitary responsiveness to GnRH, using various sheep models. Although there is good evidence for prostaglandins as common mediators for endotoxin-induced and stress axis-induced impairment of neuroendocrine reproductive processes, both mechanisms appear to have prostaglandin-independent pathways as well. At the anterior pituitary gland level, the type II glucocorticoid receptor appears to mediate corticosteroid effects. Otherwise, the identity of specific cytokines, their sites of action and the cell level mechanisms underlying the inhibition of the reproductive axis at hypothalamic and anterior pituitary levels, especially in sheep, remains largely unresolved.

## Introduction

This review is intended to provide initially a brief perspective of the immune system and its impacts on body systems in general, and on neuroendocrine pathways in particular. Thereafter, the primary objectives are to review recent research activity that has focused on mechanisms of immune stress-associated effects, largely involving corticosteroids, and cytokine-mediated effects on neuroendocrinology of reproduction with particular emphasis on studies with sheep.

## The immune system

*What is the immune system?*

The immune system can be regarded as a defence mechanism, the impairment of which has tragic consequences, as demonstrated by genetic or acquired (e.g. AIDS) severe immunodeficiency.

ciencies. Although it influences the function of most bodily systems, enables tolerance of 'self' and can reject tumours, its genesis is largely based on the need of vertebrates to cope with challenges from pathogenic organisms.

Innate immunity includes the physical epithelial barriers encountered by pathogenic organisms attempting to invade an animal and the antimicrobial factors produced by epithelia, plus macrophages and other cells that have receptors for cell-surface molecules on the organisms. This provides for interactions that may lead to phagocytosis of the invading organisms and/or activation of macrophages causing induction of an inflammatory response, and recruitment of other cell types to the site. Such pathogens may also be recognised by the complement system causing enhancement of their phagocytosis as well as activation of inflammatory responses. Antigen-specific responses are recruited from other cells attracted to the site of infection and through uptake of antigen by dendritic cells that transport antigen to lymphoid tissue where primary immune responses are initiated.

Adaptive immunity includes primary immune responses that occur when antigenic substances interact with antigen-specific lymphocytes, most commonly causing production of specific antibody molecules and proliferation of antigen-specific helper and effector T-lymphocytes. The latter cells can produce killer T cells capable of lysing infected cells. Secondary immune responses involve the formation of immunologic memory during the primary event that produces an enhanced immune response at subsequent exposure to antigen – i.e. a state of immunity and the basis of vaccination. The specificity of the immune response is determined by lymphocytes which makes them central players in the system, but a whole raft of other leukocytes plus specialised epithelial and stromal cells are involved in the immune process by providing the anatomical environment for conducting it as well as secreting chemical factors that regulate migration, growth, and/or gene activation of cells in the immune system. Antibodies are large immunoglobulin molecules that can be expressed on the cell surface of lymphocytes and secreted into the surrounding environment. Another group of chemicals that mediate the functions of cells in the immune system are small proteins referred to as cytokines. Their definition has been discussed by Turnbull and Rivier (1999) who grouped them usefully into eight families, namely: interleukins (e.g. IL-2, IL-3, etc.), tumour necrosis factors (e.g. TNF- $\alpha$ , etc.), interferons, chemokines, haematopoietins, colony stimulating factors, neurotrophins, and growth factors. A complication is that many peptides with mediatory functions are common to the immune, nervous and neuroendocrine systems. For example, GnRH, a well established hypothalamic neuropeptide with a central role in reproduction, is also produced by T-lymphocytes and affects laminin receptor expression in lymphocytes (Chen *et al.* 2002) thus having a potential role as an immunological mediator. This illustrates the difficulty, highlighted in a recent review (Shepherd *et al.* 2005), of classifying peptides using nomenclature such as 'cytokines' or 'neuropeptides' as well as confounding the linkages between immune and neuroendocrine function. Interestingly, the various signalling chemicals that have evolved within one cytokine molecular family, the class-I helical cytokines, include some members generally regarded as classical peptide hormones, e.g. leptin, growth hormone, prolactin, erythropoietin (Huisin *et al.* 2006).

#### *Immune-nervous interactions*

Involvement of the central nervous system (CNS) in regulation of immune processes at many levels is well established (e.g. Sternberg & Webster 2003; Shepherd *et al.* 2005) and the, now classical, arms of the neuroendocrine and neuronal responses to stress, i.e. the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic adrenal medulla (SAM) system, are both impor-

tant in regulation of immune responses. Both divisions of the autonomic nervous system, i.e. sympathetic and parasympathetic, modulate immune function indirectly by their various actions on blood circulation, smooth muscles, secretory glands, etc., as well as directly via receptors on virtually all immune cells, including macrophages and lymphocytes. There are other examples of neural effects on immunological processes (e.g. Shepherd *et al.* 2005), however, the focus of this review is on the converse interaction; the effects of the immune system on neuroendocrine function, specifically the reproductive neuroendocrine axis.

### **Modulation of nervous function by immune factors**

Within the CNS immune chemicals such as cytokines act like growth factors affecting neuronal cell death and survival. These cytokines and/or their receptors are synthesised directly by cells of the CNS which include resident glia or immune cells that have invaded the CNS (Sternberg & Webster 2003). Such immunoregulation is a constitutive function of these cells that is likely to be enhanced under pathological conditions or when the immune system is activated. In contrast, cytokines that reach the CNS from outside this system are likely to act, by definition, in an endocrine manner as classical hormones. For instance some cytokines act like neuropeptides and cause such centrally mediated events as fever, sleep, changes in behaviour, or activation of hormonal stress responses (Sternberg & Webster 2003). However, the occurrence of such an endocrine role of cytokines within the CNS had remained largely unproven until sufficient quantities of pure recombinant cytokines became available to test these theories directly.

Another issue related to an endocrine role of cytokines in the CNS is that of their access to brain cells. One way in which compounds such as cytokines can surmount the blood-brain barrier is by leaving the circulation at regions in the CNS where the blood-brain barrier does not exist, or the vessels are leaky. Such regions are in the circumventricular organs which include: median eminence, area postrema, subfornical organ, organum vasculosum of the lamina terminalis, and choroid plexus (Sternberg & Webster 2003). Many of these organs are located close to areas of the CNS that are linked to neuroendocrine regulatory centres, such as the hypothalamus. Access to CNS tissue can be gained also by means of active transport processes across the endothelium of CNS blood vessels (Banks 2001) or, alternatively, cytokines can signal their presence through activation of secondary messengers located at the endothelial cell membranes and achieve their effects on CNS function indirectly (Scammell *et al.* 1996; Elmquist *et al.* 1997). Another means of indirect signalling of the CNS by cytokines involves activation of sensory nervous input. For example, paraganglia cells in lymphoid tissue can signal the brain via vagal afferents when there is some change in activity of the lymphoid organs (Goehler *et al.* 2000).

### **Impacts of the immune system on body function in general – ‘immunological cost’**

Whenever the immune system of an animal is activated there is the possibility that this can be accompanied by major impacts on body function. It is now widely recognised that these impacts largely result from the immune responses themselves. A dramatic example is toxic shock syndrome following infection by streptococcal bacteria (Stevens 1995). A recent example involving an intestinal nematode parasite was provided by Greer *et al.* (2005). Lambs in the study were treated with an immunosuppressive dose of corticosteroid that was sufficient to allow establishment of a high worm burden. In spite of the greater infection of the immunosuppressed sheep, their live-weight gain and feed conversion efficiency was not affected, showing

the benefits in terms of nutrients spared from the need to maintain immune responsiveness and also indicating little actual harm caused to the sheep by the parasites *per se*. As in the case of toxic shock syndrome, most of the detrimental effects of the intestinal parasitism arose from the immune responses to the infective organism, not the organism itself. Collectively, these and similar findings lend support to the concept of an 'immunological cost', that is the metabolic cost of maintaining immunological competence or mounting an immune response (Colditz 2004) with or without the additional burden of pathology arising from tissue damage caused by severe immune responses.

Any change in nutritional status arising from activation of the immune system is likely to alter neuroendocrine function directly via changes in supply of nutrients and by actions of signalling molecules. Such molecules include leptin, which is involved in communicating nutritional status information to the reproductive neuroendocrine axis in sheep (Adam *et al.* 2003) and cows (Barb & Kraeling 2004), and ghrelin, which is associated with meal-related neuroendocrine signalling in ruminants (Sugino *et al.* 2004).

### **Immunostimulation as a stressor**

It is not surprising from evidence of its major impacts on body function that activation of the immune response cascade causes major disturbances of neuroendocrine function. Whether it is by default or by design, as it makes sense to curtail the expense of reproductive function if survival of the individual is threatened, the consequences for the reproductive axis when the immune system is activated are not unexpected. What is not so clear are the details of the mechanisms by which activation of the immune response cascade imposes its effects on the reproductive axis. Immunostimulation is one of the responses to an adverse environmental stress, such as exposure to antigens, and can be considered as being akin to responses such as those caused by thermal stress, malnutrition, psychological stress, physical injury, hypoxia or any of the stressors that activate stress responses in animals. In the current view, many or maybe all of these stressors appear to produce co-ordinated suites of responses mediated by the brain, particularly via the amygdala – the two organs of which form part of the sub cortical limbic system. These organs may be the central processors of the myriad of inputs from various stressors that in turn regulate the major responses of the body to stress. For simplicity these have been allocated into four categories, namely: (1) changes in behaviour, i.e. change of skeletal muscle function; (2) activation of the SAM system; (3) activation of the HPA axis; and (4) activation of immune responses, i.e. immunostimulation. Two of these response categories, 2 - the SAM system and 3 - HPA axis, are well-established as being regulated at various levels within the hypothalamus and there is increasing evidence (Turnbull & Rivier 1999) that the fourth category – immunostimulation – also undergoes regulatory interaction within the hypothalamus. Presumably, the motor region of the cerebral cortex plays a major part in the behavioural changes – response category 1 (above) - although these are considerably influenced by structures operating below the level of consciousness, such as in the limbic system. Activation of the SAM system causes release of adrenergic hormones which are powerful immunoregulators and activation of the HPA axis causes immunosuppression via release of glucocorticoids. This means that much of the research on effects of stress on reproduction merges with that involving the immune system.

### **Corticosteroid-mediated effects**

Studies involving the administration of the bacterial endotoxin, lipopolysaccharide (LPS), a commonly-used model of immune stress in sheep, have demonstrated a concurrent activation

of the neuroendocrine stress axis (Battaglia *et al.* 1997; Battaglia *et al.* 1998; Dadoun *et al.* 1998; Williams *et al.* 2001). This may be centrally mediated in the hypothalamus or by direct action of cytokines on the adrenal cortex (Turnbull & Rivier 1999). To test the hypothesis that cortisol mediates the inhibitory actions of endotoxin on pulsatile GnRH and LH secretion in sheep, Debus *et al.* (2002) working in Professor Fred Karsch's laboratory at The University of Michigan separated the effects of cortisol alone from those of the endotoxin-induced release of cortisol by use of metyrapone, an inhibitor of cortisol synthesis. Their work showed that although cortisol itself inhibited pulsatile GnRH and LH secretion, the marked release of cortisol provoked by administration of LPS was not required for the inhibitory effects of the bacterial endotoxin. In addition, they ruled out the possibility that their results were attributable to progesterone which was also released in response to LPS and was probably of adrenal origin because ovariectomised ewes were used. They concluded that more than one pathway must be responsible for the immune-mediated inhibition of reproductive activity, although one of these involves enhanced secretion of cortisol. Further work in Professor Karsch's laboratory with ovariectomised ewes (Breen & Karsch 2004) indicated that the site of action of cortisol in these animals is localised to the anterior pituitary gland where it lowered responsiveness to GnRH, rather than by a direct effect on hypothalamic release of GnRH. Their results showed a cortisol-induced suppression of LH pulse amplitude but no effect on pulse frequency of LH or GnRH and no effect on pulse amplitude of GnRH. However, the ovariectomised ewe model provides a very different steroid milieu from that experienced by ovarian intact ewes and eliminates potential effects of cortisol on the ovary that would alter steroid feedback at hypothalamic and pituitary gland levels. Subsequently, studies in the same laboratory have shown that stress-like elevations of plasma cortisol concentration suppressed pulsatility of LH secretion in follicular-phase ovarian intact ewes (Breen *et al.* 2005a), interfered with the preovulatory rise in plasma oestradiol concentration and blocked or delayed the preovulatory LH and FSH surges. The observation that LH pulsatility was suppressed in these intact ewes implicates the hypothalamus as a central component of this effect of cortisol. This is in clear contrast to the earlier finding with ovariectomised ewes in which there was no evidence of an effect at the hypothalamic level (Breen & Karsch 2004), suggesting a lack of sensitivity to the pulse frequency suppressing effect of cortisol in such animals. Concern that this lack of effect in ovariectomised ewes might have arisen from inadequate duration of exposure to elevated cortisol levels has been ruled out by showing that 28 hours of cortisol treatment was also without effect (Oakley *et al.* 2006). These contrasting results indicate that central responsiveness to the effects of cortisol is dependent on the presence of ovary-derived factors (e.g. ovarian steroids). However, in the case of the pre-ovulatory surge of LH, the ability of elevated cortisol concentrations to block or delay this process is not affected by the ovarian steroid milieu, as shown by Wagenmaker *et al.* (2005). Recently, Breen and Karsch (2006) demonstrated that the cortisol-induced suppression of LH pulse amplitude in ovariectomised ewes was not influenced by season of the year, which contrasts starkly with the well-known effects of season on feedback by ovarian steroids.

In support of actions of cortisol at the level of the ovine hypothalamus is the evidence for presence of the type II glucocorticoid receptor in neurons of the preoptic area and arcuate nucleus (Dufourny & Skinner 2002). This receptor is co-expressed with the progesterone receptor and the estrogen  $\alpha$ -receptor (ER- $\alpha$ ) (Dufourny & Skinner 2002). By use of RU486, an antagonist, and dexamethasone, a specific agonist, Professor Karsch and his co-workers (Breen *et al.* 2004b; Case *et al.* 2005) have provided good evidence that the type II glucocorticoid receptor also mediates the cortisol-induced suppression of pituitary responsiveness in the ovariectomised ewe, i.e. a pituitary-level site of action for this receptor.

It is clear from these studies that both components of the hypothalamic-pituitary axis are involved in mediation of stress effects on reproduction. In the anterior pituitary gland of castrated male sheep, cortisol interfered with oestrogen-stimulated increase in expression of GnRH receptor (Adams *et al.* 1999), probably via classical nuclear glucocorticoid receptors and the resulting activation of transcription processes, as shown by Maya-Nunez *et al.* (2003) in rat pituitocyte cultures. However, Professor Karsch and his colleagues recently reported a study of ovariectomised ewes where there was no effect of cortisol on pituitary content of GnRH receptor (Breen *et al.* 2005b). This result appears to conflict with the findings of Adams *et al.* (1999) and leaves this involvement of GnRH receptors at the pituitary level unresolved. Also, when the pituitary gland was disconnected from hypothalamic regulation by surgical transection, GnRH-stimulated output of LH secretion was suppressed by various stressors, showing that there is a pituitary-only component involved in the inhibitory effects of stress (Stackpole *et al.* 2003). However, because the HPA axis was disrupted, cortisol would not have been involved in mediation of this effect of stress (Stackpole *et al.* 2003).

As well as the moderately confusing picture provided by some of the work reviewed above, there has to be caution about interpretation of the role and actions of corticosteroids in the responses to stress, especially in relation to immunity and inflammation. The co-involvement of cortisol in the inhibition of reproductive function induced by bacterial endotoxin, as discussed above, may appear to be at odds with the recognised immunosuppressive/anti-inflammatory roles of glucocorticoids. Also, these corticosteroids must participate in the negative feedback regulation of the HPA axis, thus inhibiting their own secretion. The complexity of involvement of these steroids in the responses to stress was comprehensively reviewed by Sapolsky *et al.* (2000). Since then, the research group led by Masugi Nishihara at the University of Tokyo has shown that glucocorticoids counteracted the TNF- $\alpha$ -induced inhibition of LH pulsatility (Matsuwaki *et al.* 2003) and the LH surge (Matsuwaki *et al.* 2004) in rats. Also they showed that adrenalectomy actually enhanced the inhibition of LH pulsatility in ovariectomised rats exposed to a variety of stressors including infection (Matsuwaki *et al.* 2006). These workers have implicated prostaglandins as the mediators of stress-induced suppression of LH pulsatility by using combinations of adrenalectomy, corticosterone and indomethacin (a cyclo-oxygenase inhibitor) on ovariectomised rats subjected to hypoglycaemic (2-deoxy glucose) or infectious (LPS) stress (Matsuwaki *et al.* 2006). A similar role for prostaglandins in sheep had been provided earlier (Harris *et al.* 2000, see below). However, as well as the blockade by corticosterone of the stress-induced inhibition of LH pulsatility (mainly reduced pulse amplitude), this steroid counteracted the stress-induced elevation of cyclo-oxygenase immunoreactivity in the brain (Matsuwaki *et al.* 2006) showing a direct inhibitory effect of corticosteroids on the involvement of prostaglandins, by reducing their synthesis. This and related work with rats has led to the concept that prostaglandins may be common mediators of stress-induced inhibition of the reproductive axis (Maeda & Tsukamura 2006). Also these authors contend that the output of glucocorticoids resulting from an activated HPA axis initially has a protective role by suppressing prostaglandin synthesis and, hence, helping to maintain reproductive function in spite of the underlying stressor activity (Maeda & Tsukamura 2006). This concept of a reproductive support role for glucocorticoids runs counter to their generally reproduction-inhibitory effects, such as those reported in the sheep studies described above. The roles of glucocorticoids are complex and their actions in response to stress can fall into permissive, stimulatory or suppressive categories, depending on the physiological endpoint in question (Sapolsky *et al.* 2000). The protective role of glucocorticoids during the early phase of the stress response, as described by Maeda & Tsukamura (2006, above), seems to belong to the suppressive category of response.

### Cytokine-mediated effects

As mentioned above, cytokines that are released into the circulation following stimulation of cells involved in the immune response have endocrine roles in the CNS and anterior pituitary gland, some of which are directed at components of the GnRH and gonadotrophin regulatory processes. In contrast, cytokine involvement in regulation of the selection and migration of GnRH neurons during embryonic and early fetal development is most likely to be mediated by locally derived cytokines. Previously, evidence for presence of cytokines and their receptors in the brain and anterior pituitary gland has been reviewed by Sternberg (1997), Benveniste (1998) and Turnbull & Rivier (1999). The occurrence of receptors for one cytokine, fibroblast growth factor (FGF), in regions of the brain involved in regulation of reproduction has been confirmed by Gill *et al.* (2004; 2006) using immunocytochemistry. They showed the presence of FGF receptors in developing GnRH neurons of mice and the emergence of these cells to be blocked by FGF receptor antagonists. In addition they reported a stimulatory effect of FGF on neurite outgrowth and branching. Functioning of cranial neurons can also be affected by transcriptional actions of oestrogens operating via classical oestrogen receptors (ER). However, Herbison and his colleagues, using ER-knockout mice, showed that there are rapid negative feedback effects of oestrogens mediated by membrane-level, non-genomic mechanisms (Abraham *et al.* 2004). Recently it has been shown in sheep that acute suppression of LH secretion by oestradiol or an oestradiol conjugate operates via such a non-genomic mechanism (Arreguin-Arevalo & Nett 2006). In the hypothalamus, membrane-level signalling in the negative feedback pathway to GnRH neurons involves a G protein-coupled receptor (GPR54) for which major ligands are the KiSS-1 peptins (de Roux *et al.* 2003; Seminara *et al.* 2003; Dungan *et al.* 2006). These peptins and their receptor (GPR54) appear to be involved in both inhibitory and stimulatory feedback effects of gonadal steroids in the hypothalamus (Smith *et al.* 2005) and it has been speculated that KiSS-1 neurons may provide the link between steroidal feedback signals and GnRH neurons that lack ER- $\alpha$  (Tena-Sempere 2005). It is possible that inflammatory peptides such as cytokines interact with ER both at classical genomic sites and with G protein-coupled receptors at the non-genomic, membrane-level sites. As an example of the former, IL-1 $\beta$  activation of transcription, presumably via the transcription factor NF- $\kappa$ B, is antagonised by oestradiol via ER action at this promoter site (Tyree *et al.* 2002).

Downstream from the hypothalamus, gonadotropes and folliculostellate cells of the anterior pituitary gland are also implicated as targets for cytokine action (Turnbull & Rivier 1999). For instance, the pro-inflammatory cytokines, TNF and IL-1, altered the phosphorylation status of a gap junction membrane channel protein (GJA1) in cultured rat folliculostellate cells (Fortin *et al.* 2006) indicating possible modulation of cell-to-cell communication and, thus, of growth and function of these cells. This affects the growth and secretion of hormones by endocrine cells of the anterior pituitary gland and is just one example of cytokine-pituitary interactions.

As mentioned earlier, one of the components of innate immunity is activation of an inflammatory response. This is generally mediated by the so-called pro-inflammatory cytokines, primarily IL-1, IL-6, IL-8 and TNF $\alpha$ , and is defined by induction of vasodilation and increased vascular permeability. Probably, it is at the endothelial cell level in organs such as the brain where these cytokines initiate phospholipase activity, thus causing disruption of cell membrane integrity and release of arachidonic acid – a precursor for prostaglandin synthesis by the cyclo-oxygenase pathway, or of leukotrienes by the lipoxygenase pathway. Harris *et al.* (2000) showed that blockade of prostaglandin synthesis in sheep by use of the cyclo-oxygenase inhibitor, flurbiprofen, prevented endotoxin-induced fever and suppression of LH secretion, thereby implicating prostaglandins as the possible central mediators of the disturbances in hypothalamo-

hypophyseal-ovarian function following such inflammatory challenge. The various studies using sheep to investigate the different levels within the reproductive neuroendocrine axis where endotoxin achieves such disruption has been comprehensively reviewed (Karsch & Battaglia 2002). These authors argued that the advantage of their sheep model over previously reported studies was the large size of the animal which made frequent blood sampling possible, thus enabling high resolution studies of hormonal dynamics. Because of this they were able to dissociate effects of endotoxin at hypothalamic, pituitary and ovarian levels and at various phases of the ovarian cycle (Karsch & Battaglia 2002). This work has shown that endotoxin interferes with the reproductive neuroendocrine axis at both hypothalamic and pituitary levels. Endotoxin inhibits both pulsatile GnRH secretion and pituitary responsiveness to GnRH (Battaglia *et al.* 1997; Williams *et al.* 2001). The effects of endotoxin on LH pulsatility are acute, occurring within one hour (Battaglia *et al.* 1997; Harris *et al.* 2000; Williams *et al.* 2001), whereas its effects on the oestrogen-induced LH surge process appear to be installed some 10 to 20 hours before the surge would be expected to occur (Battaglia *et al.* 1999). Further work (Breen *et al.* 2004a) showed that the inhibitory effect of endotoxin on the oestradiol-induced surge of LH in the ewe operated at the level of the hypothalamus by causing blockade of the GnRH surge. Administration of the cyclo-oxygenase inhibitor flurbiprofen did not interfere with this blockade of the LH surge nor with the inhibition of LH secretion prior to the surge, although it did prevent fever. This means that, in spite of the dampening effect of endotoxin on pituitary responsiveness to GnRH mentioned above, endotoxin-induced inhibition of the LH surge in ovariectomised ewes is due to inhibition of the GnRH surge mechanism in the hypothalamus. Also, the finding that inhibition of prostaglandin synthesis by flurbiprofen did not overcome the effects of endotoxin on the LH surge in sheep is interesting as it differs from endotoxin inhibition of GnRH and LH pulsatility which were blocked by flurbiprofen (Harris *et al.* 2000). In both studies endotoxin-induced fever was blocked by flurbiprofen, showing that fever and the effect of endotoxin on pulsatility may be mediated by prostaglandins. However, the study by Breen *et al.* (2004a) indicates the existence of a separate prostaglandin-independent pathway that mediates the effects of endotoxin on the surge process. These various findings could be resolved simply as follows: endotoxin inhibits the GnRH pulse mechanism in the hypothalamus via a prostaglandin mediated pathway and inhibits the surge process, also at a central level, by a prostaglandin-independent pathway. However, the authors mentioned they had gathered initial evidence that the pulse inhibitory mechanism may also not require a prostaglandin-mediated pathway. Nevertheless, because of the differences in latency between endotoxin effects on pulse and surge mechanisms, the authors inferred that the neuroendocrine processes which mediate the endotoxin-induced effects on GnRH pulsing are "separable and fundamentally different" from those mediating the effects on GnRH surges (Breen *et al.* 2004a). These differential effects of endotoxin had been discussed earlier by Karsch & Battaglia (2002). Endotoxin-induced blockade of the GnRH surge process at a central level does not rule out existence of a similar effect on the LH surge at the pituitary level, especially as an inhibitory effect on pituitary responsiveness to GnRH has been demonstrated (Williams *et al.* 2001). However, evidence that the LH surge was of normal amplitude in ewes in which the endotoxin treatment unexplainably failed to block the GnRH surge (Breen *et al.* 2004a) strongly rules out this possibility, unless the quantity of GnRH secreted was so great that it masked any pituitary-level deficiency.

Existence of prostaglandin-independent pathways in the mediation of endotoxin effects on neuroendocrine events does not overshadow the common role of prostaglandins in stress-induced activation of the HPA axis (described above) and immunostimulation processes, but it does indicate levels of complexity in the regulatory mechanisms that await further resolution. It is not clear whether other central mediators of inhibitory actions on gonadotrophin secretion such as the endogenous opioids and corticotrophin-releasing hormone are released directly in response to endotoxin or via prostaglandin-dependent pathways, nor to what extent cytokines



released by endotoxin-activated immune cells exert direct endocrine influences in the hypothalamus and anterior pituitary gland. There is evidence that endotoxin does cause release of cytokines in sheep. Intravenous administration of endotoxin to sheep elevated circulating levels of TNF (Coleman *et al.* 1993; Perkowski *et al.* 1996; Harris *et al.* 2000) and increased expression of IL-1 $\beta$  mRNA in the choroid plexus (Vellucci *et al.* 1996). However, the role of cytokines at the pituitary gland level is complicated by *in vitro* studies of cultured ovine pituitary cells that have actually shown an increase in accumulation of LH in the media on administration of either endotoxin (Coleman *et al.* 1993) or the pro-inflammatory cytokines, IL-1 $\alpha$  and IL-1 $\beta$  (Braden *et al.* 1998). Also, the possible influence of ovarian steroids on production of cytokines or on central responsiveness to immunostimulation provides another level of complexity that is largely unresolved at present.

Virtually all the direct evidence for an inhibitory role of pro-inflammatory cytokines at the central level of regulation of reproduction has been obtained from rats, largely by intracerebroventricular administrations (reviewed by Kalra *et al.* 1998). A general analysis of cytokine signalling in the brain has been provided by Turnbull & Rivier (1999) and more recently the receptor-level mechanisms of pro-inflammatory cytokine action in the brain were reviewed by Turrin & Rivest (2004). The latter concluded that pro-inflammatory cytokines can act on endothelial cells forming the blood-brain barrier and at circumventricular organs to cause release of prostaglandin E-2 (Turrin & Rivest 2004). Another signalling pathway in the brain involves binding of cytokines to endothelial cells with activation of nitric oxide synthase and production of nitric oxide as the secondary messenger (Wong *et al.* 1996; Scammell *et al.* 1996). Both the prostaglandin-mediated and the nitric oxide-mediated mechanisms are important in induction of various brain-level responses from peripherally derived cytokines (Sternberg & Webster 2003).

Fever is one aspect of the immune response that, in the case of endotoxin administration, is mediated centrally by prostaglandins and which may have direct effects on the reproductive axis. Low dose delivery of LPS (40 ng/kg) induced fever and elevated circulating cortisol levels in sheep (Williams *et al.* 2001). However, this dose appeared to be near the threshold for effects on GnRH pulsatility because suppression of GnRH pulsing occurred in only a few of the treated ewes and only 10 out of 16 ewes had disrupted LH pulsing. This occurrence of fever without complete disruption of LH pulsing is consistent with an earlier finding (Schillo *et al.* 1990) that elevated body temperature did not alter LH pulsing in ewes. However, the latter authors reported a reduction in mean plasma LH concentration that leaves the question of direct effects of elevated body temperature on LH secretion unresolved. Further work conducted both in Professor Karsch's laboratory in Michigan and at Lincoln University, New Zealand has attempted to determine whether the endotoxin-induced inhibition of LH pulsing is attributable to the elevation of body temperature *per se* by using cyclo-oxygenase inhibitors to block endotoxin effects in ewes exposed to environmental temperatures that raised their body temperature to values equivalent of endotoxin-induced fever. The results (unpublished) give moderate support for some influence of elevated body temperature *per se* on inhibition of LH pulsatility, which provides further evidence for a prostaglandin-independent pathway for suppression of the reproductive neuroendocrine axis.

### Overview

It is well accepted that immune/inflammatory stress and activation of HPA axis inhibit the reproductive neuroendocrine axis and previous reviews of this topic (e.g. Karsch & Battaglia 2002) have shown these disruptive effects acting at several regulatory processes in this axis. In

the past three to four years there has been further resolution of these pathways, giving insight into separate avenues for direct cytokine actions at central levels and for activation of the HPA axis, with the central disruption mediated by cortisol. Also, there is a clearer view of the involvement of ovarian steroids in these events, as revealed by comparison between studies using ovariectomised and ovarian intact ewes. There are many aspects of brain function in relation to regulation of reproduction in mammals that remain shrouded in mystery. These include changes associated with puberty, seasonality, nutrition, immunostimulation and other forms of stress, and aging. It is almost certain that the mechanisms of some of these changes in brain function will be linked to each other and the continued resolution of regulatory mechanisms for any one of these will unravel and help to explain others. This argument should justify continuation of the stepwise approach to resolving individual pathways that has been demonstrated by the studies reviewed above.

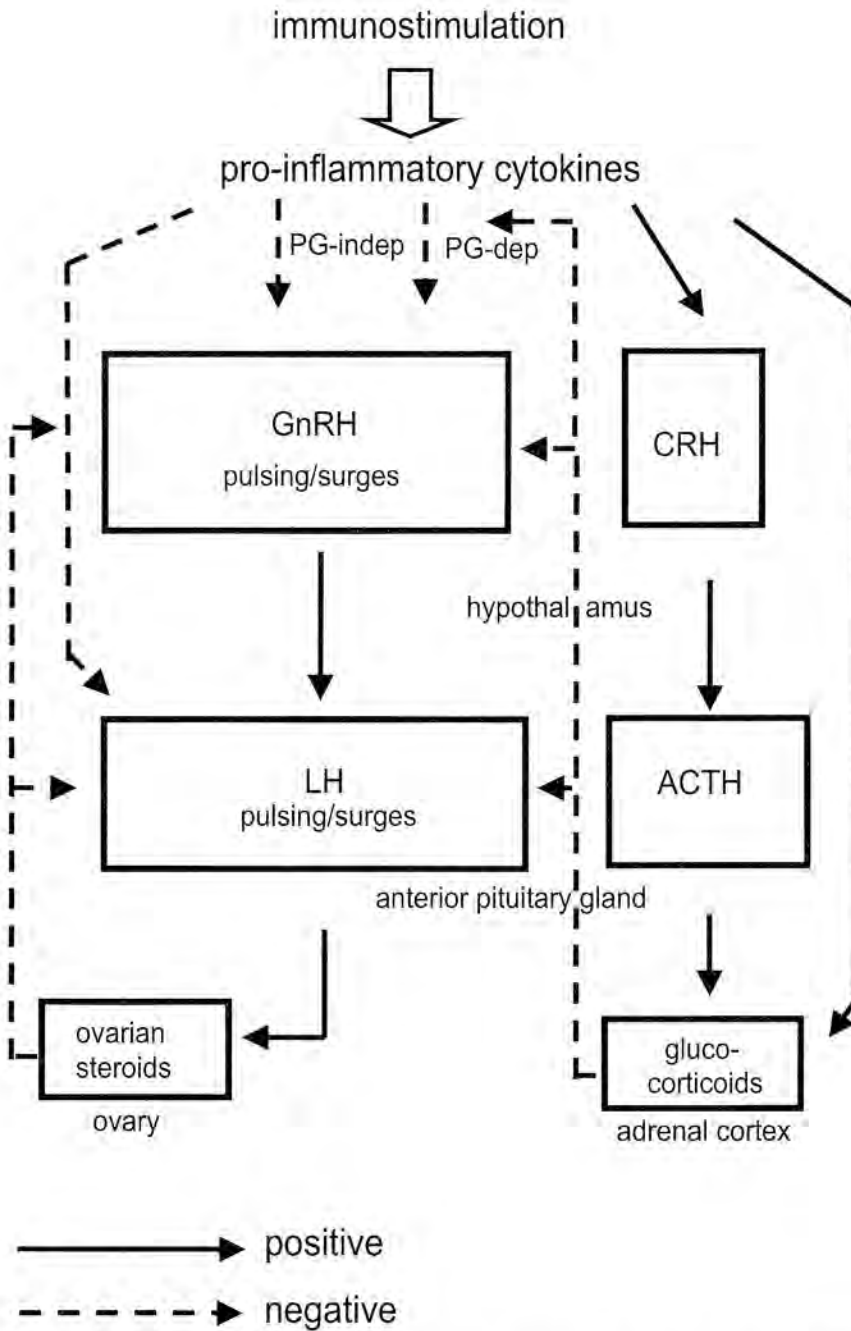
### Conclusions

It is clear that the multiplicity of pathways by which immune-neuroendocrine interactions impinge on the reproductive axis is far from resolved and there is much further investigation required to identify the specific sites, cells and cellular mechanisms that are involved. There is some uncertainty about the specific involvement of ovarian steroids in these processes. However, it seems clear that discrepancies which have arisen between results from studies using ovarian intact ewes and those involving ovariectomised ewes or steroid-replaced ovariectomised ewe models indicate an important role of ovarian steroids in the mechanisms that mediate the effects of stress-activated pathways. Ovarian steroids appear to enhance sensitivity of these pathways, which means that the presence of ovaries is required in any conceptual model of these processes. The ovary can thus be regarded as an essential component of the immune stress-reproductive neuroendocrine axis, i.e. its secretory products are involved in the central mediation of events as well as it being a target end-organ of the female reproductive system. Fig.1 provides a schema based on earlier models (e.g. see Karsch & Battaglia 2002) that incorporates linkages revealed by recent studies, primarily from those using sheep as experimental animals.

Activation of an immune response, as commonly modelled by administration of bacterial endotoxin, causes the release of pro-inflammatory cytokines as well as activation of the HPA axis with consequent release of corticosteroids. These humoral factors activate endothelial cells within CNS blood vessels or stimulate CNS neurons and anterior pituitary cells directly, leading to suppression of the GnRH pulse generating and surge mechanisms in the hypothalamus and/or inhibiting anterior pituitary responsiveness to GnRH. There is evidence for both prostaglandin mediation and prostaglandin-independent mediation of these processes and for involvement of the type II glucocorticoid receptor in the corticosteroid-mediated events. With respect to ruminant reproduction, it is noteworthy that virtually all of the recent studies are based on sheep as the animal model and much of the sheep work has emanated from a single laboratory.

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**Fig 1.** Pathways of linkages between pro-inflammatory cytokines released during immunostimulation and their disruptive effects on the reproductive neuroendocrine axis. Solid arrows represent stimulation, dashed arrows represent inhibition. N.B. PG-indep = prostaglandin-independent, PG-dep = prostaglandin-dependent

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