A conceptual model of the influence of stress on female reproduction

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Intriguingly, similar neurotransmitters and nuclei within the hypothalamus control stress and reproduction. GnRH neurone recruitment and activity is regulated by a balance between stimulation, suppression and permissiveness controlled by noradrenaline, neuropeptide Y and serotonin from the brain stem, impact from glutamate in the medial preoptic area and neuropeptide Y in the arcuate nucleus, in opposition to the restraining influences of γ -aminobenzoic acid within the medial preoptic area and opioids from the arcuate nucleus. Stress also activates neuropeptide Y perikarya in the arcuate nucleus and brain stem noradrenaline neurones. The latter project either indirectly, via the medial preoptic area, or directly to the paraventricular nucleus to release corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). Within the medial preoptic area, GnRH neurones synapse with CRH and AVP axons. Stimulation of CRH neurones in the paraventricular nucleus also activates γ -aminobenzoic acid and opioid neurones in the medial preoptic area and reduces GnRH cell recruitment, thereby decreasing GnRH pulse frequency. Oestradiol enhances stress-induced noradrenaline suppression of LH pulse frequency but when applied in the paraventricular nucleus or brain stem, and not in the medial preoptic area or arcuate nucleus. The importance of CRH and AVP in the medial preoptic area needs confirming in a species other than the rat, which uses adrenal activation to time the onset of the GnRH surge. Another stress-activated pathway involves the amygdala and bed of the nucleus stria terminalis, which contain CRH neurones and accumulate γ -aminobenzoic acid during stress.

Transmitting genes to the next generation is the most important activity of any organism. However, when conditions are not optimal, it is prudent to suspend reproduction until the environment is more favourable. In general, stress-induced adrenal stimulation delays or even inhibits the preovulatory GnRH–LH surge. In female rats, stress–reproduction interactions are complex because of a timing mechanism that increases adrenal gland activity before the GnRH–LH surge on the afternoon of pro-oestrus. Hence in rats, adrenal stimulation is normally associated with the facilitation of surge initiation. Therefore, comparisons with other species that do not present this complication are advantageous. The hypothalamus is larger in sheep than it is in rats and so anatomical targeting studies are more precise, and it is possible to collect frequent hypophyseal portal samples to delineate precise patterns of GnRH–LH secretion. Such techniques can be used to identify the critical link(s) between the mechanisms controlling reproduction and

stress responses. For example, in the context of fertility, it is pertinent to know the importance of stress-induced changes in GnRH–LH pulsatility, oestradiol profiles and the precise timing of events leading up to the LH surge.

Many of the links between reproduction and stress-control systems involve neurotransmitters. These compounds convey messages between neurones and are crucial for the control of correct hypothalamic GnRH secretion in different reproductive phases. Individual neurotransmitters are often stimulatory (for example, noradrenaline and neuropeptide Y), whereas other neurotransmitters usually exert inhibitory influence (for example, opioids and gamma amino butyric acid (GABA)). Neurotransmitters are secreted on one side of an interneurone interface (synapse) and engage with specific receptors on the opposite neurone. Furthermore, some neurones have an auto-inhibitory system, which means that presynaptic receptors are activated so that when a neurotransmitter is released, it can act back on the secreting cell and inhibit its own production.

The present article reviews recent knowledge concerning the normal regulation of the systems controlling

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Fig. 1. (a) Luteal and oestradiol-induced activation and transmission phases before the surge secretion of GnRH (derived from Evans *et al.*, 1995, 1997) in ewes. (b) Proposed changes in neurotransmitter influence during normal GnRH secretion in ewes. DA: dopamine; GABA: gamma amino butyric acid; NA: noradrenaline.

Fig. 2. (a) Changes in the endocrine milieu caused by stress in ewes (derived from Dobson and Smith, 1998, 2000). (b) Proposed changes in neurotransmitter influence during stress-induced delayed GnRH secretion in ewes. DA: dopamine; GABA: gamma amino butyric acid; NA: noradrenaline.

reproduction or stress (the hypothalamus–pituitary–ovary axis (HPO) and the hypothalamus–pituitary–adrenal gland axis (HPA)), and then examines HPO and HPA inter-relationships (in particular, the importance of oestradiol sensitivity) before placing the whole theme in the context of human and animal fertility.

Control of normal reproductive hormone profiles

Normal endocrine changes in female sheep during the follicular phase (Fig. 1) contrast with those imposed by stress (Fig. 2). The pivotal roles of different parts of the brain and hypothalamus in controlling and maintaining normal hormone profiles have been reviewed by

Herbison (1997, 1998) and Smith and Jennes (2001). Pulsatile activity is an essential intrinsic property of hypothalamic GnRH neurones and the intricate balance between GnRH synthesis and release is important to ensure that GnRH neurones are in a state of constant readiness to respond to changes in the environment (Vazquez-Martinez *et al.*, 2001). Regulation of pulsatile GnRH secretion probably involves interneurons because GnRH neurones have very few receptors for hormonal steroids. In brief, the most important site for regulation of GnRH secretion is the GnRH perikarya in the medial preoptic area of the hypothalamus (MPOA). There is still uncertainty concerning modulation of GnRH release at the other end of the axons in the median eminence (ME); similarly, debate continues over the existence of separate systems for pulsatile and surge secretion (Herbison, 1998). However, we propose that precisely controlled GnRH pulse frequency and amplitude are essential prerequisites for the timely production of appropriate GnRH–LH surge secretion. Consequently, disruption of pulsatile secretion will have deleterious effects on the production of a normal surge and will therefore reduce fertility.

Influence of the brain stem on GnRH neurones in the MPOA and modulation by oestradiol

There is a delicate balance between the positive and negative influences of oestradiol, noradrenaline and GABA in the control of GnRH perikarya in the MPOA (Figs 3 and 4). From areas within the brain stem, almost all noradrenergic cells with oestradiol receptors in regions A1 (ventrolateral medulla; VLM) or A2 (nucleus of the tractus solitarius; NTS) project axons to the MPOA to synapse with GnRH perikarya to stimulate GnRH secretion (Herbison, 1998; Scott *et al.*, 1999). Axons from noradrenaline cells in regions A1 or A2 also directly or indirectly activate GABA perikarya in the MPOA to suppress GnRH secretion. Hence, there is a balance between brain stem stimulation and suppression of GnRH secretion from the MPOA (Herbison, 1998). All GABA-ergic cells that synapse with GnRH neurones have steroid receptors and are extremely sensitive to these hormones. In the luteal phase, progesterone increases GABA tone and hence maintains very low frequency square-wave GnRH pulses (Fig. 1a). During the very early follicular phase in the presence of declining peripheral progesterone concentrations, oestradiol inhibits the autoinhibitory system of GABA cells so increased GABA concentrations are maintained in the MPOA, thereby maintaining suppression of GnRH–LH secretion (Fig. 1). Longer exposure to oestradiol during the mid-follicular phase in sheep increases noradrenaline in the MPOA culminating maximally in the late follicular phase just before the GnRH–LH surge (Robinson *et al.*, 1991). Subsequent uncoupling of noradrenaline stimulation of GABA cells follows (by an unidentified but crucial mechanism) which

Fig. 3. Location and influence of numerous neuronal nuclei in the brain of the ewe that regulate GnRH and corticotrophin-releasing hormone (CRH)–arginine vasopressin (AVP) secretion, indicating the spatial relationship between regions of the hypothalamus (medial preoptic area (MPOA), ventral medial nucleus (VMN) and arcuate nucleus (ARC) of the medial basal hypothalamus) and the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC)). PVN: paraventricular nucleus.

removes the suppression by GABA. The decrease in GABA influence and increase in noradrenaline influence lead to a surge in GnRH secretion (Herbison, 1998) and explain earlier observations in sheep that noradrenaline administered in the late follicular phase stimulates LH secretion, whereas antagonists block the LH surge (Narayana and Dobson, 1979). It must also be noted that recent studies of the MPOA using electron microscopy revealed that receptors for noradrenaline in the GnRH cell membrane are not very close to GnRH synapses in regions A1 and A2. Thus, there may be reliance on asynaptic release of noradrenaline followed by diffusion to receptors within the GnRH neurone before cell activation (Smith and Jennes, 2001). Nevertheless, the activities of GABA and GnRH perikarya in the MPOA are clearly modulated by brain stem noradrenaline neurones in response to changes in oestradiol concentration.

Influence of the medial basal hypothalamus on GnRH neurones in the MPOA and modulation by the brain stem

In addition to control by the brain stem, GnRH perikarya in the MPOA (Fig. 4) are also under the influence of afferent neurones from the arcuate nucleus (ARC) and ventromedial hypothalamic nucleus (VMN) (collectively known as the medial basal hypothalamus (MBH)). Opioid perikarya in the ARC (few of which have oestradiol receptors) project axons to the MPOA and synapse directly with GnRH cells (Goubillon *et al.*, 1999, 2002). Opioids from the ARC, noradrenaline from the brain stem and GABA neurones from within the MPOA may be involved in synchronizing the firing of different groups of neurones in the GnRH network

Fig. 4. Schematic representation of the location and influence of numerous neurons that regulate GnRH secretion (data amalgamated from observations in ewes and rats). The comma-shaped object represents a GnRH neurone; the grey shaded rectangular areas denote different portions of the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC)) and the yellow areas indicate regions in the hypothalamus (medial preoptic area (MPOA), ventral medial nucleus (VMN) and the arcuate nucleus (ARC) of the medial basal hypothalamus). The blast shapes represent neurones containing specific neurotransmitters (noradrenaline (NA), neuropeptide Y (NPY), glutamate (GLUT), *N*-methyl-D-aspartate (NMDA), gamma amino butyric acid (GABA), endogenous opioid (EOP) and dopamine (DA)). The presence of receptors for oestradiol (ER) is indicated on appropriate neurones. The interlinking black lines between areas of the brain represent known direct axons. Identified synapses are shown by red striped bars, whereas close apposition between axons and perikarya is represented by plain red bars. If direct intercellular contact remains to be proven, the axons end in an area with a purple circle. There are axons from GnRH cells to the median eminence but these have been omitted here for clarity.

(Robinson *et al.*, 1991; Goodman *et al.*, 1995, 1996). It is not certain how many of the total of 2000 GnRH neurones discharge at any one time either for each GnRH pulse or during the preovulatory surge. However, it has been hypothesized that different interneurons alter secretion rates of various groups of GnRH axons and collaborate in the co-ordinated cascade of events that culminates in surge secretion (Karsch and Evans, 1996). Within the MPOA, opioids regulate GnRH secretion (Goodman *et al.*, 1995) but as there are few endogenous opioid (EOP) receptors on GnRH cells, other interneurons may also be involved (Thind and Goldsmith, 1988). Opioid neurones also synapse with noradrenaline interneurons in the MPOA. It is of interest that a decrease in opioid activity just before the LH surge occurs synchronously with the uncoupling of noradrenaline influence on GABA cells in the MPOA (Herbison, 1998).

Thus, the MBH can modify the brain stem control of GnRH perikarya. Electrodes placed within the ARC–ME area have detected increased neuronal electrical activity after the withdrawal of progesterone in female goats. However, the frequency of electrical impulses decreases just before and during the GnRH surge (Takeuchi *et al.*, 1997).

There are other examples of MBH modulation of GnRH neurones in the MPOA. In rats, opioids restrain stimulatory catecholaminergic, glutaminergic and nitric oxide input, and enhance inhibitory GABA (Smith and Jennes, 2001). In addition, dopamine concentrations in the VMN increase just before the GnRH surge (Anderson *et al.*, 2001). Perikarya containing neuropeptide Y within the VMN–ARC also exert stimulatory influence in the MPOA (Fig. 4); neuropeptide Y is essential for GnRH surge secretion as central administration of antibodies

to neuropeptide Y blocks the LH surge in rats and sheep (Porter *et al.*, 1993). This effect could be exerted indirectly via influences on GnRH pulse frequency, or directly by interfering with any as yet unidentified 'surge mechanism'. In addition to the neurotransmitter systems already mentioned, approximately 50% GnRH cells that activate *c-fos* during the LH surge also have glutamate receptors, along with up to 80% activated GnRH cells that have *N*-methyl-D-aspartate (NMDA) receptors (Smith and Jennes, 2001). Clearly, the neuronal control of GnRH secretion involves complex but orderly interactions between the brain stem, MBH and MPOA.

Working model to explain the generation of the GnRH–LH surge

An experimental model has been proposed to facilitate closer examination of the temporal sequences leading to the GnRH–LH surge in sheep (Evans *et al.*, 1997). Here, the model is elaborated by presenting explanations in terms of the changing influence of neurotransmitters. The resultant concept indicates several possibilities, many of which are based on patchy evidence and remain to be fully tested. In the model, oestradiol implants, inserted into ovariectomized ewes 16 h after progesterone withdrawal, begin a three-stage surge induction process (Fig. 1). First, there is a signal-reading phase of 5–10 h for activation of oestradiol-sensitive neurones. Second, a signal-processing phase (from 10 to about 18 h) when message(s) are transmitted to GnRH neurones via one or more oestradiol-sensitive interneurones. A third stage involves initiation of GnRH surge release (from about 18 to 20 h). These stages approximate to the gradually merging phases of different GnRH profiles (Evans *et al.*, 1995).

Initially, there are infrequent GnRH–LH pulses (one every 4 h) in the progesterone-dominated phase that precedes the insertion of oestradiol implants. In comparison, during the follicular activation phase, pulse frequency increases and pulse amplitude decreases but square-wave GnRH pulses are discrete with barely detectable basal GnRH secretion (Fig. 1a). This pattern occurs in the presence of high GABA and opioid tone with increasing noradrenaline influence (Fig. 1b). During the early transmission phase, GABA influence declines, noradrenaline continues to increase and GnRH pulses begin to develop a more spike-like triangular shape. In the late transmission phase, within 3–4 h before surge onset, opioid influence begins to decline, GnRH pulses become more triangular and interpulse GnRH concentrations increase (Goodman *et al.*, 1995). The final surge initiation phase reveals very high GnRH concentrations and pulses are difficult to distinguish. Whether the GnRH format changes to continuous high secretion or very rapid pulses is open to debate (Evans *et al.*, 1995). Overall, it is hypothesized that GnRH neurones become progressively desynchronized and

surge secretion ensues with all neurones maximally activated and secreting GnRH.

Influence of exposure to progesterone before the follicular phase

The above neuroendocrine observations might explain the balance between the effects of oestradiol and progesterone (although the following hypotheses do require further rigorous testing). In the early follicular phase of intact ewes, there is a gradual decline in progesterone after the onset of luteolysis simultaneous with a gradual increase in oestradiol stimulated from follicles by increasing LH pulse frequency. During this phase, progesterone acts via GABA neurones to hold GnRH pulse frequency in check in the face of increasing oestradiol stimulatory influence (Robinson *et al.*, 1991). Even after removal from the peripheral circulation, progesterone has long-lasting effects. The classic progesterone receptor blocker, RU486, will advance the LH surge by 9 h when administered to intact animals even 37 h after prostaglandin-induced luteolysis (surge onset at 53 h after luteolysis versus 62 h in controls; Dobson *et al.*, 2000a). Thus, progesterone modulates the influence of oestradiol.

Early oestradiol action

When considering further details of the neuronal effects of oestradiol, it is important to recognize that almost all neurones with oestradiol receptors (70% of which contain noradrenaline) in the caudal A1 region of the brain stem project to the MPOA (Fig. 4). In addition, 30% noradrenaline cells in A1 express *c-fos* (an early indicator of neuronal activity) within 2 h of oestradiol treatment (Scott *et al.*, 1999; Rawson and Clarke, 2001). In the same time frame, oestradiol also induces *c-fos* activity in approximately 20% of neuropeptide Y cells and in a few opioid cells in the VMN. However, most of the activated neurones are of unidentified phenotype (Clarke *et al.*, 2001). Thus, it is hypothesized that, under the influence of oestradiol during the early activation phase, noradrenaline neurones from the brain stem and from within the MPOA initially activate MPOA GABA perikarya. This activation of GABA neurones by noradrenaline, along with the neuropeptide Y and opioid influence of MBH origin, decrease pulse amplitude and increase pulse frequency (Scott *et al.*, 1992; Smith and Jennes, 2001). It is proposed that, during the transmission phase, GnRH pulses begin to change shape as the effects of GABA gradually diminish (Fig. 1). In the late transmission phase, a decrease in opioid tone further prolongs pulse duration and increases GnRH secretion between pulses (Goodman *et al.*, 1995; Evans *et al.*, 1997). Eventually, at the onset of the surge, all inhibition is removed.

Clearly, GnRH secretion is regulated by a balance between stimulation, suppression and permissiveness controlled by noradrenaline from the brain stem, sudden impact from glutamate in the MPOA and neuropeptide Y from the ARC, in opposition to the inhibitory influences of GABA within the MPOA and opioids from the ARC. Furthermore, it is pertinent at this point to note that oestradiol must be administered into the VMN rather than the MPOA to generate an LH surge (Herbison, 1998).

Other roles of oestradiol in generation of the GnRH–LH surge

Removal of oestradiol implants before initiation of the surge (that is, after only 10 h exposure) influences neither the timing of surge onset nor the amplitude of the GnRH surge. However, less LH is released, indicating that there is a requirement for longer oestradiol priming at the pituitary gland for a full amplitude LH surge (Evans *et al.*, 1997). Oestradiol enhances hypothalamic GnRH synthesis and pituitary responsiveness to GnRH by increasing GnRH receptor mRNA, and thus the number of receptors, in gonadotrophs, creating greater stores of LH (Hamernik, 1995; Brooks and McNeilly, 1996; Jimenez-Linman and Rubin, 2001).

In summary, oestradiol has marked effects at the hypothalamus and the pituitary gland to produce regulated GnRH pulses and a normal, appropriately timed LH surge.

Normal HPA endocrine responses to stressors

Physiological amounts of insulin are required for normal neuronal activity but the sudden exposure to bolus insulin injections will activate the HPA axis, as will many other stressors, for example, transport, restraint or isolation. As part of this response, ACTH release from the pituitary gland is stimulated by activation of perikarya in the hypothalamic paraventricular nucleus (PVN; Figs 3 and 5). The two releasing factors from the PVN, corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP), are secreted from the ME into the hypophyseal portal system to release ACTH from the pituitary gland but the relative importance of CRH and AVP is currently controversial (Smith *et al.*, in press). More CRH than AVP is released in rats, whereas the reverse occurs in sheep. The CRH : AVP ratio may be governed by the intensity of the stressor, with CRH predominating after low doses of insulin whereas more AVP is secreted after high doses (Caraty *et al.*, 1990). However, CRH is a more potent stimulator of ACTH secretion than is AVP in sheep (McFarlane *et al.*, 1995). Nevertheless, AVP is required in stress-induced suppression of reproductive activity, as revealed by the ability of AVP antagonists to prevent the suppressive effects of insulin on LH secretion (Heisler *et al.*, 1994).

Influence of the brain stem and MPOA on PVN secretory activity

Higher up the system (Fig. 5), stressors increase the firing rate of regions in the brain stem, particularly noradrenaline–neuropeptide Y neurones in the A1 (VLM), A2 (NTS) and A6 (locus coeruleus; LC) regions. There are synaptic contacts between noradrenaline–neuropeptide Y nerve fibres and CRH perikarya in the PVN, although there have been no confirmatory retrograde or anterograde double-labelled studies to confirm the location of the perikarya of these axons (Krysiak *et al.*, 1999). In contrast, detailed tracing studies in rats have shown that noradrenaline perikarya send axons from the A1 (VLM) to the MPOA that, in turn, synapse with afferent neurones projecting to the PVN, and that these axons are activated during stress (Kawano and Masuko, 1999, 2000). It is important to establish the significance of CRH pathway interactions through the MPOA in species other than the rat that do not have an increase in corticoids associated with the GnRH–LH surge.

Involvement of receptors during negative feedback

A stimulatory role of noradrenaline in the HPA is confirmed by the sustained release of ACTH after prolonged central noradrenaline administration, in contrast to the short-lived ACTH secretion that occurs during exposure to spontaneous stressors. One explanation of these contrasting scenarios could involve immediate activation of a negative feedback system exerted by glucocorticoid receptors on CRH cells in the PVN (Fig. 5). In addition, the hippocampus contains many mineralocorticoid and glucocorticoid receptors and, although there is no direct innervation to the PVN, there are projections from the hippocampus to the ARC and VMN that exert negative feedback on PVN activity. Furthermore, axons from neuropeptide Y perikarya with glucocorticoid receptors in the ARC project to the PVN. It is reasonable that there should be an efficient negative feedback system in view of the deleterious effects of overstimulation of the HPA, that is, prolongation of enhanced glucose metabolism, altered vascular dynamics and deranged immune responses.

Other pathways modulating PVN secretion

In addition to a significant role in emotional behaviour and motivation, the rat central nucleus of the amygdala (CeA) also contains many CRH perikarya with efferent pathways to the hypothalamus through the lateral bed nucleus of the stria terminalis (BNST) and onward to the MPOA. Lesions of these tracts lower stress-induced CRH concentrations in the ME (Tellam *et al.*, 2000).

It has been suggested that CRH is a neurotransmitter as well as a neurohormone (Tellam *et al.*, 2000). CRH injected intracerebroventricularly (i.c.v.) or directly into

Fig. 5. Schematic representation of the location and influence of numerous neurons that regulate corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) secretion from the paraventricular nucleus (PVN) (data amalgamated from observations in ewes and rats). The comma-shaped object represents CRH–AVP or GnRH neurones; the grey shaded rectangular areas denote different portions of the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC)) and the yellow areas indicate regions in the hypothalamus (medial preoptic area (MPOA), ventral medial nucleus (VMN) and the arcuate nucleus (ARC) of the medial basal hypothalamus). The blast shapes represent neurones containing specific neurotransmitters (noradrenaline (NA), neuropeptide Y (NPY), glutamate (GLUT), *N*-methyl-*D*-aspartate (NMDA), gamma amino butyric acid (GABA), endogenous opioid (EOP) and dopamine (DA)). The presence of receptors for oestradiol (ER) is indicated on appropriate neurones. The presence of receptors for CRH, gamma amino butyric acid (GABA) or glucocorticoids (CRH-R, GABA-R or GR) are indicated on appropriate neurones. The interlinking red lines between areas of the brain represent known direct axons. There are axons from GnRH and CRH–AVP cells to the median eminence but these have been omitted here for clarity.

the LC increases the neuronal firing rate in this region and increases the release of noradrenaline in areas receiving projections from the LC (Fig. 5). Indeed, there is evidence to show that, in response to a variety of stressors, there is a cascade relationship between noradrenaline and CRH secretion in all areas containing CRH perikarya, that is the LC, CeA, BNST, MPOA and PVN (Koob, 1999). Thus, CRH, as a neurotransmitter, may enhance activation of the HPA as well as being involved in final ACTH output.

Reproductive hormone profiles during disruption by stressors

After presenting individual HPO and HPA control mechanisms, changes in reproductive hormone profiles

during exposure to stress will be examined, and this is followed by a discussion of the interactions involved within the brain.

Effects of stressors on tonic GnRH–LH pulsatile secretion

There are now many examples of gonadectomized animals (male and female) exhibiting a reduction in GnRH and hence LH pulsatility within 5–10 min of exposure to stress (Fig. 2a) indicating non-genomic effects on membrane polarization of interneurons (Fig. 2). It is difficult to ascertain from the available literature whether there are differences between stressors in their effects on pulse frequency or amplitude; if frequency of GnRH pulses is reduced, this may indirectly affect

amplitude because basal values will differ. The use by different laboratories of different steroid treatments before exposure to stress also complicates the issue. Furthermore, most studies have used LH measurements to reflect changes in GnRH, but it is now known that not all GnRH pulses are accompanied by LH secretion. However, changes in frequency do indicate a major effect at the hypothalamus. Indeed, the frequency, but not the amplitude or duration, of hypothalamic multi-unit electrical activity is decreased in stressed goats compared with controls (Takeuchi *et al.*, 1997).

In rats, adrenaline modulates LH frequency, whereas noradrenaline affects both frequency and amplitude (Smith and Jennes, 2001). By comparison, in non-stressed sheep, GABA neurones control pulse frequency, whereas noradrenaline and opioid neurones mediate changes in pulse amplitude (Scott and Clarke, 1993; Goodman *et al.*, 1995, 1996). As CRH predominates during 'weak' stressors and additional AVP is secreted during more intense stress (Caraty *et al.*, 1990), it is possible that CRH affects pulse frequency via GABA–noradrenaline neurones, whereas AVP responses involve opioidergic cells, particularly as, in rats, i.c.v. AVP affects pulse amplitude but not frequency (Cates *et al.*, 1999). This speculation urgently requires testing under closely controlled conditions by measuring GnRH in portal blood and by histochemical examination of multi-labelled tissues.

Effects of stress on GnRH–LH surge secretion

Acute stressors can also interrupt the occurrence of a normal GnRH–LH surge during spontaneous follicular phases in intact ewes and in steroid-treated ovariectomized animals (Fig. 2a). After exposure to the severe stressors of endotoxin or high dose insulin during the activation phase, the onset of the LH surge is delayed by approximately 10 h. Later, during the transmission phase, endotoxin has no effect on timing or other surge parameters. However, the onset is delayed by insulin and surge maximum concentrations are lower. Even the lesser stress of transport delays and reduces the magnitude of the LH surge when imposed during the late transmission or surge onset periods (Smith *et al.*, in press).

The influence of stressors at the pituitary gland has been revealed by the reduced secretion of LH in response to GnRH, both *in vivo* and *in vitro* (Phogat *et al.*, 1999), and could be one explanation for reduced maximum surge values. Reduced surge concentrations probably relate to the importance of pulsatile GnRH in maintaining the pituitary gland in a primed state. In addition, continued oestradiol exposure is curtailed during ACTH-induced reduction in LH pulsatility in the follicular phase, and this will have consequences at both the hypothalamus and pituitary gland (Dobson *et al.*, 2000b).

Mechanisms of interactions between HPA and HPO within the brain

Stress-induced changes in reproductive hormone profiles are the result of altered neurotransmitter activities mediated by different neurones in several locations.

Mediation by CRH and AVP

As the PVN is activated in all stressful situations, both CRH and AVP are prime candidates as mediators of reduced GnRH secretion. Indeed, CRH receptors have been identified on GnRH perikarya in rat immortal cell lines (Tellam *et al.*, 2000; Fig. 5). Furthermore, administration of CRH or AVP either i.c.v. or directly into the MPOA (but not the PVN, ARC or ME) reduces GnRH secretion in rats or monkeys, and reversal of the effect by naloxone indicates opioid involvement (for reviews, see Rivier and Rivest, 1991; Phogat *et al.*, 1997). Reversal of the CRH–AVP-induced suppression of GnRH by prior oestradiol treatment in monkeys indicates that the influence of steroids is balanced in different ways in different species, or that there is an oestradiol dose effect (Chen *et al.*, 1992).

Confusingly, in sheep, i.c.v. CRH administration or exposure to transport can increase GnRH–LH output, but only during periods of oestradiol negative feedback (Caraty *et al.*, 1997; Dobson *et al.*, 1999). This finding could be the result of neurotransmitter action unconnected to the stress axis, or steroid feedback mechanisms may be important. As a further complication, restraint of CRH knockout mice also decreases pulsatile and surge LH secretion indicating the presence of an opioid or AVP compensation mechanism. The importance of an AVP mechanism is emphasized by the fact that insulin has no effect on LH pulsatility in Brattleborough rats (a strain in which AVP is absent). More evidence lies in the inability of the antagonist helical CRH always to reverse the effects of CRH on LH release (Jeong *et al.*, 1999). Most of these observations can be explained by a co-operative compensatory system between CRH and AVP. One further major factor to be taken into account is that destruction of the PVN does not obliterate the suppression of LH pulsatility during stress (Rivier and Rivest, 1991). Therefore, effects in rats must be mediated at least in part by pathways other than via the PVN, for example, via the CeA or the BNST (Koob, 1999).

Interactions between the MPOA, PVN and the brain stem

Several neuronal interactions, direct or indirect, have been recognized between the MPOA and the PVN (Fig. 5). There are CRH perikarya in the MPOA and synapses occur in the MPOA between GnRH neurones and CRH-positive axons and AVP-positive axons, both of

unidentified origin in rats, monkeys and goats (MacLusky *et al.*, 1988; Thind and Goldsmith, 1988; Kikusui *et al.*, 1997). In addition, CRH neurones from the PVN project to GABA cells in the MPOA (Cullinan, 2000). Thus, indirect mechanisms operate by provoking activity in the PVN and hence increasing delivery of CRH to the MPOA. For example, the PVN receives neuronal inputs from many nuclei (Fig. 5): noradrenaline–neuropeptide Y projections containing oestradiol receptors from the regions A1 and A2; dopamine neurones from the area postrema (part of the VLM activated by glucose availability and other stressors); GABA neurones from the MPOA; and neuropeptide Y axons from the ARC. Indirectly, catecholaminergic neurones from the A1 region synapse in the MPOA with neuropeptide Y–noradrenaline afferent neurones projecting to the PVN (Kawano and Masuko, 1999, 2000).

The significance of the increased PVN influence on MPOA activity is revealed by the stress-induced concurrent activation of CRH neurones in the PVN and the reduction in the number of GnRH cells expressing *c-fos* within the MPOA, both during the period when GnRH–LH secretion declines (Briski and Sylvester, 1998). Most of the above observations are from rats and, again, it is imperative to have confirmation in another species that does not positively associate increased adrenal activity with the onset of the GnRH–LH surge.

Mediation by suppressive opioids and GABA, or stimulatory neuropeptide Y and serotonin

The influence of opioid mediation in stress-induced GnRH suppression is revealed by the ability of naloxone to reverse the decrease in pulse frequency after restraint, injection of 2-deoxy-glucose (2DG; to rapidly lower glucose availability) or insulin administration in sheep or rats, but not in monkeys (Clarke *et al.*, 1990; Chen *et al.*, 1992; Cagampang *et al.*, 1997). Insulin-induced delays in the LH surge in sheep are also reversed by naloxone, whereas the progesterone receptor antagonist, RU486, is unable to bring forward the delayed LH surge (Dobson and Smith, 2000). As there is an increase in mRNA encoding pro-opiomelanocortin (POMC) in the ARC after glucoprivation in rats, all these effects are probably mediated by opioid influence from the ARC via synapses in the MPOA. These opioid synapses occur both with noradrenaline–neuropeptide Y projections from the MPOA to the PVN, and with CRH axons from the PVN to the MPOA (Kawano and Masuko, 1999, 2000). Local opioid antagonists are effective in reversing decreased GnRH–LH pulsatility only when applied in the MPOA and not the ARC, emphasizing that it is the MPOA that is the actual site of action. Furthermore, naloxone also reverses the decline in the number of GnRH neurones recruited in the MPOA after treatment of oestradiol-sensitized ovariectomized rats with 2DG

(Briski and Sylvester, 1998). The opioid-induced low recruitment of GnRH neurones during stress probably interferes with synchronized firing of GnRH neurones, leading to disrupted LH secretion (Goodman *et al.*, 1995).

Suppressive GABA neurones have been implicated in stress-induced reductions in GnRH secretion. During chronic stress, concentrations of mRNA encoding GABA increase in the MPOA and BNST. Apart from a direct effect on GnRH neurones, as part of a negative feedback system, high GABA concentrations could exert influence via the above mentioned neuronal pathways to downregulate CRH–AVP activity in the PVN (Fig. 5). There are GABA receptors on 95% of CRH neurones in the PVN (Cullinan, 2000). Furthermore, GABA injected directly into the PVN or MBH decreases CRH and, conversely, GABA antagonists administered into the PVN increase CRH–AVP (Cole and Sawchenko, 2002). Indeed, insulin-induced effects on LH pulse frequency and amplitude are reversed in monkeys by the GABA_A receptor antagonist, alprazolam (van Vugt *et al.*, 1997). Therefore, it is clear the GABA is involved in the suppression of LH secretion (Fig. 2b) but future work is needed to examine more closely the simultaneous effects of stressors on GABA and GnRH neurone activity.

Stimulatory neuropeptide Y may have an indirect effect during stress-induced reductions in LH pulsatility. Insulin treatment increases neuropeptide Y in the ARC and PVN (Fig. 5), and administration of neuropeptide Y stimulates CRH release thus increasing peripheral opioid, ACTH and corticoid secretion (Porter *et al.*, 1993; Krysiak *et al.*, 1999). Furthermore, the decline in LH pulsatility after neuropeptide Y administration in rats is regulated by the prevailing steroid milieu, possibly as a result of oestradiol-mediated increased opioid influence. There are neuropeptide Y perikarya in the ARC with projections to the PVN (Fig. 5), but it has not been established whether they are part of the 15% of the neuropeptide Y population with oestradiol receptors (Clarke *et al.*, 2001). However, in the breeding season of sheep, administration of neuropeptide Y into the third ventricle has no effect on LH pulsatility in the presence or absence of oestradiol, although peripheral concentrations of cortisol increase (Porter *et al.*, 1993). Further studies are required to establish the precise role of neuropeptide Y in stress-induced reductions of GnRH–LH secretion.

With respect to serotonin, the possible involvement of the Raphe nuclei in the brain stem is revealed by the existence of CRH neurones that innervate this area where there are also CRH receptors. Administration of CRH inhibits serotonin release and Raphe neuronal activity (Price *et al.*, 1998). Serotonergic axons from the Raphe nuclei also project into the MPOA to enhance GnRH secretion (Smith and Jennes, 2001), and so the MPOA could be influenced by the stress-induced reduction in serotonin activity.

Influence of oestradiol on stress-induced suppression of GnRH–LH secretion

The effects of stressors on GnRH–LH secretion are intensified by gonadal steroids (oestradiol in females and testosterone in males), although conversion of testosterone to oestradiol is likely in males (Maeda *et al.*, 1994; Adam and Findlay, 1998; Tilbrook *et al.*, 2000). The sex steroids modify CRH transcription and expression (Tellam *et al.*, 2000), and CRH neurones have been identified in the MPOA in different species (see above). Indeed, in a series of elegant experiments, K-I. Maeda's group showed that oestradiol treatment sensitizes LH secretion to suppression by stressors (glucoprivation, fasting or immobilization) at doses that do not change the characteristic LH pulsatility pattern in ovariectomized rats. Catecholamines increase the number of oestradiol receptors in the hypothalamus, and this has implications for the interaction between stress and GnRH–LH suppression. Stress-induced noradrenaline suppression of LH pulse frequency is enhanced by oestradiol, but only by localized action in the PVN or A1–A2 regions and not in the MPOA or ARC (Fig. 4; Maeda *et al.*, 1994; Nagatani *et al.*, 1996; Cagampang *et al.*, 1997). Indeed, during stress, the number of noradrenaline–dopamine cells with oestradiol receptors increases in the PVN and brainstem (A1 and A2 regions) but not in the MPOA, ARC or VMN (Estacio *et al.*, 1996; Reyes *et al.*, 2001). Furthermore, α -helical CRH will reverse the effects of stressors on LH pulse frequency and amplitude in oestradiol-sensitized rats, whereas i.c.v. AVP only reduces LH pulse amplitude (Cates *et al.*, 1999). There is an oestrogen response element on the mRNA encoding CRH and an increase in this CRH mRNA occurs in the PVN after oestradiol treatment late in pro-oestrus (Herbison, 1998). Thus, there is a distinct modulating effect of oestradiol on neurotransmitter activity during stress. Again, observations must be interpreted with care in rats because of the corticoid-induced LH surge late in pro-oestrus in this species.

Other aspects of HPO and HPA interactions

Although GnRH cell bodies are most abundant in the MPOA in rats, a small population of uncertain function has been identified in the ARC in sheep. The efferent axons of both MPOA and ARC populations project into the ME close to the hypophyseal portal blood capillaries. There are receptors for noradrenaline, glutamine or neuropeptide Y at the efferent axon–capillary junction but it is thought that these neurotransmitters have minimal pre-synaptic control of GnRH release in the ME (Smith and Jennes, 2001). Furthermore, CRH neurones are not associated with GnRH rich areas in the ME. However, it is possible that changes occur in glial cell ensheathment and tanycyte architecture. These cells encasing GnRH neurones might prevent axon

terminals temporarily forming synapses with the portal capillaries. Equally, glial changes in the ARC and MPOA are associated with regulation of GnRH release. The presence of oestradiol receptors and structural changes within 5 h of oestradiol treatment indicate an involvement in reproduction but stress-associated changes still require elucidation (Herbison, 1998; Viguie *et al.*, 2001).

All stressors result in increases in corticoids and catecholamines from the adrenal gland medulla but it is unlikely that these have a major influence on LH secretion because stress effects on GnRH have been observed in adrenalectomized animals (Rivier and Rivest, 1991). Furthermore, peripheral catecholamines or cortisol may not cross the blood–brain barrier (Deaver and Dailey, 1982; Adam and Findlay, 1998). Nevertheless, prolactin and cortisol have been associated with suppression of transcription of the GnRH promoter in rats (Tellam *et al.*, 2000). In sheep, prolonged high-dose cortisol infusions interfere with LH pulses and surge secretion but, confusingly, inhibition of cortisol synthesis by metyrapone does not inhibit endotoxin-induced suppression of LH release (MacFarlane *et al.*, 2000; Karsch *et al.*, 2002).

Conclusions

In normal intact animals, oestradiol activation of GnRH–LH surge secretion involves an initial stimulatory oestradiol signal but restraining modulation is mediated by noradrenaline and opioid regulation of suppressive GABA neurones. During the later transmission phase in which oestradiol concentrations are higher, a gradual removal of opioid influence may mediate the uncoupling of noradrenaline restraint on GABA cells, resulting in a (now oestradiol-independent) more positive noradrenaline influence on GnRH–LH release. The concomitant overall increase in GnRH secretion, along with further increased oestradiol, enhances synthesis of GnRH receptors and LH β in the pituitary gland. At the end of the transmission phase, neurotransmitters control the recruitment of more GnRH neurones, culminating in co-ordinated hyperpolarization and massive outpouring of GnRH into the portal capillaries to cause secretion of the prepared stores of LH. Depending on the intensity, stress impedes the activation or transmission phases, or both, by stimulation of oestradiol-sensitive PVN activity that interferes with GnRH neurone recruitment, and hence GnRH pulsatility decreases and surge secretion is disrupted. Several reproductive situations involve suppression of GnRH–LH pulsatility mediated by increased sensitivity to oestradiol in different parts of the hypothalamus and brain stem. For example, seasonality involves the A14 and A15 regions, whereas undernutrition, fasting or other stressors involve the area postrema of the A1 region of the brain stem in collaboration with the PVN and possibly the CeA.

Therefore, in terms of human and animal fertility, how important are changes GnRH–LH pulse frequency and amplitude, oestradiol profiles and the precise timing of events leading up to the LH surge? During maturation, gametes are closely associated with steroid-producing support cells (Leydig cells in the testis or ovarian follicular granulosa cells). Interruption of LH pulsatility (by GnRH antagonists or by stressors) in the follicular phase lowers oestradiol production and reduces the subsequent rate of blastocyst formation (Dobson and Smith, 1998; Oussaid *et al.*, 1999). Oestradiol is also required for expression of behavioural oestrus, along with increased GnRH concentrations in the hypothalamus (Caraty *et al.*, 2002). Furthermore, increased CRH has behavioural effects that appear to be independent of the pituitary–adrenal axis, for example, i.c.v. administration of CRH decreases food intake and sexual behaviour in oestrogen-primed rats (Koob, 1999).

Delayed LH surges and the resulting extended follicular phases are deleterious to fertility (Dobson and Smith, 2000). Indeed, cows with 'production diseases' such as hypocalcaemia, dystocia, ketosis or lameness are less fertile, possessing smaller follicles than healthy herd-mates in the breeding period approximately 6 weeks after calving (W. J. Clarke, H. Dobson and R. F. Smith, unpublished). These smaller follicles could be the result of lowered LH pulse frequency or stress-mediated interference with gonadotrophin recruitment of early follicles. Similar studies on human patients with anxiety driven subfertility would be of interest.

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