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STEROID CONTROL OF CENTRAL NEURONAL INTERACTIONS AND FUNCTION

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Summary—Steroids have potent actions on the brain which can be categorized as; (i) fast (~ms–s), (ii) intermediate (h–days), (iii) long-term reversible (days–weeks) and (iv) long-term irreversible. Here attention is focussed on the intermediate and long-term reversible effects of steroids with emphasis on glucocorticoids and oestrogen.

Glucocorticoid negative feedback is generally classified as fast, delayed and long-term. Fast negative feedback would appear to depend mainly on a reduction in pituitary responsiveness to corticotrophin releasing factor-41 (CRF-41) and possibly arginine vasopressin (AVP). Delayed feedback is mediated by reduced AVP release into hypophysial portal blood and blockade of the ACTH response to CRF-41. Long-term negative feedback is a consequence of reduced CRF-41 and AVP release into portal blood. Lesion and electrical stimulation studies pinpoint the paraventricular nuclei as the main site at which glucocorticoids act to control ACTH release.

Oestrogen at physiologically low plasma concentrations inhibits gonadotrophin secretion. At physiologically high plasma concentrations, such as those that occur during the preovulatory surge, oestradiol-17β stimulates the biosynthesis of LHRH mRNA and LHRH and the release of LHRH into hypophysial portal blood. Oestradiol also increases pituitary responsiveness to LHRH. The action of oestrogen on LHRH neurons is probably mediated by interneurons and may involve disinhibition; this view is supported by our *in situ* hybridization studies which show that oestrogen, in its positive feedback mode, significantly reduces the synthesis of proopiomelanocortin mRNA in arcuate neurons which when active are likely to inhibit LHRH neurons. The mechanism of action of oestrogen on the pituitary gland is not yet established, but clues from the action of the priming effect of LHRH suggests that oestrogen may potentiate phosphoinositide second messenger cascades. LHRH priming involves the synthesis of a 70 kDa protein the N-terminus of which is identical to an oestrogen-induced protein in the ventromedial hypothalamic nucleus involved in lordosis, and to that of phospholipase Cα. Attention is drawn to the remarkable economy of the system by which a single steroid, oestrogen, has effects on the brain and pituitary gland which result in a co-ordinated sequence of amplifier cascades which lead first to the ovulatory surge of luteinizing hormone and then to mating behaviour, both of which are obviously essential for continuation of the species.

Long-term reversible effects of steroids are exemplified by (i) the action of testosterone, and its metabolite, oestrogen, in stimulating the synthesis of AVP in neurons of the bed nucleus of the stria terminalis, and (ii) the action of oestrogen in reducing by four-fold the amount of the steroid anaesthetic, alphaxalone, required to induce anaesthesia in the male.

These stimulatory and inhibitory actions of glucocorticoids and gonadal steroids on the brain and pituitary gland provide excellent models by means of which the molecular mechanisms of central neurotransmission in mammalian brain can be established.

INTRODUCTION

Steroids have potent actions on the brain which can be categorized as; (i) fast (~ms–s), (ii) intermediate (h–days), (iii) long-term reversible (days–weeks) and (iv) long-term irreversible. Categories (i) and (iv) are exemplified, respectively, by steroid-induced anaesthesia and steroid-induced sexual differentiation of the brain [1]. Attention here will focus on category (ii) which is exemplified by the negative feedback of glucocorticoids on adrenocorticotrophin (ACTH) secretion and the positive feedback effect of oestrogen on the hypothalamic pituitary gonadotrophin and prolactin systems, and on category (iii) which is exemplified by sex steroid control of arginine vasopressin (AVP) biosynthesis in neurons of

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the bed nucleus of the stria terminalis (BNST) and the effect of sex steroids on the potency of the steroid anaesthetic, alphaxalone.

INTERMEDIATE ACTIONS OF STEROIDS: GLUCOCORTICOID NEGATIVE FEEDBACK CONTROL OF ACTH SECRETION

Neural control of ACTH secretion is mediated mainly by corticotrophin-releasing factor-41 (CRF-41) and arginine vasopressin (AVP). Other neuropeptides such as oxytocin and angiotensin II have also been implicated as corticotrophin-releasing factors [2], but direct evidence for this is not available. Indeed, angiotensin II is not released into hypophysial portal blood (G. Fink, W. F. Ganong and I. Reid, unpublished data; [3]), and while we cannot exclude the possibility that oxytocin may potentiate the action of CRF-41 [4], we have not been able to establish a correlation between oxytocin release into portal blood and ACTH release from the pituitary gland [5–7]. Recently, we showed that atrial natriuretic factor (ANF) is released into hypophysial blood [8], and there is evidence which suggests that ANF may inhibit ACTH release [9].

The inhibition of ACTH secretion by glucocorticoids must be mediated by an action on the final common pathway CRF-41 and/or AVP neurons and/or by an action on the anterior pituitary gland to reduce pituitary responsiveness to CRF-41 and possibly AVP. The negative feedback of glucocorticoids is divided into 3 time domains; fast of about 30 min in duration, delayed of 2–4 h in duration and long-term of hours–days in duration. This classification is based on the early modelling studies of Yates and Maran [10] which showed that the fast action of glucocorticoids was rate-sensitive (i.e. the strength of inhibition was dependent on the rate of increase in plasma glucocorticoid concentrations) while the strength of delayed and long-term inhibition of ACTH secretion depended upon the level of glucocorticoids in plasma. We have studied glucocorticoid feedback directly by measuring neuropeptide release into hypophysial portal blood. Adrenalectomy (3 weeks) increased significantly the release of both CRF-41 and AVP into rat hypophysial portal blood [5]. Administration of dexamethasone, a potent glucocorticoid agonist, 2.5 h before portal blood collection reduced significantly the release of AVP but not CRF-41 into portal blood. Dexamethasone also blocked the ACTH response to CRF-41 in adrenalectomized rats [5]. Oxytocin release into portal blood was unaffected by either adrenalectomy or dexamethasone treatment.

In a second study designed to study the fast and long-term effects of corticosterone on CRF-41, AVP and oxytocin release, hypophysial portal vessel blood was collected from rats hypophysectomized 6–8 weeks before experimentation [11]. The portal vessels regenerate within a few days of hypophysectomy and vascularize the pituitary capsule which is filled with fibrous tissue that replaces the blood clot which remains after the pituitary gland has been removed [12, 13]. It is possible to collect hypophysial portal blood at any time more than 7 days after hypophysectomy [13]. Portal plasma concentrations and contents (concentrations × plasma volume) of CRF-41, AVP and oxytocin were greatly increased in hypophysectomized compared with intact animals [11]. The intravenous infusion of corticosterone (designed to test fast glucocorticoid feedback) had no significant effect on any of the three neurohormones. Subcutaneous implantation of pellets of corticosterone, known to restore peripheral plasma concentrations of corticosterone to normal, resulted after 5 days (designed to test long-term negative feedback) in a significant decrease in the output into portal blood of CRF-41 and AVP, but not oxytocin.

Taken together our two studies [5, 11] suggest that *delayed* (2–3 h) glucocorticoid feedback is mediated by blockade of pituitary responsiveness to CRF-41 and a reduction in AVP output into hypophysial portal blood. That is, in this situation AVP is the regulatory while CRF-41 is the "permissive" neurohormone. *Long-term* negative glucocorticoid feedback is due to decreased release of CRF-41 as well as AVP into portal blood. Glucocorticoids have no significant effect on the output of oxytocin into portal blood.

Pharmacological studies lead Dallman *et al.* [14] to conclude that glucocorticoid negative feedback is mediated by an action on mineralocorticoid (type I) receptors in extra-hypothalamic neurons. However, our studies on the effects of lesions of the paraventricular nucleus (PVN) [7] show that the negative feedback effect of glucocorticoids is exerted mainly on the PVN. This point is reinforced by our studies on electrical stimulation [15] which showed that PVN stimulation resulted in a 2–3-fold increase in CRF-41 release into hypophysial portal

blood; stimulation of the hippocampus had no effect on the release of CRF-41, AVP or oxytocin. This does not exclude the possibility that the hippocampus modulates the hypothalamic–pituitary–adrenal system: but, conceivably, the corticosteroid receptors in hippocampus may be more involved with functions such as learning and memory rather than in the direct control of ACTH release. Our studies with dexamethasone [5, 7], while not excluding a role for type I corticosteroid receptors, demonstrate clearly and directly that type II (glucocorticoid) receptors play a major role in the control of ACTH release.

Infusion [11] together with the pituitary cell perfusion studies [16] suggest that the fast (within 30 min) effect of glucocorticoids is exerted mainly on corticotrophs. While it has long been thought that delayed and long-term glucocorticoid negative feedback involves protein synthesis, the fast effects of glucocorticoid were thought at first to involve an extragenomic mechanism. However, the fast effect of glucocorticoids can be blocked by protein synthesis inhibitors [16] and the latency of electrophysiological effects of glucocorticoids on hippocampal cells is also of an order which is compatible with protein synthesis [17]. Very rapid effects of steroids such as those of the steroid anaesthetic, alphaxalone (3α-hydroxy-5α-pregnane-11,20 dione), are known to occur and appear to act by way of GABA receptors increasing the chloride current [18, 19]; however, so far there is no evidence that glucocorticoids have this effect in hypothalamic pituitary control.

POSITIVE FEEDBACK OF OESTROGEN ON THE HYPOTHALAMIC PITUITARY, GONADOTROPHIN AND PROLACTIN CONTROL SYSTEMS

The spontaneous ovulatory surge of luteinizing hormone (LH) is due to a positive feedback cascade in which a surge of oestradiol-17β acts on the brain to trigger a surge of LHRH, and on the anterior pituitary gland to increase pituitary responsiveness to LHRH [1]. The increase in pituitary responsiveness to LHRH can be further potentiated by progesterone. Luteinizing hormone-releasing hormone has the unique capacity to increase responsiveness (by at least 7-fold *in vivo*) to itself. This property of LHRH, which we termed the priming effect of LHRH, is probably important further to potentiate pituitary responsiveness to LHRH and to co-ordinate the increased release of LHRH and pituitary responsiveness to LHRH so that both reach a peak at the same time thereby ensuring a massive spontaneous, ovulatory surge of LH.

In the present series of studies we sought to answer the following questions: (i) as well as stimulating the LHRH surge does oestradiol also stimulate the synthesis of LHRH mRNA; (ii) how does oestradiol-17β stimulate LHRH biosynthesis and release; (iii) what is the mechanism of the priming effect of LHRH and does it have anything in common with the action of oestradiol-17β?

Does oestradiol-17β stimulate LHRH biosynthesis?

To determine whether oestradiol-17β stimulates the biosynthesis of LHRH we [20] used the same paradigm as that used to prove that oestradiol stimulated the LHRH surge [21]. That is, rats were ovariectomized on the morning of dioestrus in order to eliminate the spontaneous surge of oestradiol-17β and injected s.c. with either oestradiol benzoate (OB) or vehicle (0.2 ml sesame oil). The animals were killed between 1600 and 1700 h of the next day (presumptive pro-oestrus). LHRH mRNA was determined by *in situ* hybridization in serial coronal sections of the hypothalamus taken from just anterior to the diagonal band of Broca to the anterior arcuate nucleus. *In situ* hybridization was carried out with a 30 mer, ^{32}P-labelled, oligonucleotide probe complementary to LHRH mRNA. As assessed by grain counts, the concentrations of LHRH mRNA in perikarya in the medial preoptic area, diagonal band of Broca and medial septum area were significantly greater in OB compared with matched pairs (in terms of day of experiment and simultaneous processing for *in situ* hybridization and grain counts) of oil-treated control animals. Plasma LH concentrations confirmed that an LH surge had occurred at the time that brains were taken for LHRH mRNA determination in OB but not in the oil-treated rats. Thus, oestradiol in its positive feedback mode stimulates the synthesis of LHRH mRNA.

The increased synthesis of LHRH mRNA correlates with increased hypothalamic content of LHRH which can be detected in the adult [22], but is seen much more clearly during the first pro-oestrus [23]. In the latter, the content of LHRH in the medial preoptic area and in the remainder of the hypothalamus rose significantly between 1800 h at 2 days before

vaginal opening (equivalent of dioestrus) and 1200 h at 1 day before vaginal opening (equivalent of pro-oestrus) and then fell significantly to reach low levels at 1800 h of pro-oestrus. The fall of hypothalamic LHRH content during the afternoon of pro-oestrus coincides with the spontaneous surge release of LHRH into hypophysial portal blood [23].

How does oestradiol stimulate LHRH biosynthesis and release?

As in the case of the oestrogen-stimulated LHRH surge [21] the above findings [20] begged the question of how oestradiol exerted its effect, in that Shivers *et al.* [24] have demonstrated that LHRH neurons do not contain oestradiol receptors. The action of oestradiol-17β must, therefore, be mediated by interneurons. On the basis of several pharmacological studies, including our own on the effect of monoamines on LHRH release into portal blood [25], we have postulated several obvious interneuronal mechanisms which could mediate the stimulatory action of oestradiol-17β on LHRH biosynthesis and release [1]. Of these mechanisms the most parsimonious consists of an opioid and dopaminergic "clamp" whereby arcuate proopiomelanocortin (POMC) containing neurons and dopaminergic (DA) neurons inhibit LHRH neurons. Both DA and POMC neurons are known to project to the external layer of the median eminence where these terminals are close to those of LHRH neurons. Proopiomelanocortin (β-endorphin) terminals also represent about 9% of all synaptic input to LHRH neurons [26]. The hypothesis [1] is that in the presence of low levels of oestradiol-17β arcuate POMC and DA neurons inhibit LHRH biosynthesis and release; when plasma oestradiol-17β concentrations rise to surge levels POMC and DA neurons are switched off and as a consequence LHRH neurons are disinhibited leading to LHRH biosynthesis and release. Since DA is known to inhibit prolactin release, the inhibition of DA neurons by oestrogen could also result in the spontaneous preovulatory prolactin surge which occurs concurrently with the ovulatory gonadotrophin surge in rat and man [1, 27]. This hypothesis is probably grossly oversimplified in that other neurotransmitters/neuromodulators are likely to be involved in the control of LHRH biosynthesis and release. Nonetheless, the hypothesis provides the basis for the systematic investigation of how oestradiol stimulates the LHRH and prolactin surge. The answer to this question is obviously important for our understanding of the mechanisms that control LHRH biosynthesis and release, but, as well, is likely to provide important principles for the understanding of neurotransmission in mammalian brain.

Two approaches have been/are being used to tackle our hypothesis, both of which utilize *in situ* hybridization to determine mRNA levels in neurons under several experimental conditions. The aim of the first set of experiments was to determine the effect of oestradiol on POMC mRNA in the arcuate nucleus. For this purpose the experimental paradigm was the same as that used in our earlier study on the effect of oestradiol on LHRH mRNA levels [20]. Our results, based on pairs of oil compared with OB-treated animals, showed that in oestrogen-treated animals there is a significant reduction in POMC mRNA levels in the anterior region of the arcuate nucleus [28]. This region of the arcuate nucleus corresponds precisely with the region in which Wise *et al.* [29] found a significant reduction in POMC mRNA on the afternoon/evening of pro-oestrus. The fact that oestrogen can significantly reduce POMC transcription within 60 min [30] suggests that the reduced levels of POMC mRNA are due to inhibition of POMC gene transcription rather than to increased POMC mRNA degradation. These results together with those of Wise *et al.* [29] are compatible with the possibility that the stimulation of LHRH biosynthesis and release by oestrogen positive feedback may be mediated, in part, by disinhibition of LHRH neurons as a consequence of inhibiting POMC biosynthesis in some arcuate neurons. However, before this speculation is accepted it will be necessary to determine whether (i) the arcuate neurons in which POMC mRNA is reduced project to LHRH neurons, and (ii) whether the reduction of POMC mRNA levels is also reflected in a reduction in the release of POMC products, and especially β-endorphin, at nerve terminals on LHRH neurons.

Our second approach is to use the paradigm we employed to investigate the role of monoamines in the release of LHRH into hypophysial portal blood [25]. In this paradigm the effects of monoamine agonists and antagonists were tested on the surge of LHRH/LH induced in prepubertal rats by injecting pregnant mare serum gonadotrophin (PMSG); the latter is presumed to stimulate the LHRH surge by

generating a surge of oestrogen. We showed that the LHRH surge is induced in part by activation of α_1 noradrenergic receptors [25], a finding consistent with studies in which LHRH output has been measured indirectly by assay of ovulation or LH release [31, 32]. The role of α_1 noradrenergic receptors in LHRH mRNA synthesis is now being examined by *in situ* hybridization as outlined above [20].

In summary, as assessed by *in situ* hybridization, oestrogen stimulates the biosynthesis of LHRH mRNA in the rostral diencephalon and significantly reduces synthesis of POMC mRNA in neurons of the rostral arcuate nucleus. This increase in LHRH mRNA correlates with increased hypothalamic content of immunoreactive LHRH. These findings are consistent with the hypothesis that oestrogen stimulates LHRH biosynthesis and release by inhibiting POMC neurons which in turn leads to the disinhibition of LHRH neurons. Inhibitory mechanisms are of course common within the central nervous system. In the present case, corroborative evidence for our hypothesis comes from metabolic studies which showed that [^{14}C]2-deoxyglucose utilization was markedly reduced in the arcuate nucleus, median eminence and preoptic area around the time of the spontaneous ovulatory LH surge [33].

What is the mechanism of the LHRH priming effect and does it have anything in common with the action of oestradiol-17β?

The priming effect of LHRH is briefly discussed here because recent studies have pointed to a possible common mechanism of action of LHRH and oestrogen. The priming effect of LHRH (for definition see above) differs from the simple releasing action of LHRH in that it (i) cannot be mimicked by K^+ depolarization or Ca^{2+} ionophores, (ii) is independent of normal extracellular Ca^{2+} concentrations, (iii) involves an elongation and change in orientation of the microfilaments, (iv) involves the movement of secretory granules towards the plasma membrane of immunoidentified gonadotrophs ("margination") and (v) involves the synthesis of a new protein [1, 34–37]. We showed that the new protein induced by LHRH had a relative molecular mass of 70 kDa [36]. In their studies of the potentiation of lordosis behaviour in the female rat, Mobbs *et al.* [38] found that the most prominent protein stimulated by oestrogen in the ventromedial nucleus of the hypothalamus (VMH) also had a relative molecular mass of 70 kDa. The similar properties of the two proteins on 2-dimensional gel electrophoresis and the fact that both proteins are induced within the same time domain suggested that the LHRH priming protein in pituitary and the oestrogen induced protein in the VMH may be identical and might mediate the effects of both hormones. More detailed gel electrophoresis revealed that these 70 kDa proteins (termed hormone-induced protein—HIP 70) are derived from more acidic 70 kDa forms. How LHRH and oestrogen bring about a shift in PI of the acidic to the more basic 70 kDa forms is not known, but this shift is consistent with single dephosphorylation [39]. The amino acid residue sequences at the amino terminals of the acidic and basic forms of HIP 70 are identical to one another and with that of phospholipase Cα (PLC-α) [39, 40]. The functional significance of this is that PLC generates the second messenger cascades which result in the formation of prostaglandins, diacylglycerol and inositol triphosphate (IP_3) all of which have been implicated in mediating the actions of LHRH. Thus, for example, we have shown that LHRH priming involves a massive increase in (i) the amount of IP_3 in pituitary tissue, and (ii) the efflux of Ca^{2+} from pituitary slices (suggestive of increased release of Ca^{2+} from intracellular stores, possibly by IP_3) [41]. LHRH is also known to stimulate lordosis behaviour about 1 h after injection into the mid brain central grey. Thus HIP 70 may be an important component of a mechanism common to both LHRH and oestrogen which is involved in massive potentiation of pituitary responsiveness to LHRH and also of lordosis behaviour.

LONG-TERM REVERSIBLE EFFECTS OF STEROIDS

Steroid effects on AVP neurons of the bed nucleus of the stria terminalis

De Vries *et al.* [42–44] were the first to demonstrate that there is a marked sex difference in the vasopressinergic innervation of the rat brain outside the classical hypothalamic–neurohypophysial system. These sex differences are most pronounced in the lateral habenular and lateral septum in which there is a dense plexus of AVP containing fibres which project from perikarya in the BNST. We

confirmed that in the mouse, as in the rat, the density of neurophysin II (i.e. AVP-containing) nerve fibres in the lateral septum and lateral habenular was greater in the male than in the female [45]. Although the male–female difference in the density of neurophysin-containing terminals in the lateral habenular and other regions is established early in life, this feature is *reversible* in that castration results in a reduction in density of these terminals. Furthermore, no immunoreactive neurophysin II fibres could be seen in the lateral habenular of the hypogonadal mouse, a mutant which is totally deficient in hypothalamic LHRH and as a consequence of which has atrophic gonads which do not secrete testosterone [46]. The presence of neurophysin II-containing fibres in the lateral habenular can be induced in hypogonadal mice by the insertion of a hypothalamic graft from normal mice into the third ventricle. This graft innervates the hypophysial portal vessels with LHRH-containing terminals. LHRH stimulates the pituitary gland to secrete normal amounts of gonadotropin which stimulates the secretion of testosterone which in turn stimulates the male pattern of neurophysin II-containing fibres in the habenular [45]. The administration of testosterone or oestrogen but not 5α-dihydrotestosterone (5α-DHT) to male hypogonadal mice also results in the normal development of neurophysin II fibres in the lateral habenular. The androgen-insensitive testicular-feminized mouse (Tfm) also has no detectable neurophysin II fibres in the lateral septum or habenular. Oestrogen administration induces the presence of AVP-containing fibres in the Tfm lateral habenular and lateral septum. Since oestrogen appears to be as effective as testosterone in inducing AVP-containing fibres in the lateral habenular and lateral septum and since 5α-DHT is ineffective it may be assumed that testosterone exerts its effects by way of conversion to oestrogen by the aromatizing enzyme complex which is known to be present in relatively high concentrations in hypothalamus. Although not remarkable, since many of the central actions of testosterone are probably dependent upon its conversion to oestrogen, this conclusion has important implications for our understanding of the deficiency of the Tfm mutant in that exceedingly large quantities of testosterone had to be given for a longer period to Tfm mice in order to generate AVP-containing fibres in the habenular and septum (C. Mayes, J. McQueen, H. M. Charlton and G. Fink, unpublished data). This suggests that in addition to the defect in androgen receptors, the Tfm mutant also has a defective aromatase complex. Whether this defect is coupled to or simply a consequence of the genetic defect in androgen receptors remains to be determined. In turn these studies show that the absence or presence of neurophysin II (AVP-containing) fibres in the lateral habenular and lateral septum is a reversible effect which depends upon the presence of normal amounts of oestrogen or aromatizable androgens. The reason for the substantially higher concentrations of AVP fibres in male compared with female brain suggests that the amount of oestrogen in the male brain is higher than in the female presumably because the amount of testosterone available for conversion to oestrogen by aromatase exceeds significantly the combined effect of small amounts of androgen and relatively small amounts (by comparison with testosterone in the male) of oestrogen in the female.

Our recent studies were designed to determine whether the action of the gonadal sex steroids was due to stimulation of AVP biosynthesis as assessed by determination of AVP mRNA by *in situ* hybridization (R. Rosie, H. Wilson and G. Fink, in preparation). Briefly, male rats were castrated at 3 days after birth and at 21 days of age implanted with either an empty or testosterone propionate (TP) containing silicone elastomer capsule. The animals were killed at 43 days of age and the brains processed for *in situ* hybridization using a ^{35}S-labelled 49 mer oligonucleotide probe complementary to the 5′ end of the glycopeptide coding domain of AVP mRNA. In animals implanted with empty capsules no AVP mRNA could be detected in the BNST: animals exposed to TP had detectable amounts of AVP mRNA in the BNST although the concentration was substantially less than in the PVN, supraoptic (SON) or suprachiasmatic nuclei (SCN). There was no obvious difference in the level of AVP mRNA in the PVN, SON or SCN in animals implanted with testosterone compared with empty capsules. AVP mRNA could be detected in the BNST of 43-day-old intact male but not intact female rats, a finding which is in keeping with the lower concentrations of immunoreactive AVP and neurophysin II in female compared with male rats and mice [42–45]. Our findings, which agree with similar studies carried out in adult rats [47] show that the action of testosterone and presumably oestrogen is to stimulate the

synthesis of AVP mRNA in BNST neurons. The vasopressinergic neurons of the BNST would appear to play a crucial role in "social memory" [48].

Sex steroid effects on the potency of the steroid anaesthetic alphaxalone

Shortly after we first employed alphaxalone anaesthesia for studies of the ovulatory surge of LHRH [49] a number of workers (and especially a referee of one of our papers) drew our attention to the fact that alphaxalone, injected i.p., was not an effective anaesthetic in male rats. This led us to examine formally whether there was a sex difference in response to alphaxalone and if so whether this was due to sexual differentiation of the brain or to a reversible effect of sex steroids [50]. We found that the dose of alphaxalone, administered i.p., necessary to produce surgical anaesthesia in male rats increased with age until in the adult the dose required is four times that in the female. This difference is not due to sexual differentiation of the brain because the dose of alphaxalone required to anaesthetize androgenized (treated with TP on postnatal day 4) rats was not significantly different from that in normal female rats. Similarly, male rats castrated on postnatal day 4 (which prevents masculinization of the brain) required the same high dose of alphaxalone, when adult, as normal males. The sex difference could also not be attributed to sex differences in metabolic clearance rate of alphaxalone [50]. The sex difference was not abolished by castration alone, but was significantly reduced by administering oestradiol-17β (by way of s.c. implanted, silicone elastomer capsules) for 10 but not for 3 days. In addition to providing another example of how sex steroids can determine the functional plasticity of the brain, these findings pointed towards the fact that the anaesthetic action of alphaxalone was activated by a specific receptor rather than simply a generalized change in cell membrane structure which at the time was the prevailing concept of the mechanism of action of anaesthetics. Whether sex steroids affect $GABA_A$ receptors on which alphaxalone would appear to exert its major action [18, 19] remains to be determined.

CONCLUSIONS AND HYPOTHESES

The first question that might reasonably be asked is whether the classification of steroid action set out in our introduction is valid. After all, it may be more rational simply to classify steroid action as genomic and extragenomic. However, the latter classification is not helpful for the detailed analysis of how steroids exert their effects; indeed it will be obvious that the four categories based on a temporal classification may require further subclassification in order to establish fundamental principles of steroid action.

Duration and strength (tissue and plasma concentrations) of steroid action are major determinants in the effect that a steroid has. Oestrogen at higher physiological concentrations tends to have positive effects. Thus, the preovulatory surge of oestradiol-17β results in an increased synthesis of LHRH mRNA and LHRH followed by a surge release of LHRH into hypophysial portal blood. It is tempting to conclude, but has yet to be proven, that the surge release of LHRH depends upon, or is at least tightly coupled with, the increased synthesis of LHRH. In any case this action of oestradiol is not due to a direct action on LHRH neurons, but is mediated by interneurons. At present we are testing the hypothesis that the stimulatory action of oestradiol is due to disinhibition of the LHRH neurons as a consequence of oestrogen inhibition of neurons, such as arcuate POMC neurons, that terminate on, and probably inhibit, LHRH neurons. Support for this hypothesis is derived from several experiments including our own which show that oestrogen reduces significantly the level of POMC mRNA in certain arcuate neurons. The stimulatory action of oestrogen is likely to involve several different neuronal systems (e.g. opioid, GABAergic, DA and noradrenergic) which may either serve as a "fail safe" back-up for one another or may all be required to act synergistically. The preovulatory surge of oestradiol is also essential for the 20–50-fold increase in pituitary responsiveness to LHRH at the time of the LH surge. The mechanism of action of oestradiol in increasing pituitary responsiveness to LHRH is not yet established, but evidence from studies on the priming effect of LHRH suggest that like LHRH itself physiologically high plasma concentrations of oestradiol-17β may potentiate enzyme systems, specifically phospholipase C, which are known to be involved in the major second messenger cascades that mediate the action of LHRH. Oestrogen is also essential for lordosis behaviour and for increased motor

activity that both reach a peak at the time of ovulation early on oestrus [1]. There is a remarkable economy in this system in which the same steroid secreted by the ovary activates in a precisely timed manner the mechanisms (amplifier cascades) required for the ovulatory surge of LH and for mating. The same hormone also triggers a surge of prolactin: in the rat this hormone is important for maintaining the corpora lutea—in man, the purpose of the mid-cycle prolactin surge is not yet known.

The LH surge is one of the few positive feedback mechanisms that occur physiologically and, arguably, it may be considered the most important neuroendocrine signal since on its regular occurrence depends the continuation of the species. It is perhaps hardly surprising, therefore, that there appear to be several back-up systems by means of which oestrogen can trigger LHRH synthesis and release and that LHRH has the unique property of increasing pituitary responsiveness to itself (priming effect). The economy of the system is even more remarkable in that oestradiol at low physiological levels inhibits gonadotrophin release. The inhibitory (negative feedback) action of oestradiol is poorly understood even though the massive effects on gonadotrophin secretion of breaking the negative feedback loop by ovariectomy is perhaps the most reproduced and reproducible of experiments. Intravenous injection of oestradiol-17β in ovariectomized rats inhibits LHRH release into hypophysial portal blood within minutes [51]. The long-term effects of oestrogen or its precursor, testosterone, on the AVP neurons of the BNST are also stimulatory, and so together with the effects on the neuronal networks that activate the LHRH surge and mating provide an excellent experimental model for studying how oestrogen stimulates (either by inhibiting or facilitating individual neuronal systems) several brain functions which are likely to be interrelated.

In contrast to gonadal steroids, the actions of adrenal corticosteroids on the hypothalamic–pituitary–adrenal system appear always to be inhibitory. Our measurements of neurohormone release into hypophysial portal vessel blood show that (i) the neural control of ACTH secretion is mediated mainly by CRF-41 and AVP, (ii) that delayed negative feedback is due mainly to a reduction of AVP output and a blockade of the ACTH response to CRF-41, and (iii) that long-term negative feedback is due to inhibition of both AVP and CRF-41 release. Fast (within minutes) negative feedback would appear to be due mainly to a reduction in pituitary ACTH response to CRF-41 and AVP.

Because of their potent actions on brain–pituitary functions, and because so much is already known about steroid receptors and their interactions with steroid response elements on genes in general, the study of steroid action on the brain and pituitary offers a powerful method of establishing key principles about the molecular mechanisms that underlie central neurotransmission. The next phase in this area of research will need to determine (i) the degree to which gene transcription and translation are involved, (ii) whether the latter is concerned mainly with the production of new enzymes, receptors or second messenger and cytoskeletal elements or tertiary messengers such as oncogene products (e.g. *c*-fos and *c*-jun) which could determine membrane and/or receptor actions, and (iii) the extent to which the action of steroids may be extragenomic. The extragenomic actions of steroids could involve (i) a direct action on plasma membranes, (ii) an action on membrane "receptors" or ion channels, (iii) effects on enzymes, as has been postulated for the action of catechol oestrogens on monoamine synthesizing enzymes, and (iv) direct interference by protein–protein interactions with the binding of the *c*-fos/*c*-jun (AP-1) complex to DNA [52, 53].

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