Regulation of Neuropeptide Gene Expression by Steroid Hormones

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Contents

Abstract

Steroid hormones modify several brain functions, at least in part by altering expression of particular genes. Of interest are those genes that are involved in cell–cell communication in the brain, for instance neuropeptide genes and genes that code for enzymes involved in synthesis of neurotransmitters. Steroid regulation of mRNA levels for several genes has been reported, including the genes coding for the neuropeptides vasopressin, corticotropin releasing factor, luteinizing hormone-releasing factor, pro-opiomelanocortin; somatostatin, prepro-enkphalin, and the enzyme tyrosine hydroxylase. Steroid control of releasing factor genes is consistent with classical neuroendocrine concepts of negative feedback. Steroid-induced plasticity of gene expression is sometimes in evidence, with the presence or absence of a particular steroid inducing expression of a neuropeptide gene in neurons that under other conditions do not express the gene.

As a means of gaining some insight into the mechanism of action of steroid hormones, several groups have determined some of the neuropeptide profiles of neurons that contain receptors for steroid hormones. Marked heterogeneity is found, in that often only a subpopulation of phenotypically-similar neurons, even within a single brain area, contains receptors for a given steroid.

Index Entries: Steroid hormones; neuropeptide genes; gene expression; releasing factor genes; steroid receptors.

Introduction

Steroid hormones—deriving from either the gonads (estrogens, progestins, and androgens) or the adrenal (glucocorticoids and mineralocorticoids)—have profound effects on neuronal functions. The regulation of the release of several hypothalamic releasing or inhibiting hormones by steroids has been well documented (Fink, 1979). Gonadal steroids are also well known to modulate several sexual and social behaviors (for a review, *see* Pfaff, 1980). Regulation of food intake (for a review, *see* Nance, 1976) and locomotor activity (Young and Fish, 1945) by steroid hormones has also been well documented. Finally, various aspects of human behavior, such as mood (Bäckström, 1983; Sanders and Reinisch, 1985) and sensitivity to visceral and somatic sensations (Henkin, 1970) are modulated by steroid hormones.

In all systems examined to date, steroid hormones exert major regulatory actions, at least in part by altering expression of certain genes (for a review, *see* Yamamoto, 1985). For instance, estrogen and progesterone regulate a set of genes expressed in the chick oviduct (Lawson et al., 1982); the products of these genes help to produce the physiological response of the oviduct to these steroids. Similarly, estrogen induces expression of the vitellogenin gene in the frog oocyte (Brock and Shapiro, 1983). It is anticipated that steroid hormones will be found to regulate expression of certain genes in the central nervous system as well. Indeed, pharmacological studies have indicated that the functional outcomes of steroid hormone actions on the brain are often prevented by manipulations that interfere with synthesis of RNA or protein. For instance, the induction of lordotic responsiveness in female rats by estrogen and progesterone can be blocked by administration of cycloheximide, anisomycin, or actinomycin-D (for a review, *see* Meisel and Pfaff, 1984).

In organs that are relatively homogeneous, such as the chick oviduct or the frog oocyte, much progress has been reported in the elucidation of batteries of genes regulated by steroid hormones. However, in the central nervous system, with an anatomical complexity far surpassing that of any other organ, progress toward understanding the molecular response to steroid hormones has been much slower. Herein lies the crux of the problem: Which genes are regulated by steroid hormones acting on neurons? Despite seemingly insurmountable difficulties in approaching this question, the question is pregnant with the promise of insight into the relationships between genes and neuronal functions, including behavior.

This review is divided into two parts. First will be a consideration of the efforts to date elucidating the regulation of specific mRNA levels in defined brain regions by steroid hormones. Second will be an evaluation of studies that provide insight into the mechanism of action of steroid hormones on gene expression, specifically by determining whether neurons that express a particular gene have receptors for a particular steroid hormone. As will become evident, the nervous system does not always operate in a manner that would seem to be the most logical.

Regulation of Specific mRNA Levels in the Brain by Steroid Hormones

Before reviewing the work in this area, it would be useful to clarify the difficulties facing an investigator approaching this problem. Twofold perplexities confront the investigator. First, which genes should be examined for regulation by steroid hormones? This question becomes confounded by the complexity of gene expression in the nervous system; a larger percentage of the genome is transcribed in the nervous system than in any other organ (Van Ness et al., 1979; Chikaraishi, 1979). Second, the anatomical complexity of the nervous system, where phenotypically different neurons are intermingled in a poorly understood organization, de-

mands that the analysis be carried out in well-defined brain regions, if not at the level of the individual neuron. Fortunately, advances in technology over the last several years provide the opportunity to quantify a large number of specific mRNAs in total RNA extracted from small and well-defined brain regions, or in individual neurons using the technique of *in situ* hybridization.

Advantages for examining mRNA levels rather than protein or peptide product levels are multiple. First, mRNA levels provide a more direct measurement of the regulation of a particular gene. Second, measurements of protein or peptide levels in a given region of the brain, especially in homogenates, are difficult to interpret because of the differential distribution of protein products in neuronal cell bodies, dendrites, axons, and terminals. Although some of these difficulties can be overcome by quantitative immunocytochemistry (for instance, Shivers et al., 1983a), interpretive difficulties still lie in attempting to distinguish between decreased synthesis and increased release. Finally, the renowned instability of many peptides is contrasted with a rather remarkable stability of specific mRNA levels postmortem (for instance, Garcia and Harlan, 1987). This stability sets the stage for analysis of specific mRNA levels in human brains postmortem.

Throughout this review, the term gene expression will be used in its most expansive meaning, i.e., what is meant by increased gene expression is increased levels of the mRNA transcribed from the gene. In the vast majority of studies on regulation of mRNA levels in the brain, no attempt has yet been made to distinguish between alterations in transcription rate and alterations in mRNA stability. "Snapshots" of mRNA levels are much more readily obtained than are "videos" of rates of transcription and degradation. Recent technological developments may provide a means toward obtaining information regarding rates of transcription, while maintaining analysis at the single-cell level. One approach is by performing *in situ* hybridization using a probe complementary to a unique sequence contained in an intron of the gene of interest. The assumption is that the transient presence of intronic sequences indicates the level of transcription of the gene. Fremeau et al. (1986) have used this approach to study glucocorticoid regulation of proopiomelanocortin transcription in the intermediate lobe of the pituitary. A limitation of this approach is the low abundance of intron-containing messages per cell. A technique that may facilitate detection of low-abundance messages has recently been suggested by Tecott et al. (1987). An oligonucleotide complementary to a portion of the mRNA of interest (preferably toward the 3' end) is hybridized to the mRNA *in situ*. This duplex serves as a primer for reverse transcriptase, which synthesizes a labeled cDNA in the cell, in the presence of radiolabeled nucleotides. Whereas these authors report promising results on pro-opiomelanocortin mRNA localization in the pituitary, the technology is still in a state of development and may require considerable technical changes before it is generally useful.

Most of the studies to date on the regulation of specific mRNA levels by steroid hormones have focused on mRNAs encoding neuropeptides or enzymes involved in synthesis of neurotransmitters from amino acids. Of the various types of genes that could be examined for regulation by steroid hormones, these genes would seem to be of particular relevance because of their involvement in communication among neurons. One of the major neurotransmitters derived from amino acids is dopamine; the rate-limiting enzyme for synthesis of this neurotransmitter is tyrosine hydroxylase (TH). Using the sensitive technique of nuclear runoff assays, which provides an index of the synthesis rate of a particular mRNA, Blum et al. (1987) have reported a complex action of estradiol on TH gene expression in the arcuate nucleus of the hypothalamus. Injection of estradiol into ovariectomized rats produced a marked decrease in TH

transcription rate (40% of control at 20 min and 5% of control at one h). In the presence of continuous estradiol exposure, TH transcription rates gradually increased over the next several days, reaching 70% of control on d four and showing no difference from control on d 14. However, by d 14 (but not on d one and four), TH mRNA levels were significantly reduced. This marked difference in the time-course of changes in transcription rates and mRNA levels points out the need to examine both parameters. Since these dopaminergic neurons in the arcuate nucleus are major regulators of pituitary prolactin release (for a review, *see* Neill, 1974) and may play roles in the regulation of secretion of other pituitary hormones, these data may provide insight into mechanisms by which estrogen can regulate pituitary function by acting on particular hypothalamic neurons. Numerous reports indicate that steroid hormones can regulate levels or turnover rates of neurotransmitters such as dopamine, norepinephrine, serotonin, and acetylcholine (for a review, *see* McEwen et al., 1979). Whether these effects of steroid hormones can be attributed to alterations in expression of the genes encoding enzymes involved in the synthesis of these neurotransmitters has yet to be investigated systematically. For instance, estrogen has been implicated in basal ganglia functioning, in part by altering dopamine turnover (for a review, *see* Van Hartesveldt and Joyce, 1986). Future work in this area might prove fruitful.

Several neuropeptide genes have also been investigated for possible regulation by steroid hormones. A classic model of neuropeptidergic neurons consists of the magnocellular neurons of the hypothalamic paraventricular system, which secrete either oxytocin or vasopressin, along with a number of neuropeptides colocalized with these neuropeptides (for a review, *see* Swanson and Sawchenko, 1983). Since vasopressin can act as a corticotropin releasing factor, under some conditions (*see* Reisine et al., 1986), classical concepts of negative feedback actions of glucocorticoids would predict that vasopressin mRNA levels would be decreased by administration of glucocorticoids, and increased following adrenalectomy. This prediction has been verified in at least two laboratories (Davis et al. 1986; Young et al., 1986b). As will emerge as a common theme in the steroid regulation of neuropeptide gene expression in the brain, there is marked heterogeneity in the response to the steroid. Davis et al., (1986) found that adrenalectomy resulted in a twofold increase in vasopressin mRNA levels in the medial parvocellular division of the paraventricular nucleus, but no changes were found in other vasopressinergic neurons in this nucleus or in the supraoptic and suprachiasmatic nuclei. By combining *in situ* hybridization with immunocytochemistry, these researchers were able to demonstrate that adrenalectomy stimulates vasopressin mRNA expression in a different population of neurons, those that contain corticotropin releasing factor. This finding confirmed previous work based on immunocytochemistry (*see* Swanson et al., 1986). More recent work (Baldino et al., 1987) indicates that both glucocorticoids and synaptic input from the lateral septum can regulate vasopressin gene expression in the paraventricular nucleus. Unilateral septal lesions resulted in a 56% increase in the number of neurons containing vasopressin mRNA. This "new" population of vasopressinergic neurons corresponded in distribution to oxytocinergic neurons.

The concept of negative feedback regulation in neuroendocrinology would predict that expression of the gene coding for corticotropin releasing factor (CRF), the major regulator of adrenocorticotropin (ACTH) release from the pituitary, would also be increased by adrenalectomy. This prediction was verified by Young et al. (1986a), who reported a 90% increase in CRF mRNA levels in the paraventricular nucleus following adrenalectomy. In addition, adrenalectomy produced a 63% increase in the area of labeled neurons, presumably reflecting expres-

sion of CRF mRNA in a different population of neurons in the paraventricular nucleus. No change in CRF mRNA levels was detected in the dorsal supraoptic nucleus. Quantitation of CRF mRNA levels in dissected hypothalami by Northern analysis revealed a 52% increase following adrenalectomy, a finding in keeping with the prior report of Jingami et al. (1985). As suggested by Young et al. (1986a), inclusion of CRF neurons that do not respond to adrenalectomy may result in an apparent diminished effect when analyzing results on Northern blots rather than by *in situ* hybridization.

Another neuropeptide classically implicated in neuroendocrine regulatory mechanisms is the decapeptide luteinizing hormone-releasing hormone (LHRH), which is the major regulator of pituitary secretion of luteinizing hormone (LH) and to lesser extent follicle-stimulating hormone (*see* Fink, 1979). A single gene in the haploid genome codes for this neuropeptide (Adelman et al., 1986). Conflicting data have been presented on the regulation of LHRH gene expression by gonadal steroids. Classical concepts of negative feedback action of androgen in male rats would predict that removal of the negative feedback by castration would result in an increase in LHRH mRNA levels and that testosterone administration to castrated rats would decrease LHRH precursor mRNA levels. However, Rothfeld et al. (1987a) reported that testosterone administration to castrated male rats resulted in an increase in LHRH precursor mRNA levels, as determined by *in situ* hybridization with an oligomer complementary to human LHRH precursor mRNA. It is conceivable that the relative insensitivity of this probe—given the mismatches between the rat and human sequences in this part of the gene (Adelman et al., 1986)—resulted in an incomplete analysis of the response to testosterone.

The neuroendocrine control of LHRH release is more complicated in the female than in the male, because of the steroid-induced facilitation of LHRH release (as-well as steroid-induced-inhibition of LHRH release) under certain conditions in the female rat, but not in the male rat (for reviews, see Harlan et al., 1979; Gorski, 1984). This increased complexity makes predictions of effects of steroids on LHRH gene expression difficult to postulate. Perhaps it is not surprising that conflicting data have been reported. Zoeller et al. (1986) reported that estrogen treatment of ovariectomized rats decreased LHRH precursor mRNA levels by about twofold, but only in a medial group of LHRH neurons; lateral LHRH neurons were not affected. This result is consistent with the classical concept of estrogen-induced negative feedback regulation of LHRH neurons. On the contrary, Rothfeld et al. (1987b) reported that estradiol treatment of ovariectomized rats leads to a small, but significant *increase* in LHRH precursor mRNA levels. Since LHRH release is quite dynamic—being timed to a circadian clock, manipulated by steroid hormones, and driven by an oscillator with a period measured in minutes—several factors may be critical in determining the level of LHRH precursor mRNA at any given time. To address some of the problems, Zoeller and Young (1987) measured LHRH precusor mRNA levels, by quantitative *in situ* hybridization, in brains of female rats at different times during the afternoon of proestrus. They found a significant increase in LHRH precursor mRNA levels only *after* the afternoon LH surge, suggestive of an increased synthesis of LHRH precursor mRNA designed to replenish LHRH peptide levels after the LHRH surge. Although the magnitude of the reported increase was small (23%), it is important to keep in mind that the total amount of hypothalamic LHRH released on the afternoon of proestrus is only about 2% (Sherwood et al., 1980).

The tripeptide thyrotropin-releasing hormone (TRH) is another neuroendocrine peptide derived from a precursor produced by a single gene (Lechan et al., 1986). Although the hormones (T3 and T4) secreted from the thyroid in response to thyroid stimulating hormone (TSH)

released from the pituitary by TRH are not steroids, the classical concepts of negative feedback action of T3 and T4 would predict that TRH precursor mRNA would be increased in thyroid-deficient rats. This prediction has recently been borne out by Koller et al. (1987) and by Segerson et al. (1987), who demonstrated that the goitrogen propylthyiouracil increased TRH precursor mRNA, and this increase was blocked by administration of thyroxin. The thyroid hormone receptor(s) involved in this feedback action on the brain are of interest, since a recent report (Thompson et al., 1987) indicates the presence of multiple forms of thyroid hormone receptors, with one form highly enriched in brain.

Besides these releasing factor peptides, whose genes appear to be regulated in a manner consistent with classical concepts of negative feedback, there are additional neuropeptide genes that are regulated by steroid hormones. Recent work of Werner et al. (1987) indicates that the somatostatin gene in the hypothalamus is regulated by gonadal steroids in both male and female rats. This effect may be related to the growth-inhibiting action of estrogen in rats. The opiate peptide pro-opiomelanocortin (POMC) has also been reported to be regulated by estrogen. Wilcox and Roberts (1985) reported that estrogen treatment resulted in a 40% decrease in hypothalamic levels of the mRNA for this single copy gene (Drouin and Goodman, 1980) which is the precursor to ß-endorphin, ACTH, and several other neuropeptides (Eipper and Mains, 1978). Subsequent work by Schachter et al. (1986) replicated this finding by *in situ* hybridization. Estrogen had no effect on the number of neurons containing POMC mRNA, but resulted in a 30% decrease in the number of reduced silver grains per labeled neuron.

Another opiate peptide gene regulated by steroid hormones is the single-copy gene (Rosen et al., 1984; Yoshikawa et al., 1984) that encodes preproenkephalin (PPE), precursor to *met*-enkephalin, *leu*-enkephalin, and peptide E. In mapping the locations of neurons that express the preproenkephalin gene, Harlan et al. (1987a) discovered that large numbers of PPE neurons are found in brain areas containing neurons with estrogen receptors. This relationship was particularly evident in the ventrolateral division of the ventromedial hypothalamic nucleus, where up to 75% of the neurons express the PPE gene (Harlan et al., 1987a) and approximately 40% of the neurons contain estrogen receptors (Morrell et al., 1986). This strong anatomical overlap suggests that estrogen may regulate PPE gene expression. Indeed, such a regulation has been demonstrated (Romano et al., 1988). Treatment of ovariectomized rats for two w with estradiol resulted in a threefold increase in PPE mRNA levels in the hypothalamus. This effect was found primarily in the ventrolateral portion of the ventromedial hypothalamic nucleus, where estrogen treatment resulted in a significant increase in both the number of PPE mRNA copies per neuron and the number of neurons expressing the PPE gene (Romano et al., 1988). This latter finding is somewhat reminiscent of the effect of adrenalectomy on the number of paraventricular neurons expressing the CRF or vasopressin genes.

The ventrolateral portion of the ventromedical nucleus is the primary site at which estrogen acts to facilitate lordosis (for a review, *see* Pfaff, 1980). Pathways by which this effect of estrogen is mediated include projections to the midbrain central gray. Immunocytochemical studies indicate that enkephalinergic neurons in the ventromedial hypothalamic nucleus project to the midbrain central gray (Yamano et al., (1986). Although *met*-enkephalin does not appear to alter lordosis when applied to the central gray (Sirinathsinghji et al., 1983), it is conceivable that other peptides derived from PPE may play a role in lordosis.

Estrogen is not the only steroid that can regulate PPE mRNA levels; at least three studies indicate that glucocorticoids and/or stress can alter PPE gene expression in the brain. In extensive mapping of the locations of PPE neurons

throughout the rat central nervous system, Harlan et al. (1987a) noted that in the vast majority of brain areas, there is a good qualitative match between the number of neurons expressing the PPE gene (labeled by insitu hybridization) and the number of neurons containing peptides derived from PPE (labeledby immunocytochemistry), although in most cases many more neurons were labeled by *in situ* hybridization than were reported to be labeled by immunocytochemistry. However, one brain structure was markedly different: the hippocampus. Harlan et al. (1987a) found only a few neurons in the hippocampus expressing the PPE gene, whereas several labs using immunocytochemistry reported that many neurons in the hippocampus contain immunoreactive PPE peptides (Gall et al., 1981; Stengard-Pedersen et al., 1983; McGinty et al., 1984; Chavkin et al., 1985). Although a number of methodological differences could account for this discrepancy, one major procedural difference seemed to stand out. Immunocytochemical localization of hippocampal cell bodies containing immunoreactive PPE peptides was always done following administration of fairly substantial quantities of colchicine; the *in situ* hybridization experiments were performed in the absence of colchicine treatment.

Can colchicine treatment alter neuropeptide gene expression? If so, how? Although one can postulate direct effects of colchicine on gene expression, another alternative is that colchicine treatment might activate the pituitary-adrenal stress response, and the resulting high levels of glucocorticoids might alter PPE gene expression, especially in the hippocampus, which is a major site of action of glucocorticoids (Fuxe et al., 1985; Sapolsky et al., 1986). To determine whether glucocorticoids could regulate PPE mRNA levels in the hippocampus, Harlan and Garcia (1987) injected male rats subcutaneously with corticosterone (the major glucocorticoid in the rat) or vehicle daily for two w, in an effort to mimic a chronic stress situation. This treatment with corticosterone resulted in a significant ($p < .01$) increase in PPE mRNA levels in the hippocampus, with no significant increase in the hypothalamus, amygdala, or caudate-putamen. These data are consistent with the possibility that colchicine treatment may activate adrenal release of glucocorticoids, which act on the hippocampus, increasing PPE gene expression and resulting in a large number of neurons that contain immunoreactive PPE peptides.

In this study, corticosterone treatment resulted in a near doubling of the PPE mRNA levels in the caudate-putamen, though this did not quite reach statistical significance ($p < .07$). In a related study, Chao et al. (1987) reported that adrenalectomy significantly decreased PPE mRNA levels in the caudate-putamen and that this effect could be reversed by treatment with corticosterone. Despite substantial methodological differences between these two studies, both studies suggest increases of PPE mRNA levels in the caudate-putamen following glucocorticoid treatment. The relative paucity of neurons in this brain region containing glucocorticoid receptors (Fuxe et al., 1985) suggests the possibility of a rather indirect effect of glucocorticoids on PPE gene expression in the caudate-putamen.

Recent work by Lightman and Young (1987) suggests that glucocorticoids, or other stress related hormones, may alter PPE gene expression in the paraventricular nucleus of the hypothalamus. Following either stress or naloxone-induced opiate withdrawal, PPE mRNA levels in the paraventricular nucleus were increased more than 10-fold. The increased PPE mRNA levels may be an important step in the activation of the hypothalamic-pituitary-adrenal stress response.

These studies indicating regulation of the PPE gene by glucocorticoids are bolstered by in vitro studies. The glioma line C6 expresses both PPE mRNA and glucocorticoid receptors. Yoshikawa and Sabol (1986a) reported that glucocorticoids facilitate the augmented expres-

sion of the PPE gene following norepinephrine or other activators of adenylate cyclase. Similar results were reported for the neuroblastoma x glioma cell line NG108-15 (Yoshikawa and Sabol, 1986b), when glucocorticoids were found to act alone or synergistically with norepinephrine. It is interesting to note that there is a partial sequence similarity between the proposed glucocorticoid response element (Yamamoto, 1985) and regions 5' to and in intron A of the rat PPE gene (Yoshikawa and Sabol, 1986b; Rosen et al., 1984).

Another neuropeptide gene that might be regulated by estrogen is the gene that encodes preprotachykinin (PPT), the precursor to substance P and substance K (Nawa et al., 1983; Krause et al., 1987). The primary transcript of this gene is differentially processed into three transcripts (Krause et al., 1987). *In situ* hybridization studies have shown that neurons in the ventrolateral portion of the ventromedial hypothalamic nucleus express the PPT gene (Harlan et al., 1987b,c). Thus, there is an anatomical basis for suspecting that estrogen might alter PPT gene expression in this brain region. Although there is some evidence against the proposed estrogenic regulation of PPT gene expression in the hypothalamus (Romano et al. 1987), the presence of estrogen receptors in many hypothalamic neurons containing substance P (*see* below) would suggest that further inquiry into this question is warranted. Fibers and terminals immunoreactive to substance P are found in the midbrain central gray (Ljungdahl et al., 1978), where release of substance P facilitates lordosis (Dornan et al., 1987). Within this context, possible estrogen-induced activation of PPT gene expression in the hypothalamus may have behavioral relevance.

These actions of estrogen on neuropeptide gene expression in the hypothalamus may be a manifestation of a more global effect of estrogen on these neurons. Estrogen acts on a subset of hypothalamic neurons to elicit a cascade of intracellular reorganization culminating in neurons that are capable of synthesizing and packaging for release large amounts of neuropeptides (Cohen and Pfaff, 1981; Carrer and Aoki, 1982; Cohen et al., 1984; Jones et al., 1985a). As part of this intracellular activation, estrogen increases ribosomal RNA levels (Jones et al., 1986). The outcome of such intracellular activation presumably is increased synaptic release of certain neuropeptides, including peptides derived from PPE and from PPT. The functional roles of these synaptically-released peptides have not been fully determined. Involvement of *met*-enkephalin, via interaction with opiate receptors, has been implicated in the control of several hypothalamic releasing peptides (for a review, *see* Olson et al., 1981). An involvement of substance P, synthesized in ventromedial hypothalamic neurons and possibly transported to terminals in the midbrain central gray, in the neural control of the estrogen-dependent behavior lordosis has been reported. Increased synthesis of substance P in response to estrogen treatment would clearly be consistent with a role of this neuropeptide in facilitation of lordosis.

Steroid Receptors in Phenotypically-Identified Neurons

With this substantial documentation of effects of steroids on gene expression in the brain, we can also begin determining some of the basic mechanisms of action of these steroids. As a beginning, it is important to determine which phenotypically-identified neurons contain receptors for steroid hormones. As we will see, two themes emerge from such studies. First, steroid hormones can exert actions on neurons that do not contain receptors for the steroid. Presumably, steroid hormones act on receptor-containing neurons that interact with the phenotypically-identified neurons in question. Second, much heterogeneity is found in populations of phenotypically-similar neurons with re-

gard to presence of steroid hormone receptors, i.e., often only a subset of neurons that express a certain gene, even when found in a single brain region, have receptors for a particular steroid.

Some methodological considerations are in order. Two general procedures have been used to determine the neuropeptide profile of neurons that contain steroid hormone receptors. First, the traditional approach to localization of neurons containing steroid receptors, that is steroid autoradiography, has been combined with immunocytochemistry of neuropeptides. This procedure is technically difficult and often requires a compromise of both autoradiographic and immunocytochemical approaches. Moreover, the technique requires photodevelopment of sections prior to immunocytochemistry, a procedure that in some cases destroys the antigenicity of the neuropeptide to be localized. Also, the technique must be performed in animals in which the endogenous source of the steroid has been removed. However, this approach is often the best available. A second approach is to perform double immunocytochemistry, using antibodies to a neuropeptide and to a steroid receptor. This second approach has provided some information on cell types containing glucocorticoid receptors. However, technical considerations have limited the usefulness of antibodies to receptors for estrogen, progesterone, and androgen. A third potential approach to this question would be to combine immunocytochemistry for a steroid receptor with *in situ* hybridization for a neuropeptide mRNA, or by combining immunocytochemistry for a neuropeptide with *in situ* hybridization for a steroid receptor mRNA.

By combining steroid autoradiography and immunocytochemistry, Sar and Stumpf (1980) were the first to report the presence of estrogen receptors in neuropeptide neurons, specifically a small percentage of paraventricular neurons that contain vasopressin. These results were subsequently extended to indicate that some oxytocin neurons also concentrate estrogen (Rhodes et al., 1982). Given the substantial amount of evidence indicating that estrogen and progesterone regulate neurons that produce luteinizing hormone-releasing hormone (LHRH), it would seem parsimonious if LHRH neurons had receptors for estrogen and/or progesterone. However, Shivers et al. (1983b) determined that LHRH neurons do not have receptors for estrogen, and Fox et al. (1986a) found that these neurons do not have receptors for progesterone. These results, coupled with the rather small and inconsistent effects of estrogen on LHRH mRNA levels reviewed above, suggest that the major effects of gonadal steroids on LHRH neurons may be in regulation of LHRH release, rather than synthesis.

The presence of estrogen receptors in certain neurons containing opiate peptides is suggested, but remains controversial or undetermined. Three groups have reported estrogen receptors in hypothalamic neurons containing peptides derived from pro-opiomelanocortin. Morrell et al. (1985) reported that only a small percentage (4%) of β-endorphin immunoreactive neurons contain estrogen receptors, whereas Shivers et al. (1984) found that about a third of these neurons contain estrogen receptors, and Jirikowski et al. (1986) reported that about 26% of these neurons contain receptors for estrogen. Morrell et al. (1985) also reported that 6% of the hypothalamic dynorphin neurons concentrate estrogen. No studies have yet been reported on the percentage of hypothalamic PPE neurons that contain estrogen receptors. However, studies of localization of neurons containing PPE mRNA indicate that, in estrogen-treated animals, up to 85% of the neurons in the ventrolateral portion of the ventromedial hypothalamic nucleur express the PPE gene (Romano et al., 1988). As previously noted, about 40% of the neurons in this same region have estrogen receptors (Morrell et al., 1986). Thus, some PPE neurons must have estrogen receptors. However, the exact percentage still re-

mains to be determined. Indeed, if estrogen changes the number of neurons expressing a particular neuropeptide gene, then the current method for determining the overlap between estrogen-concentrating and neuropeptide-containing neurons may be inadequate, since steroid autoradiography must be performed in ovariectomized rats.

Other studies have indicated that some somatostatin neurons in the periventricular hypothalamus contain estrogen receptors (Sar and Stumpf, 1983). As noted above, Werner et al. (1987) reported that estrogen can regulate somatostatin mRNA levels in the hypothalamus. About 30% of the arcuate hypothalamic neurons that contain tyrosine hydroxylase (and are thought to be dopaminergic) contain estrogen receptors (Sar, 1984). This finding is consistent with the observations described above on the regulation of tyrosine hydroxylase mRNA by estrogen. Some of the catecholaminergic neurons in the brainstem have receptors for estrogen (Sar and Stumpf, 1981). Possible genomic effects of estrogen on these catecholaminergic neurons have yet to be determined. Akesson and Micevych (1988b) provided strong evidence that neurons that produce cholecystokinin (CCK) do not contain estrogen receptors. Within the preoptic area and anterior hypothalamus of the rat, some GABAergic neurons reportedly concentrate estradiol (Flügge et al., 1986). Quite relevant for the results discussed above, Akesson and Micevych (1988a) have reported that about 50% of the neurons that produce substance P have receptors for estrogen.

Little is known about the cell types that have receptors for glucocorticoids. Using a morphometric procedure and antibodies to CRF and to the glucocorticoid receptor, Agnati et al. (1985) reported that many of the CRF neurons in the paraventricular nucleus have glucocorticoid receptors. Thus, the negative feedback action of glucocorticoids on CRF gene expression, reviewed above, may be mediated directly on the CRF neurons. Glucocorticoid receptor immunoreactivity has also been detected in several monoaminergic neuronal groups in the brainstem and hypothalamus (Harfstrand et al., 1986). The phenotypic identities of neurons with glucocorticoid receptors in the hippocampus remain to be determined.

Some studies have also been carried out to determine the phenotypic identities of hypothalamic neurons that contain progesterone receptors. Combining steroid autoradiography for the synthetic progestin ligand R5020 with immunocytochemistry, Fox et al. (1986b) found that the vast majority of tyrosine hydroxylase neurons in the arcuate nucleus concentrated the progestin. Under a variety of technical approaches, she found that at least 85% of the arcuate TH neurons have progesterone receptors. In contrast, none of the TH neurons in the nearby zona incerta had progesterone receptors. Progesteone concentration by arcuate TH neurons may help explain several aspects of the regulation of pituitary prolactin release by progesterone, since lactotrophs in the pituitary did not have progesterone receptors (Fox et al., 1986b). Although no studies have yet explored regulation of hypothalamic TH mRNA by progesterone, Wang and Porter (1986) reported that progesterone treatment decreased TH protein levels in the median eminence, which is the projection site of TH neurons of the arcuate nucleus. The finding that at least 85% of the arcuate TH neurons contain progesterone receptors, coupled with the previous report (Sar, 1984) that about 30% of the arcuate TH neurons concentrate estradiol, indicates that at least some of the arcuate TH neurons have receptors for both steroids. To my knowledge, this is the first indication that some neurons have receptors for both estrogen and progesterone. The exact percentage of arcuate TH neurons with receptors for both steroids has yet to be determined.

Since LHRH is a key peptide in the neural regulation of reproduction (Shivers et al., 1983c), one might predict direct effects of steroids on

LHRH neurons. However, Fox et al. (1986a) found that none of the LHRH neurons examined had progesterone receptors. Perhaps this is not surprising, since Shivers et al. (1983b) had found previously that essentially none of the LHRH neurons had estrogen receptors. As indicated above, the regulation of the LHRH gene by gonadal steroids may not be particularly marked. However, it is important to keep in mind that the steroid-induced facilitation of LH (and LHRH) release on the afternoon of proestrus, or artificially induced in ovariectomized rats, is dependent upon synthesis of RNA and protein (Jackson, 1972, 1973). This does not necessarily imply, though, that the steroids regulate expression of the LHRH gene; the steroids may be regulating expression of other genes, whose products regulate LHRH release, directly or indirectly. Although the identities of these products have yet to be determined, a candidate may be the opiate peptide β-endorphin (Gabriel et al., 1983; Bhanot and Wilkerson, 1984) or other opiate peptides. Note that POMC (precursor to β-endorphin) gene expression in the hypothalamus is influenced by estrogen (Wilcox and Roberts, 1985), and possibly by progesterone, since Fox et al. (1986a) found that approximately 30% of the hypothalamic POMC neurons concentrate progestin. Whether these progestin-concentrating POMC neurons are the same as those that concentrate estrogen is unknown at this time.

Summary

Two themes emerge from the work reviewed here. First, steroid-induced plasticity of neuronal phenotype is often, but not always, in evidence. Adrenalectomy stimulates CRF and vasopressin gene expression in populations of neurons that do not normally express these genes. Similarly, estrogen treatment induces expression of preproenkephalin or preprotachykinin genes in neurons that do not express these genes in ovariectomized rats. Further investigation into this issue may provide new insight into the mechanisms of differentiation and plasticity of neurons. A second theme is that marked heterogeneity exists in the response to steroid hormones. Within a population of neurons differentiated to express a given phenotype and located in a particular brain region, a subpopulation may contain receptors for a certain steroid. For instance, about a third of the POMC neurons in the hypothalamus contain progesterone receptors. Perhaps these subpopulations are functionally distinct or are similar in neuroanatomical connectivity patterns. For instance, it is conceivable that those POMC neurons with progesterone receptors all project to a particular brain region. Analysis of the hypothesis would require a triple-label approach, combining detection of POMC (probably by immunocytochemistry), detection of progesterone receptors (by steroid autoradiography, immunocytochemistry, or *in situ* hybridization), and determination of projection pathways (by use of retrograde tracers).

As with many newly-developing fields, we are still at the stage of descriptive anatomy regarding steroid hormone regulation of gene expression in the brain. Several factors help to outline the enormity of this descriptive task. First, the multiplicity of neurotransmitter and neuropeptide candidates provides a rich field for systematic examination of possible regulation of gene expression by steroids hormones. Second, potential regulation by several different steroid hormones—of gonadal or adrenal origin—can be examined; moreover, each steroid may have a different temporal mode of action. Third, the anatomical complexity of the nervous system demands a single-cell analysis, especially in light of the existence of subpopulations of steroid-responsive neurons. The enormity of the task before us is awe inspiring. Can we limit the search to a more defined and manageable operation?

One way of limiting the scope of the search is to devote acute attention to the information provided by neuroanatomy and by physiological and behavioral analysis. Prior to launching an investigation into possible steroid regulation of a particular mRNA or into whether a particular phenotype has receptors for a given steroid, there should be a reasonable justification, based on neuroanatomical, behavioral, or physiological data. However, we have already seen examples where our concepts of logic or parsimoniousness may hamper our vision and steer us away from experiments with only minimal apparent justification.

Perhaps a more generalized approach may be useful. Techniques are available for comparing cDNA libraries from stimulated and unstimulated cells. For instance, genes regulated by nerve growth factor have been determined, using differential screening techniques (Milbrandt, 1987). A similar approach could be made in the hypothalamus or other brain regions containing many neurons with steroid receptors. Such an approach may help to limit the scope of the search for steroid-regulated genes in the brain.

A second generalized approach to regulation of gene expression by steroids is to determine steroid-induced alterations in content of proteins or peptides separated by two-dimensional gel electrophoresis or HPLC. Several labs have begun studies employing these techniques (Scouten et al., 1985; Jones et al., 1985b; Rodriguez-Sierra et al., 1986; Mobbs et al., 1988). For instance Mobbs et al. (1988) approached this question by infusing ^{35}S–methionine and ^{35}S–cysteine into the hypothalamus of ovariectomized rats with or without systemic treatment with estradiol. After an appropriate amount of time to allow for incorporation of the amino acids into protein and transport of some of the labeled proteins by fast axoplasmic transport to terminals in several brain regions, including the midbrain central gray, the rats were killed and the brains were microdissected using a punch technique. Individual microdissections were processed for two-dimensional gel electrophoresis of proteins. Labeled proteins were detected autoradiographically. Two major advantages of this in vivo labeling approach over the other studies that have labeled proteins in vitro (Jones et al., 1985b; Rodriguez-Sierra et al., 1986) are that the hypothalamic neurons are not disrupted form their normal neuronal inputs and that studies on the transport of labeled proteins from the hypothalamus to other brain regions can be attempted. Indeed, Mobbs et al. (unpublished) found that the pattern of labeled proteins on two-dimensional gels from hypothalami labeled in vivo is quite different from that labeled in vitro.

Whereas studies employing in vitro labeling have indicated that quantities of several proteins are altered by estrogen, Mobbs et al. (1988) found that only one protein appeared to respond to estradiol following in vivo labeling. This protein, with an apparent molecular weight of approximately 70 kilodaltons (kD) and p*I* of 5.9 was nearly always present in estrogen-treated rats and nearly always absent without estrogen treatment. A second protein of 70 kD molecular weight and a p*I* of 5.8 varied in intensity inversely with respect to the 70 kD 5.9 p*I* protein. A reasonable interpretation, which remains to be verified, is that the two spots are the same protein which is post-translationally modified in response to estradiol treatment, so that the p*I* varies. Several lines of evidence were obtained, suggesting that the protein is transported from the hypothalamus to the midbrain central gray by a fast axoplasmic transport system. The function(s) of this protein or proteins remains to be determined.

This generalized approach to proteins regulated by steroid hormones has several limitations. First, the technique seems to be fairly insensitive; Mobbs et al. (1988) were able to separate reproducibly only about 250 proteins. Moreover, these proteins are presumably those proteins with the highest synthetic rates at the

time of labeling; steroid hormones may regulate synthesis of specific proteins whose synthetic rates are not sufficient for the proteins to be detected autoradiographically. The two-dimensional gel electrophoresis approach is limited to proteins with molecular weights between about 10 and 100 kD and with pI's between about 4.5 and 7.0. Steroid hormones may regulate proteins, and especially peptides, which are not resolved on these gels. However, proteins that are regulated by steroid hormones may be detected by this approach. The high degree of protein purification provided by two-dimensional gel electrophoresis may allow the development of antibodies from protein excized from the gel, thus allowing further characterization of the steroid-regulated protein.

It is important to devote some thought to the impact on the neuron of altering gene expression of its (major) neuropeptide. For instance, estrogen treatment increases preproenkephalin mRNA levels approximately threefold in neurons of the ventrolateral portion of the ventromedal hypothalamic nucleus (Romano et al., 1986). What does this mean for the neuron? Is all of this mRNA translated into preproenkephalin? Is all the precursor cleaved to small peptides? Are all of the peptides transported to release sites? Are they all released? Does the efficiency of any of these processes change in response to estrogen, or even in response to the rather sudden change in the mRNA levels? These questions become even more acute when one considers the rapid and very large (more than 10=fold) increase in preproenkephalin mRNA levels following stress or opiate withdrawal, as reported by Lightman and Young (1987). These types of questions need to be addressed now that some of the agents that regulate neuropeptide gene expression have been identified.

Finally, it is appropriate to question the usefulness of the entire endeavor. Will the information gained from this analysis of steroid regulation of gene expression in the brain be worth the effort? Of course, this question can be answered only by historians of science. However, the promise of insightful benefit is alluring. Understanding how steroids control expression of particular genes in the brain may ultimately yield a deeper understanding of the relationship between genes and behavior and may provide insight into the biochemical basis of human psychological processes. For instance, clinical depression is often associated with abnormalities in the neuroendocrine control of the hypothalamic-pituitary-adrenal axis (Meltzer et al., 1987). Steroid hormones derived from the gonads also influence mood, as in premenstrual tension (Bäckström, 1983), and can affect performance on repetitive or cognitive tasks (Sanders and Reinisch, 1985). A person's individual glucocorticoid response to a stressful situation appears to be related to his or her overall performace and especially to the "fit" between one's occupation and one's psychological makeup (Rose et al., 1982). A deeper understanding of the genes regulated by steroid hormones acting on the brain may yield new insight into our response to stress and our general outlook on ourselves as individuals coping in a continuously-changing environment.

Acknowledgments

I would like to thank Meredith Garcia for critically reviewing this manuscript and Debbie Lauff for word processing. Research was supported by NS-24148.

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