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Plenary Lecture

Competition for DNA Steroid Response Elements as a Possible Mechanism for Neuroendocrine Integration

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For the analysis of a simple steroid-dependent mating behavior, careful response definition, complete neural circuit delineation and placement of estrogen-responsive cells within this circuit have been accomplished. Molecular studies of two relevant genes have emphasized DNA/RNA hybridization assays and DNA binding techniques. For both the rat preproenkephalin gene and the gene for the progesterone receptor, a strong induction by estrogen, tissue specificity of expression and a sex difference in regulation are prominent phenomena. On the rat preproenkephalin promoter, estrogen (ER) and thyroid receptors may compete for a DNA binding site. Likewise, progesterone (PR) and glucocorticoid receptors may compete for the same sites. On the rat PR gene, interactions between ER and AP-1 binding proteins are of special interest. Such interactions could underlay competitions and synergies between steroid hormones and neurally signalled events in the environment.

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While recent interest in neurobiology has often focused on the concept of neural plasticity, an equally valid perspective emphasizes the constraints within which the central nervous system (CNS) operates in order to manage its biologically adaptive operations. Consider the case of reproductive physiology. Assuming that the peripheral preparations for reproduction such as gametogenesis and implantation will occur successfully, we depend upon steroid sex hormones not only to coordinate peripheral with central preparations for reproduction but also to drive CNS activities. The biological requirements for reproduction which constrain these neuroendocrine preparations include, most obviously, the need to deliver the gametes—and thus to successfully perform mating behavior—which in turn demands that the organism recognize and respond appropriately to external signals from the potential mating partner. In addition, the brain must sense and evaluate both internal and external signals with respect to the adequacy of food supplies, water and salt, temperature, day length, time of day, the adequacy of nesting material and the assurance of a relative absence of stress.

In the case of a simple mammalian mating behavior, we have shown how internal signals, steroid hormones, can interact in the forebrain with external signals from the mating partner in order to control the occurrence and amplitude of the required mating behavior, lordosis [1]. Specific cellular targets for steroid hormones such as estradiol, discovered with steroid autoradiography [2] comprise a recognizable limbic–hypothalamic system. Most of these identified limbic and hypothalamic cellular groups appear to bind steroid sex hormones in a manner general across vertebrates. Some other neuronal groups are species specific, with the apparent task of controlling species specific mating behaviors such as birdsong [3, 4]. With respect to the external signals, a circuit for the sensory and hormonal control of lordosis behavior was worked out, the first for a mammalian behavior. Several principles emerged. Firstly, the neural circuit for lordosis behavior, the primary mating response among female quadrupeds,

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showed that it is possible to analyze mechanisms for vertebrate behavior in such detail—it comprised an "existence proof". Within the circuit, modular construction was obvious. Neural modules in the lordosis behavior circuit matched embryologic divisions of the neuraxis, with a clear job for the hypothalamic module being to add the feature of steroid dependence [5]. On the sensory side of the circuit, the sparing economic distribution of sensory information was demonstrated. On the motor side, both a hierarchical structure and economic use of specific motor control information were clear.

Thus, questions about the full range of neuroendocrine integrative mechanisms can be approached from a background of firm knowledge about female reproductive behavior circuitry. Major findings include the universality of many features of steroid sex hormone binding and nuclear accumulation in the brain, as mentioned above, across vertebrates and the remarkable conservation of many neuroendocrine molecules and biochemical reactions across vertebrates and conservation from peripheral reproductive organs to the CNS. An excellent example is the decapeptide LHRH (GnRH). This molecule, which has preserved its important features across vertebrates, acts in the brain to synchronize mating behavior with peripheral preparations for reproduction [6, 7]. The neurons which produce GnRH are born, surprisingly, in the epithelium of the olfactory pit and migrate across the nasal septum into the basal forebrain [8]. Failure of GnRH neurons to migrate into the brain accounts for Kallmann's syndrome, hypogonadotrophic hypogonadism coupled with anosmia [9].

We can also list several genes whose products or activities are increased by estradiol and which drive lordosis behavior. This pattern of results allows the inference that the effects of estradiol on those gene products comprise molecular mechanisms by which estradiol drives lordosis behavior [5]. The transcriptional systems so far identified include muscarinic receptors, adrenergic α-1 receptors, the progesterone receptor, the opioid peptide preproenkephalin, oxytocin and its receptor and GnRH itself. A firm conclusion which follows from this list of genes is that even for an individual steroid hormone such as estradiol and a simple behavior such as lordosis, we are not dealing with a one hormone–one gene–one behavior system.

From this list of genes, we emphasize recent work on the molecular controls over preproenkephalin expression and synthesis of the progesterone receptor in brain tissue.

Admitting that the full range of neuroendocrine control mechanisms for reproduction is not yet known, what are the candidates? Clearly, changes in extrahypothalamic portions of the circuitry can play a role [1]. And even within the hypothalamus, traditional mechanisms involving recognized neurotransmitters and neuropeptides are importantly involved [5]. The purpose of this chapter is to consider whether interactions among transcription factors on the promoters of behaviorally relevant hormone controlled genes might occur—and such interactions on introns and 3′ untranslated regions should also be considered. That is, it seems possible that competition for binding sites on DNA, with particular attention to those protein–protein configurations which are transcriptionally active, and potential cooperative protein–protein interactions, when present in hypothalamic neurons could underlay some of the synergies and combinatorial logic which are required for biologically adaptive neural management of reproduction. We have begun to approach this question using protein/DNA binding assays with nuclear extracts from hypothalamic tissue with an emphasis on controls over the genes for preproenkephalin and the progesterone receptor.

GENE FOR PREPROENKEPHALIN (PPE) EXPRESSED IN HYPOTHALAMIC NEURONS

The gene for preproenkephalin is expressed in a diverse set of neural cell groups in the rat brain [10] and merits extensive discussion.

The regulation of PPE gene expression in neuronal tissues

The PPE gene is widely expressed throughout the CNS and peripheral tissues, including the adrenal gland. In the CNS, enkephalin-containing peptides are formed at every level of the neuroaxis, including cells in the cortex all the way down to cells in the spinal cord. Fiber projections have not been clearly mapped, but both local and long tract systems may be present [10–12]. The endogenous opioid peptides have been postulated to function as neurotransmitters, neuromodulators and/or neurohormones [11] and have been implicated in the modulation of pain, reproductive behavior, motor behavior, mental disorder, cardiorespiratory function, immune responses and gastrointestinal and neuroendocrine functions [11–15].

The regulation of PPE gene expression has been intensively studied in both CNS and adrenal tissues. Since the adrenal gland is considered as a peripheral neuronal ganglion, and is a major source of endogenous opioid peptides and allows manipulation by surgery, explantation or hormonal factors in a manner not easily accomplished in CNS neurons expressing the PPE gene [16–18], a lot of work has been concentrated on this tissue. Growing evidence indicates that the expression of PPE gene is regulated by transsynaptic activity and steroid hormones in both CNS and adrenal tissue. In the rat adrenal, increases in transsynaptic activity by stress result in an increase in PPE gene expression, which is mediated via cholinergic neurotransmission [19–21]. Paradoxically, a decrease in transsynaptic activity in the rat adrenal by denervation, ganglionic blockade, or explanation to organ culture also produces an increase in PPE gene expression

[16, 18, 21–23]. Furthermore, both the basal level and induction of PPE gene expression in adrenal tissues are dependent on glucocorticoids [20, 23–25]. These results also reflect to some extent the PPE gene expression in the CNS. In the CNS, transsynaptic dopaminergic activity from substantia nigra exhibits tonic inhibition of striatal PPE gene expression [26–28], whereas, transsynaptic dopaminergic activity from the frontal cortex displays positive regulation of striatal PPE gene expression [29]. Seizure activity increases PPE mRNA in the hippocampus and hypertonic saline and opiate withdrawal stresses induce PPE gene expression in hypothalamus [20, 21, 30]. Steroid hormones, such as glucocorticoids, estrogens and progesterone have also been demonstrated to regulate PPE gene expression in CNS. In the striatum, the expression of the PPE gene is regulated by glucocorticoids [31], and estrogen and progesterone produce synergistic induction of PPE gene expression in the female hypothalamus [32]. The molecular mechanisms of regulation of PPE gene expression by neurotransmitters and hormones are not well understood, although a lot of effort has been made. It has been shown that the immediate–early genes like c-*fos* can induce PPE gene expression in some systems. However, it is also clearly demonstrated that the induction of c-*fos* (or AP-1) and PPE gene expression can be dissociated both in *in vivo* and *in vitro* systems [20, 21, 33].

As part of the effort to understand the mechanisms involved in the regulation of the proenkephalin gene, we undertook a study to look at the possible role of c-*fos* in the increase of proenkephalin mRNA in response to β-adrenergic and glucocorticoid stimulation of C6 glioma cells [34]. In glioma cells, the results prove that changes in proenkephalin and c-*fos* mRNA can be dissociated from one another. Combined treatment with isoproterenol and dexamethasone resulted in a large increase in proenkephalin message levels while c-*fos* mRNA decreased. The opposite situation was also evident in that treatment of C6 glioma cells with TPA resulted in a dramatic increase of c-*fos* transcripts without subsequent elevation of proenkephalin mRNA. It is clear from these data that newly synthesized c-*fos* protein is not the rate-limiting determinant of augmented proenkephalin mRNA abundance. It is also obvious that increased levels of c-*fos* mRNA are not always followed by increased amounts of proenkephalin mRNA, even when these changes occur inside the same cell.

At least three major phenomena of interest for discerning possible transcriptional controls have been revealed for PPE mRNA in the forebrain. First is the hormone effect. In a robust fashion, estradiol treatment leads to an induction of PPE mRNA in ventromedial hypothalamic neurons [35]. This induction is rapid, within 1 h, and can be potentiated by progesterone [36]. It is tightly correlated with the occurrence of lordosis behavior [37] and is likely causal with respect to this behavior, acting through delta opioid receptors [15].

A second phenomenon concerns tissue specificity. With respect to basal expression, neurons in some brain regions express PPE RNA at much higher levels than others [10]. Even with respect to estrogen induction, the regulation is tissue specific: in the same experiments where estrogen up-regulated mRNA in the ventromedial hypothalamus, there was no change in the amygdala or in the caudate.

Third is genetic sex specificity. The same estrogen treatment which could induce PPE gene expression in genetic females was not effective in the hypothalamus of genetic Sprague–Dawley males [38]. This was not simply because the genetic male ventromedial hypothalamic neurons could not express the gene, since basal levels of PPE mRNA were just as high as in genetic females. Nor was it the case that the genetic male required testosterone, because that steroid had no effect either. How do these phenomena arise at the mRNA level? Protein/DNA binding assays as well as transcriptional assays are required, wherever possible using actual hypothalamic neuronal nuclear proteins.

Gel shift assays for protein/DNA binding to potential estrogen response elements (EREs) in hypothalamic cells

Electrophoretic mobility shift assay (EMSA) is a sensitive, simple and powerful tool to reveal protein–DNA interactions in neurons [39]. A suitable EMSA is available for determination of ERE binding activity in both neuronal and non-neuronal tissues [39]. By the use of EMSA with consensus ERE oligonucleotides, specific estrogen receptor (ER)–ERE interactions were detected in the hypothalamus of both female and male rats [39]. Actually, the ERE binding activity in male rat hypothalamus was comparable to that in female rat hypothalamus (unpublished observation). Thus, the differential effect of estrogen on hypothalmic PPE mRNA induction between male and female rats [38] can not be accounted for by a simple difference in ERE binding activity, suggesting some other factor(s) are involved, which we are currently investigating in our lab. The rapid induction (within 1 h) of PPE gene expression by estrogen in female hypothalamus [32] suggests that estrogen may directly activate the PPE gene transcription. Indeed, by use of EMSA with a polymerase chain reaction (PCR) fragment or an oligonucleotide, which contains a putative ERE site from the promoter region of PPE gene, specific ERE binding activity was observed in the female hypothalamus. These *in vitro* data imply that *in vivo* estrogen may bind to its receptor and form a complex with DNA in the promoter of the PPE gene to induce gene expression. Functional analysis is going on in our lab to identify the functional ERE in the PPE gene.

However, the action of steroid hormones on gene regulation is not a simple one ligand–one receptor–one DNA response element relationship. In fact, the core DNA sequences of both ERE and glucocorticoid response elements (GRE) are two major elements

which may subserve interactions between members of the nuclear steroid receptor superfamily. We have observed that a potent thyroid response element (TRE) oligonucleotide (DR4) was a very strong *competitor* to the ERE binding activity in hypothalamic nuclear extracts, suggesting that thyroid receptor (TR) may also bind to ERE (unpublished observations). Furthermore, by use of a vitamin D_3 receptor (VDR) binding domain [40], we have demonstrated that VDR can also bind to ERE as either a monomer or as a homodimer. These DNA–protein interactions are further complicated by possible protein–protein interactions, for instance, the interaction between steroid receptors with retinoic acid X-receptor and with other transcription factors like AP-1 proteins [41], and the TR interaction with triiodotyronine receptor-auxiliary proteins (TRAPs) [42]. The complexity of steroid hormone receptor–DNA interactions may rationalize some of the wide spread and multiple actions of steroid and thyroid hormones on gene expression including their behavioral consequences.

The up-regulation of the progesterone receptor (PR) by estrogen and its down-regulation by progestins at both the protein and mRNA levels has been amply demonstrated in human breast cancer cells, in avian oviduct, in mammalian uterus, and in specific brain regions of female rats (e.g. ventromedial hypothalamus) [43–46]. Because of progesterone response element (PRE)/GRE sequences on the PPE gene, it may be that PR and glucocorticoid receptor (GR) essentially *compete* in a biologically significant fashion for control over PPE expression.

Recent experiments in our laboratory have shown that nuclear extracts from different regions of female rat brains exhibit specific binding to a consensus PRE, as demonstrated by "gel shift", i.e. EMSA. Abundant protein/DNA binding to a consensus PRE was present in nuclear extracts from ventromedial hypothalmus and cerebellum, as well as in extracts from uterus. Moreover, the amount of binding could be regulated by estrogen and progesterone. Estrogen treatment of ovariectomized female rats increased PRE binding in nuclear extracts from VMN and uterus, while progesterone treatment following estrogen exposure decreased PRE binding in these tissues [47]. Using monoclonal antibodies to human PR and GR, we found that both PR and GR were present in the extracts and that each receptor type contributed to the signal detected in the EMSA. PR and GR bind to identical response elements, suggesting important competitive interactions in controlling PPE gene transcription. Thus, it should be noted that, like estrogen and progesterone, glucocorticoids have also been shown to regulate PPE expression and that the PPE gene contains at least three consensus DNA binding sequences for the PR and GR. These findings offer intriguing possibilities for the regulation of PPE expression by PR and GR through competition and/or cooperative interaction with control elements in promoter regions of the PPE gene.

Transcriptional assays using hypothalamic neuronal nuclear proteins

The tissue specific regulation of the rat PPE gene by steroid hormones may be accomplished by several mechanisms. For example, specific steroid hormone receptors might interact directly with their cognate DNA response elements in the promoter of the gene or the receptors may complex with other transcription factors which directly control promoter functions. At present, the specific regions of the PPE gene that are modulated by estrogen, progesterone, and dexamethasone have yet to be elucidated. In an effort to determine the targeted gene sequences as well as the factors necessary to regulate PPE transcription, we are developing an *in vitro* transcription assay using nuclear extracts from ventromedial hypothalamus and other brain regions. By examining the efficiency of transcription from different portions of the promoter region of the PPE gene in the presence of nuclear extracts from hormone treated rats, we hope to further define the conditions which control PPR expression *in vivo*. Initial studies showed that Hela cell nuclear extracts (a gift from R. Bernstein and Professor Roeder) could drive transcription from a plasmid containing a $+1300--2700$ base fragment of the rat PPE gene (a gift from S. Sabol). Further experiments have resulted in transcription, though at reduced efficiency, from this construct in nuclear extracts from caudate/putamen and hypothalamus derived from estrogen treated female rats. Thus, the development of this assay has hopefully provided a tool to better study complex, functionally important interactions of factors from brain tissues with specific promoter domains of targeted genes such as PPE.

Another approach to transcriptional analysis utilizes neurotrophic viral vectors. The defective herpes simplex viral vector was developed several years ago by Niza Frenkel and colleagues as a unique cloning and amplifying vector. Direct manipulation of gene expression in the adult mammalian neuron *in vivo* would be of great use both as a tool for basic research and as a potential modality for treatment of certain neurologic diseases. For the past several years, a major effort of our group as focused upon delivery of foreign genes into brain tissue of living, adult mammals. We reported the first successful *in vivo* expression of a functional foreign gene in the adult mammalian brain following transfer via an HSV1 amplicon-based defective viral vector [48]. In this approach, a small percentage of the genome of wild-type herpes is selected to include only those two sequences required for replication: the HSV ori and the cleavage/packaging sequence. These are cloned into a plasmid which also carries a promoter active in rat brain and a reporter gene such as Lac Z. Grown in the presence of a temperature-sensitive helper virus, at the permissive temperature (31 °C), the helper supplies the proteins needed for DNA repli-

cation of the experimental plasmid and for the neurotropic viral envelope. The use of HSV1 defective viral vector for *in vivo* gene transfer into neurons provides new opportunities for neurobiology. One novel use of the defective HSV vector would be as a vehicle for the analysis of promoter function *in vivo*. This technique combines the advantages of plasmid-based systems which have been widely utilized for promoter analysis in tissue culture, with the ability to assay promoter functions *in vivo*. The PPE promoter was chosen for *in vivo* promoter analysis because of the complex patterns of endogenous PPE expression and regulation in the nervous system. The vector dvHENK.2700 which contains 2.7 kb of the PPE promoter followed by the β-galactosidase gene, was delivered directly into hypothalamic and caudate regions followed by systematic treatment of estrogen. Our preliminary data indicated that estrogen stimulated PPE promoter by a 10-fold increase in ventromedial hypothalamus where endogenous PPE gene is expressed and regulated by estrogen [32], while estrogen had no effect on PPE promoter function in the caudate. This result suggested that an estrogen responsive promoter region might be localized within the 2.7 kb of PPE chosen. PPE promoter deletion analysis will answer this question.

While PPE promoter analysis *in vivo* and foreign gene manipulation in the brain through defective HSV vectors has proceeded well, it is nevertheless the case that the *in situ* PCR technique will play an important role in these applications as a monitor of viral vector within neurons by detecting very small amounts of viral DNA [49]. Considering all of our tools, the histochemical staining of β-gal, *in situ* PCR and *in situ* hybridization, we can measure the expression and regulation of a transgene in rat brain at three different levels, the viral gene, its transcript and protein product.

EXPRESSION OF GENE FOR PR IN BRAIN TISSUE

With *in situ* hybridization histochemistry, Romano *et al.* [49a] were able to find medial hypothalamic neurons expressing the gene for the PR, itself a transcription factor. The distribution of these cells on the hypothalamus matched what would have been predicted from progesterone binding, and this close agreement represents one form of validation of the *in situ* hybridization result. Strikingly, pretreatment with estradiol benzoate markedly increased the amount of mRNA for the PR in hypothalamic neurons [49a], which resulted from the estrogen treatment recruiting hypothalamic neurons to express the gene to the point of where the number of labeled cells following estrogen treatment was about four times as great as in the ovariectomized control animals. Another validation of the specificity of the hybridization technique is that the effect of estrogen on PR is opposite to that of the mRNA for the ER itself [50, 51].

In the same animals, estrogen treatment did not influence PR mRNA in the dorsal medial hypothalamus or in the amygdala.

Interestingly, the same estrogen treatment given to genetic females was ineffective in increasing PR mRNA in castrated male rats [45]. It was not the case that genetic males simply required testosterone treatment, since that was not effective either. Nor was it the case that hypothalamic neurons in the genetic male were incapable of expressing the PR gene because, if they were simply left intact, the number of neurons expressing this gene in the hypothalamus was substantial.

In turn, the induction of progestin binding is tightly correlated with effects of estradiol on female reproductive behavior [52]. A new approach allows us to confirm that this correlation really shows a causal role for PR synthesis in female reproductive behavior. Technical features of antisense application directly in mammalian brain have been discussed [53]. Application of antisense DNA reagents against PR mRNA directly to the ventromedial hypothalamus of female rats 12 but not 24 h following estradiol treatment was able to greatly reduce lordosis behavior and was able to decrease the occurrence of proceptive behavior (which is especially progesterone sensitive) by 80% [54]. Thus, the *in situ* hybridization evidence regarding PR mRNA coupled with the antisense DNA results show for the first time the causal linkage from the synthesis of a particular transcription factor to the occurrence of a specific behavior [49a, 54].

Gel shift assay

Three major phenomena with respect to PR mRNA in hypothalamic tissue must be explained: tissue specific transcription, estradiol induction, and sex differences. How do these phenomena arise? Search of the available sequences did not yield perfect consensus ERE on the PR gene. However, we know that estradiol can work through imperfect partial sequences.

One example is the rat creatine kinase B gene (CKB). Results showed that the rapid elevation of CKB mRNA in the rat uterus after estrogen treatment was mediated through a motif, aGGTCAgaaCACCCt, which locates at -550 upstream from the transcription start site. Despite having three deviations from the ERE consensus, the sequence is capable of binding ER. It is also necessary and sufficient for conferring estrogen responsiveness [55, 56]. Non-consensual EREs were also found in rat prolactin, human oxytoxin, human pS2, human c-*fos*, and rabbit uteroglobin genes, all have one, two, or three deviations, respectively [57].

A transcriptionally active ERE identified by Savouret *et al.* [59] provided the launching platform for gel shift assays using uterine and hypothalamic nuclear extracts. Hypothalamic cytoplasmic proteins bound to a consensus ERE as well as the PR ERE-like sequence

with high affinity and limited capacity, but experiments with ER antibodies clearly showed a difference between hypothalamic and uterine DNA binding as well as a difference between the consensus ERE and the physiological PR sequence [58]. Continued work with nuclear proteins indicated unusual binding to this potential ERE on the PR gene sequence which might include binding to an AP-1 like component of this sequence which itself does not satisfy the criteria for classical AP-1 binding. That is, the only functional ERE found in the rabbit PR gene has two deviations from the consensus ERE and overlaps with the translational start site [59]. This potential ERE overlaps with a motif, TGACTGA, matching 6 out of 7 bases of the consensus TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element, TGACTCA, also called an AP1 binding site. The ERE–AP1 complex in the PR genes is well conserved among species (adapted from Ref. [56]):

HUMAN	AGTCGTCATGACTGA
RAT	GGTCGTCATGACTGA
MOUSE	GGTCGTCATGACTGA
RABBIT	GGTCGACATGACTGA
Translation start site	ATG
ERE consensus	GGTCANNNTGACC
AP1 consensus	TGACTCA

The list above presents a comparison of the putative ERE–AP1 complex of PRs from different species. Sequences from human, rat, mouse, and rabbit PRs are aligned. Mismatches to the consensus ERE are underlined. Mismatches to the consensus AP1 site are in boldface. This ERE–AP1 complex is overlapped with the first translational start site is indicated.

Preliminary gel shift studies (Wu-Peng *et al.*, unpublished data) show that hypothalamic nuclear extracts capable of binding the consensus AP1 site specifically formed complexes with the ERE–AP1 element. An excess of the fragment itself was able to effectively compete binding. However, the consensus AP1 sequence itself can only compete at very high concentration. The result suggests that AP1 may not bind to the ERE–AP1 element classically and independently. Protein–protein interaction may be involved as found in other systems [60, 61]. Another possibility is that ER and AP1 compete for binding to the element since the AP1 binding site resembles the ERE half site. Indeed, possible competition for this element on the PR gene exists not only between ER and AP-1 proteins, but also with thyroid hormone receptor, VDR, RXR-β and COUP-TF. Further work in our lab on possible cooperative or competitive relations among these proteins using gel shift techniques and subsequent transcriptional analyses are expected to yield interactions of importance for reproductive neuroendocrine signalling.

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