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Title: Biochemical and Behavioral Characterization of Steroid Receptors in Neuronal Membranes

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Frank L. Moore

It is well established that steroid hormones modulate brain function behaviors through intracellular receptors that act as ligand-dependent transcription factors, regulating specific genes. Recent evidence indicates that steroids can also elicit rapid neuronal responses independently of gene expression, presumably through steroid receptors in neuronal membranes. However prior to this thesis, there was little direct evidence for steroid receptors in neuronal membranes.

This thesis investigates the receptor mechanisms mediating the rapid suppression of male reproductive behavior following stress or corticosterone administration in an amphibian, Taricha granulosa. The rapidity of this behavioral response is more consistent with a membrane-bound corticosterone receptor than with intracellular receptor-mediated events.

Radioligand binding studies, using [3H]corticosterone, reveal that a
genuine, high-affinity receptor for corticosterone exists in neuronal membranes. These binding sites are most enriched in synaptic membrane fractions and have a pharmacology distinct from intracellular receptors. Receptor autoradiography indicates the highest densities of receptors are found in the neuropil, rather than over cell bodies, in limbic brain regions.

Behavioral studies demonstrate that these membrane-bound receptors most likely mediate the stress-induced suppression of behavior; the potencies of steroids to bind to these receptors are highly correlated with their potencies to inhibit male behavior.

Additional radioligand binding studies demonstrate that this corticosteroid receptor is distinct from the GABA<sub>A</sub> receptor complex. Select steroids, but not corticosterone, are potent modulators of GABA<sub>A</sub> receptors in Taricha brains and mammalian brains, suggesting that this mechanism of steroid action has been highly conserved and is physiologically important.

The signal transduction mechanism associated with the corticosteroid receptor, like many neurotransmitter receptors, appears to involve G proteins. [3H]Corticosterone binding was negatively modulated by guanyl nucleotides in a manner consistent with a G protein-coupled transmembrane receptor.

Together, these studies provide the first evidence for a G protein-coupled steroid receptor in neuronal membranes and the best evidence that membrane-bound steroid receptors are behaviorally relevant. These findings also support the conclusion that steroids regulate brain function through multiple mechanisms of action, genomic and nongenomic.
Biochemical and Behavioral Characterization of Steroid Receptors in Neuronal Membranes

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PREFACE


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BIOCHEMICAL AND BEHAVIORAL CHARACTERIZATION OF STEROID RECEPTORS IN NEURONAL MEMBRANES

CHAPTER I: GENERAL INTRODUCTION

Genomic versus Non-genomic Actions of Steroids in the Central Nervous System

One significant breakthrough in modern cellular biology has been the discovery of the molecular mechanism that mediates many of the cellular responses to steroid hormones--the intracellular steroid receptor. The intracellular steroid receptors belong to a family of receptors for gonadal and adrenal steroids, thyroid hormones, vitamin D metabolites, retinoids, as well as a number of as yet undetermined ligands in vertebrate and invertebrate cells (Evans, 1988; Fuller, 1991). In the now classical model of steroid hormone action, steroids diffuse across plasma membranes and bind to cytosolic or nuclear receptors in target cells. The binding of ligand to receptor facilitates the binding of the activated receptor complex to consensus sequences on DNA molecules, the hormone response elements, thereby altering gene transcription (Yamamoto, 1985; Green and Chambon, 1988; Carson-Jurica, 1990; Beato, 1991). Thus, the classical steroid receptors act as ligand-dependent transcription factors, and the responses of cells to steroids results from steroid-directed changes in protein synthesis.

Steroids have profound influences on the brain, ranging from the life-long organizational effects of gonadal steroids on sexually dimorphic brain regions that
occur during development (Phoenix et al., 1959; Arnold and Gorski, 1984) to cyclic, transient effects of gonadal and adrenal steroids on neuronal activity and behavior (Weeks, 1991; Silver et al., 1992; Pfaff, 1989; McEwen et al., 1991). These actions of steroids can be ascribed to the positive and negative regulation of gene expression, including genes for neurotransmitter receptors, neuropeptides, G proteins, and enzymes for neurochemical metabolism (reviewed in McEwen et al., 1986; McEwen, 1991; Costa and Paul, 1991). However, in addition to the well-characterized, classical steroid effects on the regulation of gene expression, there is a subclass of effects produced by gonadal and adrenal steroids that cannot easily be accounted for by the classical model of steroid action.

Fifty years ago, Seyle discovered that steroids can have potent anesthetic and anti-convulsant effects (Seyle 1941, 1942). Since that time there have been numerous observations of steroid-induced changes in neuronal excitability (Kelly et al., 1977; Teyler et al., 1980; Nabekura et al., 1986; Majewska et al., 1986; Havens and Rose, 1988; Hua and Chen, 1989; Wong and Moss, 1991; Dubrovsky et al., 1990; Ffrench-Mullen and Spence, 1991), or neurosecretion (Keller-Wood and Dallman, 1984; Dluzen and Ramirez, 1989; Borski et al., 1991) that occur with a latency of seconds to very few minutes. The rapidity of these actions would seem to preclude a genomic mechanism of action. However, latency alone may not be sufficient to distinguish between genomic and nongenomic actions of steroids, especially when responses occur with a latency of minutes, rather than seconds or hours (McEwen, 1991). Recognizing this, many
rapid steroid effects have been demonstrated to occur when access to intracellular steroid receptors is blocked (Godeau et al., 1978; Hua and Chen, 1989; Dluzen and Ramirez, 1989), in brain regions or brain preparations lacking intracellular receptors (Edwardson and Bennett, 1974; Drouva et al., 1985; Smith et al., 1987; Su et al., 1988; Lan et al., 1990; Wilson et al., 1989; Inoue and Kuriyama, 1989; Sokolovsky et al., 1981), or in the presence of protein synthesis inhibitors (Nabekura et al., 1986; Schumacher et al., 1990).

These rapid, non-genomic events are presumed to be mediated through steroid recognition sites on neuronal membranes. However, despite the evidence that steroids can act at the level of neuronal membranes, the existence of physiologically relevant steroid receptors has remained largely inferred (McEwen et al., 1988), and the receptor and transduction mechanisms remained unknown. At the beginning of the studies described herein, there was only one paper demonstrating saturable binding sites for steroids to brain membranes (Towle and Sze, 1983). They found that gonadal and adrenal steroids saturably bound to partially purified synaptic membranes, but not with high affinity ($K_d = 120$ nM for corticosteroids), such that the physiological relevance of these binding sites was doubted (Schumacher, 1990). Two other radioligand binding studies have been recently published, describing steroid binding to neuronal membranes. Ke and Ramirez (1990) demonstrated that progesterone, when conjugated to iodinated bovine serum albumin, bound with low nanomolar affinity to neuronal membranes, but the results could not be replicated with progesterone alone.
Majewska et al. (1990) found that a "neurosteroid" (see below), dehydroepiandrosterone sulfate, bound with micromolar affinity to neuronal membranes. The methodology employed in that study was questionable; the use of a filtration assay to quantify low-affinity ligand binding to membranes is suspect.

Binding sites for steroids on cell surfaces of non-neuronal tissue have also been described (Suyemitsu and Terayama, 1975; Koch et al., 1978; Pietras and Szego, 1979; Blondeau and Baulieu, 1984; Bression et al., 1986; Trueba et al., 1987; Gametchu, 1987; Patino and Thomas, 1990; Ping et al., 1991; Ibarrola et al., 1991) that might account for some of the rapid actions of steroids that occur outside of the central nervous system (reviewed in Duval et al., 1983; Schumacher, 1990). As with the rapid effects of steroids on neuronal activity, the non-genomic steroid effects in peripheral tissue were also largely ignored as the details of the transcriptional model of steroid action were elaborated.

Interest in the rapid effects of steroids on brain function was aroused when a potential molecular mechanism was discovered. Investigations into the actions of anesthetic steroids revealed that steroids can allosterically modulate receptors for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain (Harrison and Simmonds, 1984). Subsequently it was discovered that physiological concentrations of endogenous steroid metabolites are also potent modulators of GABA_\text{\textalpha} receptors (Majewska et al., 1986). Finally, a biosynthetic pathway for steroid compounds was discovered in the brain (reviewed in Baulieu, 1991), including a pathway for the synthesis of "neurosteroids" that are active at
the GABA<sub>A</sub> receptor. A number of labs are currently attempting to establish a physiological role for endogenous GABA<sub>A</sub> receptor-active steroids (for review, Paul and Purdy, 1992). However, as demonstrated in Chapters 3 and 4, not only can select steroids modulate the GABA<sub>A</sub>/benzodiazepine receptor chloride ionophore complex, but other neuroactive steroids appear to use cell-surface receptors besides sites on the GABA<sub>A</sub> receptor.

The Problem

Reproductive behavior in all vertebrates is regulated through interactions among external cues and a suite of steroids, neurotransmitters, neuropeptides and their receptors (Fig. I.1). In addition, the same brain regions--the preoptic area, amygdala, hypothalamus, for example--appear to have key roles in regulation of reproductive behavior in all vertebrates. Therefore, the basic regulatory mechanisms that govern reproductive behaviors appear to have been highly conserved among vertebrates (Moore, 1990). Because of the relative simplicity of behavioral patterns and brain organization in non-mammalian vertebrates, and their robust behavioral responses to neurochemical manipulation, "lower" vertebrates may constitute powerful model systems for understanding the mechanisms that regulate reproductive behaviors.

In most vertebrates stress and reproduction are reciprocally related (Greenberg and Wingfield, 1987; Sapolsky, 1987). This is true for the amphibian Taricha granulosa (rough-skinned newt). For example, in Taricha,
stress suppresses the circulating levels of testosterone; this response may be due to the suppression of GnRH release by the adrenal (interrenal) steroid corticosterone (Moore and Zoeller, 1985). Acute stress also inhibits the reproductive behavior of male *Taricha*, the amplexic clasping of females; corticosterone is a causal factor in this behavioral response (Moore and Miller, 1984). The behavioral response to corticosterone appears to involve GABA, inasmuch as the stress- or corticosterone-induced suppression of clasping behavior is blocked when males are pretreated with a GABA synthesis inhibitor or treated concurrently with a GABA$_A$ receptor antagonist (Boyd and Moore, 1990; Moore and Orchinik, 1991).

These behavioral and endocrinological studies using *Taricha* invoke questions of the molecular mechanisms of steroid action because the onset of the response to stress or corticosterone is rapid; male clasping behavior is suppressed within 30 minutes of injection (Moore and Miller, 1984). Further examination of the response latency revealed that male sexual behavior is significantly suppressed, relative to saline-injected animals, within 8 minutes of intraperitoneal injection of corticosterone (Orchinik et al., 1991a). More recent work indicates that the excitability of *Taricha* hindbrain neurons involved in clasping behavior is altered within 2 minutes of topical application of corticosterone (Rose et al., 1991; Orchinik et al., 1991b). Behavioral and electrophysiological responses that are this rapid are inconsistent with the classical model of steroid-directed changes in protein synthesis.
Hypotheses and Objectives

The insufficiency of the classical model of steroid hormone action to account for the rapid behavioral and neuronal responses to corticosterone in *Taricha* provided the motivational basis for the studies undertaken in this thesis. The major objective of the studies described herein was to identify and characterize receptor and transduction mechanisms that could account for the rapid behavioral responses to corticosteroids.

The first hypothesis, the subject of Chapter 2, was that a physiologically relevant, high-affinity receptor for corticosteroids exists in neuronal membranes. The rapidity of the response to corticosterone dictated that the receptor be localized on the cell surface. Although there was no precedent for a high-affinity receptor in neuronal membranes for any naturally-occurring steroid, the exquisite sensitivity of *Taricha* males to corticosterone injection (significant inhibition of behavior by intraperitoneal injection of 1 μg) necessitated a high-affinity recognition site. Chapter 2 describes the pharmacological studies performed to satisfy basic criteria in validating a genuine, neurochemical receptor system.

A related hypothesis was that the corticosteroid recognition site on neuronal membranes played a role in the suppression of reproductive behavior. This hypothesis was tested in Chapter 2 in a series of behavioral experiments designed to determine if the affinity of steroids for the receptor is correlated with their potencies to suppress clasping behavior. This question was also addressed indirectly in receptor autoradiographic studies--functional receptors should be
localized in brain regions involved in the regulation of male sexual behavior.

The remaining hypotheses related to the transduction mechanisms associated with non-genomic steroid effects. The first of these hypotheses tested was that the potent steroidal ligands for mammalian GABA<sub>A</sub> receptors modulate GABA<sub>A</sub> receptors in *Taricha* brains in a similar manner. If so, this would provide strong evidence that this mechanism of steroid action has been conserved through vertebrate evolution, and therefore is likely to be important physiologically. Experimental tests of this hypothesis are described in Chapter 4.

As a corollary to this hypothesis, I tested the hypothesis that the corticosteroid receptor in neuronal membranes is a steroid recognition site on the GABA<sub>A</sub> receptor. This was a reasonable hypothesis because the behavioral response to corticosterone involves an interaction with the GABA<sub>A</sub> receptor system, and we found that other steroids can modulate GABA<sub>A</sub> receptor function in *Taricha* brain membranes. This hypothesis, which we eventually rejected, is addressed in a cursory manner in Chapter 2, but in depth in Chapter 4.

After rejecting the hypothesis that the rapid responses to corticosterone are mediated through a recognition site on the GABA<sub>A</sub> receptor, we examined an alternative hypothesis—that the corticosterone receptor utilizes guanyl nucleotide binding proteins (G proteins) in neuronal membranes in signal transduction. More than a hundred receptors for hormones and neurotransmitters have been identified that couple to G proteins (Simon et al., 1991), although none have been receptors for steroids. We performed radioligand binding studies to determine if
ligand binding to the corticosteroid receptor is modulated in a manner consistent with a G protein-coupled receptor. These studies are detailed in Chapter 3.

Together the studies in this thesis provide strong evidence that the stress-induced suppression of reproductive behavior in Taricha males is mediated by corticosteroid receptors that are coupled to G proteins in neuronal membranes. These receptors are not associated with the GABA\textsubscript{A} receptor complex, but GABA\textsubscript{A} receptors in Taricha brains are modulated by steroids very much like mammalian GABA\textsubscript{A} receptors. This second non-genomic mechanism of steroid action appears to have been highly conserved and therefore is probably an important molecular mechanism. These studies indicate that, in addition to classical intracellular receptor-mediated transcriptional events, steroids regulate vertebrate brain function and behavior through the use of multiple receptor and transduction mechanisms associated with neuronal membranes.
Fig. I.1. Model for the regulation of male *Taricha* reproductive behavior.

Clasping behavior is regulated by the integration of external cues with multiple excitatory and inhibitory neuromodulators. As in the mammalian brain, reproductive behavior is facilitated by actions of gonadal steroids. Steroids facilitate behavior, in part, through the regulation of the activity of neurotransmitter and neuropeptides, and their receptors. In response to inhibitory stimuli, such as stress, the hypothalamic-pituitary-adrenal axis is activated. Corticosterone, released from the adrenals, antagonizes the excitatory pathways regulated by arginine vasotocin (the amphibian homologue to arginine vasopressin) and gonadotropin releasing hormone (GnRH). The behavioral effects of corticosterone also involve the inhibitory neurotransmitter GABA. These neurochemical signals and regulatory mechanisms have been highly conserved in vertebrate evolution.
Excitatory Stimuli

Testosterone

Vasotocin

GnRH

Inhibitory Stimuli

Behavioral Response

CORT

GABA

Fig. I.1
CHAPTER II. A CORTICOSTEROID RECEPTOR IN NEURONAL MEMBRANES

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Abstract

Steroids may rapidly alter neuronal function and behavior through poorly characterized, direct actions on neuronal membranes. The membrane-bound receptors mediating these behavioral responses have not been identified. \[^3\text{H}\]Corticosterone labels a population of specific, high-affinity recognition sites (\(K_d = 0.51\) nM) in synaptic membranes from an amphibian brain. These binding sites were localized by receptor autoradiography in the neuropil, outside the regions of perikarya. The affinities of corticoids for this \[^3\text{H}\]corticosterone binding site were linearly related to their potencies in rapidly suppressing male reproductive behavior. Thus, it appears that brain membranes contain a corticosteroid receptor that could participate in the regulation of behavior.
In the classic model of steroid hormone action, steroids bind to intracellular receptors, which act as ligand-dependent transcription factors that regulate gene expression (Yamamoto, 1985; Justafsson et al., 1987; Carson-Jurica et al., 1990). In addition to these well-known actions of steroid receptors, steroids may alter brain function through nongenomic mechanisms (Schumacher, 1990; Majewska, 1987; Duval et al., 1983). For example, in rats, short-term exposure to the gonadal steroid progesterone is associated with rapid changes in behavior, and this effect occurs in the absence of new protein synthesis (Schumacher et al., 1990). Both gonadal and adrenal steroid hormones can alter neuronal firing activity within milliseconds to minutes of administration (Kelly et al., 1977; Nabekura et al., 1986; Riker et al., 1982; Saphier and Feldman, 1988), and these responses can occur in brain regions lacking classic steroid receptors (Smith et al., 1987) or if steroid access to intracellular receptors is blocked (Hua and Chen, 1989). These events appear to be mediated by direct steroid action on neuronal membranes, but there is little information concerning steroid binding to membrane-bound recognition sites in the brain (Towle and Sze, 1983; Ke and Ramirez, 1990; Majewska et al., 1990).

To investigate the possibility that glucocorticoid receptors occur on neuronal membranes, radioligand binding studies were performed on synaptic (P2) membranes from brains of an amphibian, *Taricha granulosa*, known to have
rapid behavioral responses to corticosterone (CORT) (Moore and Miller, 1984; Boyd and Moore, 1990; Moore, 1990). Equilibrium saturation binding experiments\(^1\), \(^2\) indicated that \(^{3}\text{H}\)CORT binding to brain membranes was specific, saturable, and of high affinity (\(K_d\), 0.51 nM) (Fig. II.1A). Kinetic experiments indicated that specific binding of \(^{3}\text{H}\)CORT was relatively rapid and reversible (Figs. II.1, B and C). The \(K_d\) value calculated from kinetic data (0.16

\(^1\) Whole brains from males were homogenized in cold 0.32 M sucrose containing 5 mM Hepes (pH=7.45), centrifuged at 1,000g (15 min) and the resulting supernatant centrifuged at 30,000g (40 min). The P2 pellet was frozen, then resuspended in 150 volumes (weight to volume) cold buffer (25 mM Hepes, 1 mM EDTA free acid, 60 \(\mu\)g/ml bacitracin, pH=7.45) for 3 hours to dissociate endogenous ligand. After centrifugation at 30,000g (30 min), the final pellet was frozen, resuspended in assay buffer (25 mM Hepes, 0.5 mM EDTA salt, 200 mM NaCl, pH=7.45) to a protein concentration of 450 to 550 \(\mu\)g/ml. Protein concentration was determined by Bio-Rad microassay with a bovine serum albumin standard.

\(^2\) Incubations (45-55 \(\mu\)g protein in 300 \(\mu\)l) were terminated after 2 hours by vacuum filtration and a 9 ml wash with buffer over Whatman GF-C filters. Radioactivity bound to filters was measured by liquid scintillation spectroscopy. Nonspecific binding was determined by addition of 10 \(\mu\)M unlabeled CORT. Specific binding was typically 75% to 80% of total binding. All binding experiments were performed in triplicate and were repeated at least twice, with similar results.
nM) was close to the $K_d$ estimated from equilibrium saturation data. The affinity of this site differs from the low affinity (120 nM) of the $[^3H]$CORT binding site described in the synaptosomal fraction from rats (Towle and Sze, 1983).

The specific binding of $[^3H]$CORT was temperature sensitive—greatest at 30°C, but eliminated when assays were performed at 60°C. The specific binding was also inhibited in a concentration-dependent manner by treatment of the membranes with the protease trypsin (0.001 - 1.0 mg/ml). Together, these data suggest that $[^3H]$CORT binds to a proteinaceous recognition site in the P2 fraction.

The binding site was highly specific for CORT (Table II.1) and did not have a pharmacological signature that resembled that of mammalian (McEwen et al., 1986; De Kloet and Reul, 1987; Funder and Sheppard, 1987) or amphibian (Medhi et al., 1984; Lange and Hanke, 1988) intracellular corticoid receptors. Neither the mineralocorticoid aldosterone, the glucocorticoid dexamethasone, nor

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3 Specific binding was low at 2°C, moderate at 15°C and 45°C, negligible at 60°C. Binding studies were routinely performed at 15°C, in the physiological temperature range of Taricha.

4 Experiments in which perfused and nonperfused brains were compared indicated that it was unlikely that $[^3H]$CORT binding activity was due to interaction with plasma corticosteroid binding proteins.

5 Amphibian intracellular corticoid receptors have high affinity ($K_d < 10$ nM) for aldosterone and/or dexamethasone as well as CORT.
any other Type I or Type II corticoid receptor ligands we tested displayed high affinity for this membrane-associated binding site. Furthermore, the [3H]CORT binding site did not appear to be associated with the gamma-aminobutyric acid (GABA$_A$) receptor. The steroid metabolites, 5α-pregnan-3α,21-diol-20-one (5α-THDOC) and 5α-pregnane-3α-ol-20-one (3α-OH-DHP), are potent modulators of mammalian (Majewska et al., 1986; Gee, 1988; Puia et al., 1990) and Taricha (Orchinik and Moore, 1989) GABA$_A$ receptors, but 5α-THDOC inhibited [3H]CORT binding with only modest affinity, while 3α-OH-DHP was devoid of activity (Table II.1). In addition, nonsteroidal GABA$_A$ receptor ligands did not displace [3H]CORT binding to membranes. The inability of GABA$_A$ receptor ligands to alter [3H]-CORT binding is consistent with the inability of CORT to alter GABA$_A$ receptor ligand binding or GABA-stimulated chloride flux in rodent (Gee et al., 1987; Harrison et al., 1987; Turner et al., 1989; Morrow et al., 1990) and Taricha brains (Orchinik and Moore, 1989).

Because the P2 fraction contains mitochondria as well as pre- and post-synaptic membranes (Whittaker, 1969), and [3H]CORT binds to adrenal and liver (Satre and Vignais, 1974; Cozza et al., 1990; Leschenko and Sergeyev, 1987) mitochondria, we examined [3H]CORT binding to synaptosomal and mitochondrial fractions prepared by discontinuous sucrose gradient centrifugation. As a marker for synaptic membranes, we measured the binding

6 Synaptosomes, mitochondria, nuclei were prepared from fresh brains as described in Whittaker (1969). The
of the muscarinic cholinergic antagonist, quinuclidinyl benzilate, [3H]QNB. As a mitochondrial marker, we measured succinate cytochrome C reductase activity (Feyereisen et al., 1985). The succinate cytochrome c reductase assay indicated that mitochondria were well separated from synaptic membranes (homogenate, 1.2; mitochondrial fraction, 10.6; synaptosomal fraction, 2.8 units/minute·mg protein).

Our data indicate that [3H]CORT binds to synaptic membranes. The specific binding of [3H]CORT was most enriched, more than 11-fold, in the synaptosomal fraction (ratio of [3H]CORT specific binding activity relative to binding activity in homogenate: crude cytosolic, 0.06; nuclear, 0.59; mitochondrial, 7.04; synaptosomal, 11.50). Furthermore, [3H]CORT binding activity paralleled the enrichment of muscarinic receptor specific binding in the synaptosomal fraction (ratio of [3H]QNB specific binding activity relative to [3H]QNB binding activity in homogenate: crude cytosolic, 0.06; nuclear, 0.68; mitochondrial, 3.06; synaptosomal, 11.20). The minimal amount of [3H]CORT "crude cytosolic" fraction resulted from a 48,000g (45 min) centrifugation of the initial homogenate. All fractions were resuspended in assay buffer to a protein concentration of 600 µg/ml.

7 P2 fraction resuspensions were incubated with 0.5 nM [3H]QNB + 10 uM scopalamine (to determine nonspecific binding).

8 The greater enrichment of [3H]CORT binding in the mitochondrial fraction relative to [3H]QNB suggests there
binding in the nuclear and crude cytosolic fractions demonstrated that under these conditions [3H]CORT binding to intracellular receptors is negligible, thus providing further evidence that [3H]CORT binding activity in the synaptosomal fraction is not attributable to intracellular receptor contamination.

To further substantiate the presence of CORT binding sites in synaptic membranes we performed in vitro receptor autoradiography. Sections of Taricha brain were incubated with [3H]CORT in the presence of unlabeled ligands specific for intracellular receptors. Under these conditions, [3H]CORT specific binding sites were detectable, with the greatest density in distinct regions of neuropil in areas including the amygdala, preoptic area, and hypothalamus (Fig. II.2). These [3H]CORT binding sites were located almost exclusively outside regions of

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may be binding sites for CORT in neuronal mitochondria, as well as in synaptic membranes.

9 Thaw mounted 25-μm brain sections were incubated for 30 min at 22°C with assay buffer containing 200 nM dexamethasone and ZK91587, then incubated for 2 hours with 2 nM [3H]CORT in buffer containing 200 nM dexamethasone and ZK91587. Nonspecific binding was determined in alternate sections by the addition of 10 μM CORT. The reaction was terminated by two 3-min washes in ice cold buffer. Sections were dried under cool air, apposed to 3H-sensitive film for 2 months, and stained with toluidine blue for histology. The autoradiogram was analyzed by computer-assisted densitometry (DUMAS). Specific binding was determined by subtraction of nonspecific binding from the aligned total binding sections.
perikarya. The localization of binding sites in neuropil, areas rich in synaptic terminals, provides corroborating evidence for CORT binding sites on synaptic membranes.

A series of behavior experiments were designed to determine if $[^3H]$-CORT binding sites could be behaviorally relevant receptors. Stress can suppress the sexual behavior of male *Taricha*; this rapid response is dependent upon the adrenal (interrenal) steroid CORT and is mimicked by CORT injection (Moore and Miller, 1984; Boyd and Moore, 1990; Moore, 1990). Male sexual behavior was suppressed within 8 min of intraperitoneal injection, compared to vehicle-injected controls (Fig. II.3A). A behavioral response this rapid is consistent with the presence of CORT receptors on synaptic membranes. Sexual behavior was inhibited by a low dose of CORT (2.5 nmol; Fisher Exact Test, $P < 0.005$), consistent with a high-affinity recognition site for CORT. We established dose responses for a series of steroids as inhibitors of male sexual behavior.

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10 Adult male *Taricha* in breeding condition were collected locally and experiments conducted in the field. Males (mean weight, 22 g) received intraperitoneal injections (0.1 ml) of steroid or vehicle (amphibian Ringers with 8% EtOH/2% dimethyl sulfoxide). Testing was initiated 5 min after injection, when stimulus females were added to tanks holding males. Males displaying sexual behavior (dorsal amplexic clasping of female) were removed from tanks. Females received 500 µg progesterone by injection 24 hours before tests to enhance attractivity (Moore, 1978).
behavior (Fig. II.3B). The potencies of corticoids to rapidly inhibit sexual behavior were linearly related to their potencies to inhibit [³H]CORT binding (slope ± SE = 0.58 ± 0.06; P < 0.003). These data suggest that this binding site, or pharmacologically similar receptors in the spinal cord or peripheral tissue, could be involved in stress-induced suppression of reproductive behavior.

In conclusion, our binding studies reveal the presence of a specific, high-affinity ligand-receptor interaction (Limbird, 1986; Weiland and Molinoff, 1981) in synaptic membranes. Autoradiographic studies provide further evidence for the localization of CORT binding sites in synaptic membranes (in brain regions known to regulate sexual behaviors). The linear relationship between the potencies of compounds to interact with this CORT receptor and to inhibit behavior suggests that this receptor may mediate CORT regulation of sexual behavior.\(^{11}\)

\(^{11}\) CORT induces sexual behavior in female rats within 5 min of intravenous injection through unknown mechanisms (Kubli-Garfias, 1990).
Table II.1. Potency of steroids as inhibitors of the specific binding of \[^{3}H\]CORT to crude synaptosomal membranes. $K_i$ values and slopes determined by nonlinear regression analysis (LIGAND); data are mean ± SEM values. Inhibition reported as percent inhibition of basal \[^{3}H\]CORT specific binding at a concentration of 1 $\mu$M competitor (Sigma, Steraloids, New England Nuclear; RU 38486 courtesy of Roussel-UCLAF, France); 0.5 nM \[^{3}H\]CORT. The highest concentration of solvent used (0.3% EtOH/0.1% dimethyl sulfoxide) was added to control tubes. Potent modulators of GABA$_A$ receptors designated by *.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
<th>Inhibition (%)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>0.11 ± .006</td>
<td>100</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td>Cortisol</td>
<td>3.75 ± .56</td>
<td>100</td>
<td>0.92 ± 0.08</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>293 ± 13</td>
<td>69</td>
<td>1.06 ± 0.06</td>
</tr>
<tr>
<td>5α-THDOC*</td>
<td>297 ± 14</td>
<td>70</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>RU 28362</td>
<td>569 ± 40</td>
<td>36</td>
<td>1.14 ± 0.24</td>
</tr>
<tr>
<td>Progesterone</td>
<td>759 ± 113</td>
<td>37</td>
<td>0.99 ± 0.10</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1,138 ± 63</td>
<td>32</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td>ZK91587</td>
<td>&gt;5,000</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>&gt;5,000</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>RU 38486</td>
<td>&gt;5,000</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3α-OH-DHP*</td>
<td>&gt;5,000</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table II.1
Fig. II.1. Binding of [3H]CORT to crude synaptosomal membranes. (A) Equilibrium saturation binding. P2 fraction resuspensions were incubated with increasing concentrations of [3H]CORT (101.6 Ci/mmol; NEN, Boston, MA.). Specific binding (filled circles) equals total binding (not shown) minus nonspecific binding (open circles). Estimates of $K_d = 0.51 \pm 0.04$ nM and $B_{max} = 146 \pm 4.4$ fmol/mg protein obtained by non-linear regression analysis (Lundon 1). The data were best fit by a one-site model. Inset: Scatchard replot of data was linear; Hill coefficient $= 0.98$ (LIGAND). (B) Association of [3H]CORT specific binding to brain membranes. P2 fraction resuspensions were incubated with 0.5 nM [3H]CORT for increasing intervals. Data were fit by a one site model (LIGAND); $k_{obs} = 0.054 \pm 0.005$ min$^{-1}$. (C) Dissociation of [3H]CORT from neuronal membranes. P2 fraction resuspensions were incubated for 2 hours with 0.5 nM [3H]CORT prior to addition of 100 $\mu$M cold CORT. Data best described with a one site model; $k_1 = 0.013 \pm 0.001$ min$^{-1}$. 
Fig. II.1

[Graph showing the relationship between Free \[^3\text{H}\]-CORT (nM) and Bound \[^3\text{H}\]-CORT (dpm).]
Fig. II.1

$[\text{H}^3] \text{-CORT Specific Binding}$

(fmol/mg protein)

$k_{\text{obs}} = 0.054 \text{ min}^{-1}$

Time (minutes)

---

26
Fig. II.1

$[\text{H}]$-CORT Specific Binding (fmol/mg protein)

$k_{-1} = 0.013 \text{ min}^{-1}$
Fig. II.2. Distribution of putative, membrane-bound [$^3$H]CORT binding sites in the preoptic area (POA). Section (left) showing darkly stained perikarya of POA neurons. Autoradiogram of identical section (right) shows localization of [$^3$H]CORT specific binding sites in the neuropil surrounding perikarya of POA neurons.
Fig. II.3. Inhibition of male sexual behavior. (A) Latency of response to CORT. Males were injected with 32 nmol (11 μg) CORT or vehicle, n= 14. Arrow indicates time of addition of females to tanks. Data reported as cumulative percentage of claspers at 1 min intervals. CORT injected males were significantly inhibited (*) within 3 min of testing (Fisher Exact Test, P = 0.025).

(B) Linear relationship between potency of steroids to inhibit [3H]CORT binding and potency to inhibit sexual behavior. Males were injected with one of 5 - 7 doses of steroid, or of vehicle (n= 24 for each dose of each steroid; except RU 28362, n=14). Data recorded as number of claspers in 20 minute tests (except cortisol; 60 minute tests, performed late in the breeding season). ED50 values to inhibit male clasping behavior versus controls were obtained by probit analysis (Tallarida and Jacob, 1979).
Fig. II.3

Male Sexual Behavior (Percent Claspers)

- Vehicle
- CORT (32 nmol)

Minutes After Injection

*
Inhibition of Sexual Behavior

(log ED50, nmol)

Inhibition of $[^3]$H[CORT Binding
(log $K_i$, nM)

Fig. II.3

0
1
2
3
4

-1
0
1
2
3
4

B

Dexamethasone

RU 28362

Aldosterone

Cortisol

Corticosterone
Acknowledgements

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CHAPTER III. GUANYL NUCLEOTIDES MODULATE BINDING TO STEROID RECEPTORS IN NEURONAL MEMBRANES

(corticosteroid receptors/G proteins/stress/sexual behavior)

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Abstract

The recently characterized corticosteroid receptor on amphibian neuronal membranes appears to mediate rapid, stress-induced changes in male reproductive behaviors. Because the transduction mechanisms associated with this receptor are unknown, we performed radioligand binding studies to determine whether this steroid receptor is negatively modulated by guanyl nucleotides. The binding of $[^3H]$corticosterone to neuronal membranes was inhibited by nonhydrolyzable guanyl nucleotides in both equilibrium saturation binding and competition studies. The addition of guanyl nucleotide plus unlabeled corticosterone induced a rapid phase of $[^3H]$corticosterone dissociation from membranes that was not induced by the addition of unlabeled ligand alone. Furthermore, the equilibrium binding of $[^3H]$corticosterone and the sensitivity of the receptor to modulation by guanyl nucleotides were both enhanced by Mg$^{2+}$. These results are consistent with the formation of a ternary complex of steroid, receptor, and guanine nucleotide-binding protein that is subject to regulation by guanyl nucleotides. Therefore rapid signal transduction through corticosteroid receptors on neuronal membranes appears to be mediated by guanine nucleotide-binding proteins.
Introduction

Steroid hormones modulate behavior and neuroendocrine function through the regulation of neuronal protein synthesis, an action mediated by intracellular steroid receptors (Evans, 1988; Carson-Jurica, 1990). However, not all effects of steroid hormones on brain function involve these genomic mechanisms (Schumacher, 1990). Evidence for nongenomically-mediated actions of steroids includes changes in neuronal excitability or neurosecretion that occur within seconds of steroid administration, when de novo protein synthesis is inhibited, when access to intracellular receptors is blocked, or in regions of the brain where intracellular steroid receptors have not been identified (Kelly et al., 1977; Nabekura et al., 1986; Smith et al., 1987; Hua and Chen, 1989; Dubrovsky et al., 1990). These actions of steroids that occur independently of classical intracellular receptors may have important roles in the regulation of behavior. For example, progesterone induces reproductive behavior in female rats, in part, through the modulation of hypothalamic oxytocin receptors; this rapid steroid effect is independent of de novo protein synthesis (Schumacher et al., 1990).

The binding sites for steroids on the membranes of neurons (Towle and Sze, 1983; Ke and Ramirez, 1990), as well as other cells (Suyemitsu and Terayama, 1975; Pietras and Szego, 1979; Koch et al., 1978; Blondeau and Baulieu, 1984; Gametchu, 1987; Patino and Thomas, 1990), might mediate some rapid cellular responses to steroids. However, little is known about the
transduction mechanisms associated with these recognition sites. Only a few
general types of transduction mechanisms account for the multitude of cellular
responses initiated by cell surface receptors: a receptor may be an integral part of
a ligand-gated ion channel, a transmembrane-regulated enzyme, or a
transmembrane protein that couples to guanine nucleotide-binding regulatory
proteins (G proteins) (Hollenberg, 1991). It is now well established that some
steroids can directly modulate the functioning of a ligand-gated ion channel, the
GABA_A receptor (Majewska et al., 1986; Lan et al., 1990). It appears that
steroids may utilize alternate mechanisms as well. We recently characterized a
high-affinity corticosteroid receptor in neuronal membranes from an amphibian
brain (Orchinik et al., 1991a) which appears to be physiologically relevant in
mediating the stress-induced suppression of male reproductive behavior (Moore
and Miller, 1984; Boyd and Moore, 1990; Moore and Orchinik, 1991). This
receptor is not associated with the GABA_A receptor chloride channel complex
(Orchinik et al., 1991a). Therefore, we have conducted studies to determine
whether the transduction mechanism utilized by this steroid receptor involves G
proteins.

Members of the superfamily of G protein-coupled transmembrane
receptors exhibit common structural and regulatory motifs (Raymond et al.,
1990). One characteristic of these G protein-coupled receptors is that the binding
of hormone or neurotransmitter to the receptor is subject to heterotrophic negative
modulation by guanine nucleotides (Birnbaumer et al., 1990; Gilman, 1987).
Therefore to address this question with respect to the membrane-bound corticosteroid receptor, we performed a series of radioligand binding studies to determine if [³H]corticosterone binding is sensitive to regulation by guanyl nucleotides. The data support the conclusion that signal transduction through this corticosteroid receptor in neuronal membranes is mediated by G proteins.
Methods

Animals. Adult male rough-skinned newts (*Taricha granulosa*) were collected locally (Benton Co., OR). Animals (mean weight 20 g) were maintained in the lab in large tanks under a lighting and temperature regimen that approximated natural conditions. Animals were maintained and sacrificed in accordance with I.A.C.U.C. guidelines.

Membrane preparation. Brains were rapidly removed and homogenized in 40 volumes (original weight to volume) of cold 0.32M sucrose containing 5 mM Hepes (pH=7.45). The whole brain homogenate was centrifuged at 1,000 x g (15 min), and the resulting supernatant was centrifuged at 30,000 x g (40 min; 4°C). The P2 pellet was frozen and thawed, then resuspended in 150 volumes (original weight to volume) of cold buffer (25 mM Hepes, 10 mM EDTA free acid, 60 µg/ml bacitracin, pH=7.45) for 2-3 hrs at 4°C to dissociate endogenous ligands and remove endogenous cations. The suspension was centrifuged at 30,000 x g (30 min). The resulting pellet was washed in 150 volumes of 25 mM Hepes buffer (pH=7.45) and centrifuged again at 30,000 x g for 30 minutes. The final pellet was resuspended in approximately 900 µl assay buffer/brain to a protein concentration of 250-350 µg/ml. Assay buffer contained 25 mM Hepes and 10 mM MgCl₂ (unless specified otherwise), pH=7.45. Protein concentration was determined by the Bradford method using Pierce Coomassie protein assay reagent (Rockford, Ill.), and BSA standard.
**Radioligand binding assays.** The binding of radiolabeled corticosterone (CORT) to crude synaptic membranes was initiated by the addition of 100 µl \[^3H\]CORT (0.75 nM final concentration) to 100 µl of the membrane preparation and 100 µl of inhibiting compound or buffer. For equilibrium saturation binding experiments a range of \[^3H\]CORT concentrations, from 0.03 - 5.0 nM, were used. The assays were incubated for 2 h at 30°C (21), unless specified otherwise, and the reactions terminated by rapid filtration and 9 ml rinse with cold buffer (25 mM Tris, pH=7.45) over Whatman GF-C filters, using a Brandel harvester (M-24R). Radioactivity bound to the filters was quantified by standard liquid scintillation spectroscopy. Non-specific binding was defined as that occurring in the presence of 1 or 10 µM unlabelled CORT. At the concentration of \[^3H\]CORT used, specific binding was typically 80% of total binding. The data from saturation and competition experiments were analyzed by nonlinear regression analysis, using LUNDON I Saturation Analysis Software (Lundon Software, Cleveland, OH) and EBDA (Elsevier-Biosoft, Cambridge, UK), respectively.

Kinetic experiments were performed to study the guanine nucleotide-induced dissociation of \[^3H\]CORT from membrane receptors. \[^3H\]CORT (0.75 nM) was allowed to equilibrate with *Taricha* brain membranes for 2 h as above. Dissociation was initiated by the addition of 25 µl of 25 µM unlabelled CORT or CORT plus 1 mM guanosine 5’-O-(3-thiotriphosphate) (GTP-γS). The estimates of the kinetic parameters were obtained using a non-linear least squares curve
fitting program KINETIC (Elsevier-Biosoft).

All binding experiments were performed in triplicate and were repeated at least twice with similar results.

Materials. [³H]Corticosterone, [1,2,6,7-³H(N)]-, specific activity 85-88 Ci/mmol, was purchased from New England Nuclear. Unlabeled CORT and guanine nucleotides were obtained from Sigma.
Results

The specific binding of $[^3H]CORT$ to *Taricha* neuronal membranes was enhanced in a concentration-dependent manner by the addition of MgCl$_2$ (Fig. III.1). The EC$_{50}$ of Mg$^{2+}$ for stimulation of $[^3H]CORT$ binding was $0.51 \pm 0.17$ mM. This enhancement of binding appeared to be cation specific; NaCl and CaCl$_2$ did not appreciably alter $[^3H]CORT$ specific binding (data not shown). In subsequent assays, 10 mM MgCl$_2$ was included in the assay buffer. Equilibrium saturation experiments (Fig. III.2) indicated that $[^3H]CORT$ bound to a single population of high affinity recognition sites with a $K_d$ of $0.14 \pm 0.01$ nM and a $B_{max}$ of $183 \pm 4$ fmol/mg protein. The specific binding of $[^3H]CORT$ was reduced by GTP-γS, a nonhydrolyzable analog of GTP (Fig. III.2). In the presence of 100 μM GTP-γS, the saturation isotherm was best fit by a one-site model yielding estimates of $K_d = 0.21 \pm 0.02$ nM and $B_{max} = 140 \pm 3$ fmol/mg protein; the increase in $K_d$ was not significant ($T = 3.13; P = 0.089$).

In titration experiments (Fig. III.3), the nonhydrolyzable guanyl nucleotides GTP-γS and guanyl-5′-yl-imidodiphosphate [Gpp(NH)p], inhibited up to 88% of the specific binding of $[^3H]CORT$ in a concentration-dependent manner; IC$_{50} = 43.2 \pm 11$ μM and $104 \pm 65$ μM, respectively. The inhibition of $[^3H]CORT$ binding by GTP-γS was temperature sensitive; GTP-γS was less potent at 15°C (IC$_{50} = 234$ μM), than at 30°C. The rank order potency of nucleotides to inhibit $[^3H]CORT$ binding was GTP-γS > Gpp(NH)p > > GDP>
GMP >> ATP. The potency of GTP (data not shown) was variable between experiments, but was generally similar to GDP. The limited potency of the endogenous nucleotide is presumably due to the high level of GTPase activity in neuronal membranes (Birnbaumer et al., 1990; Leid et al., 1988; Keen et al., 1991).

As shown in Fig. III.4, the ability of guanine nucleotides to inhibit \[^3H\]CORT binding was enhanced by MgCl\textsubscript{2}. The efficacies of GTP-\(\gamma\)S and Gpp(NH)p as modulators of \[^3H\]CORT binding were markedly decreased when MgCl\textsubscript{2} was excluded from the assay buffer.

To determine the effects of guanyl nucleotides on dissociation kinetics, we compared the dissociation of \[^3H\]CORT initiated by unlabeled CORT alone with that initiated by GTP-\(\gamma\)S plus CORT (Fig. III.5). Dissociation of \[^3H\]CORT initiated by CORT alone was monophasic, yielding a dissociation rate constant (\(k_1\)) of 0.014 ± 0.0006 min\(^{-1}\). In contrast, dissociation of \[^3H\]CORT initiated by the simultaneous addition of CORT plus GTP-\(\gamma\)S was biphasic (Fig. III.5). The initial rapid phase of dissociation was described by a \(k_1 = 0.515 ± .134\) min\(^{-1}\), in which 22% of \[^3H\]CORT dissociated. The subsequent slow dissociation was described by a rate constant (\(k_2 = 0.015 ± .0004\) min\(^{-1}\)) similar to the \(k_1\) obtained by the addition of CORT alone. The addition of GTP-\(\gamma\)S alone induced a rapid dissociation of approximately 20% of \[^3H\]CORT binding within 2 minutes (data not shown).

Considering that coupling of membrane receptors to G proteins may be
dependent upon the oxidation state of cysteine residues (Fraser, 1989; Boege et al., 1991), we performed GTP-γS titration experiments in the presence or absence of 100 μM dithiothreitol, a disulfide bond reducing agent. The equilibrium binding of [³H]CORT was slightly decreased, but the potency of GTP-γS to inhibit [³H]CORT binding was dramatically reduced, when membranes were homogenized and assayed in buffers containing dithiothreitol (Fig. III.6).
Discussion

Our results provide evidence for a novel molecular mechanism for the rapid modulation of brain function and behavior by steroid hormones. The data support the conclusion that signal transduction through the recently described corticosteroid receptor in neuronal membranes is mediated by guanyl nucleotide-binding proteins. Of the more than one hundred different G protein-coupled receptors that have been identified (Simon et al., 1991), none have been steroid hormone receptors. However, the current findings that ligand binding to the CORT receptor was negatively modulated by guanyl nucleotides in equilibrium saturation, titration and kinetic experiments, provides strong evidence that this receptor is allosterically regulated by heterotrimeric G proteins. Given these findings, it appears that there are multiple receptor and transduction mechanisms that steroid hormones may utilize to modulate neuronal activity, including the regulation of transcription via soluble intracellular receptors, the direct modulation of ligand-gated ion channels, and the regulation of cellular effector mechanisms via G protein-coupled receptors.

Since the discovery that GTP regulates the binding of glucagon (Rodbell et al., 1971), it has become clear that the negative modulation of agonist binding by guanyl nucleotides is a generalized phenomenon among G protein-coupled receptors (De Lean et al., 1980; Birnbaumer, 1990). This regulation is believed to be due to a heterotropic interaction between nucleotide binding to G protein
and agonist binding to receptor. In the absence of receptor-bound ligand, G proteins exist as heterotrimers with GDP bound. The binding of agonist to the receptor facilitates the formation of an agonist-receptor-G protein complex and the dissociation of GDP. GTP is exchanged for GDP; this promotes dissociation of the G protein into $\alpha$ and $\beta\gamma$ subunits which may activate effector molecules, and dissociation of the receptor from the G protein-receptor complex. When dissociated from G proteins, the free receptor displays low affinity for agonists (Birnbaumer et al., 1990; Gilman, 1987). Dissociation of the ligand from the receptor, and the hydrolysis of bound GTP, allows for continued activation of the cycle in response to hormone or transmitter.

The results from each of the experiments presented herein are consistent with the formation of a receptor-G protein complex which is subject to allosteric regulation by guanyl nucleotides. The binding of $[^3H]CORT$ to Taricha brain membranes was inhibited in a concentration-dependent manner by guanyl nucleotides. As in most G protein-coupled receptor systems, the nonhydrolyzable nucleotides had greater efficacy in inhibiting ligand binding than the hydrolyzable compounds, and the adenosine nucleotide was without effect. Also, GTP-$\gamma$S induced a rapid phase of $[^3H]CORT$ dissociation from membrane receptors that was not induced by CORT alone, consistent with a nucleotide-induced shift in the affinity state of the CORT receptor. We also found that MgCl$_2$ enhanced the specific binding of $[^3H]CORT$, an effect not induced by CaCl$_2$ or NaCl. In many receptor systems, Mg$^{2+}$ facilitates formation of the high affinity state of the
receptor, presumably by binding to the G protein (Birnbaumer et al., 1990; Gilman, 1987). Therefore, the increase in binding elicited by Mg²⁺ likely reflects a conversion from the low- to the high-affinity state of the receptor for CORT. The EC₅₀ value (0.51 mM) for this Mg²⁺ effect is similar to the Kₐ of Mg²⁺ for hormone receptor-mediated activation of Gₛ (Iyengar and Birnbaumer, 1982).

The inhibition of [³H]CORT binding by GTP-γS in saturation isotherms was described by an apparent decrease in the number of binding sites, rather than a decrease in affinity. The increase in Kₐ (from 140 to 210 pM) was modest, similar to the affinity shift induced in GABAₐ receptors by GTP (Hill et al., 1984), for example. However, the rapid dissociation of [³H]CORT binding induced by GTP-γS suggests a more pronounced affinity shift, at least in a subpopulation of CORT receptors (Fig. III.5). In many systems, guanyl nucleotides induce a 50- to 100-fold decrease in receptor affinity, usually measured as a decreased ability of agonists to displace radiolabeled antagonist binding. No antagonists to the CORT receptor are currently available (Orchinik et al., 1991b), and the highest concentration of [³H]CORT used was 5 nM, which effected a 97% occupancy of high-affinity recognition sites. Therefore, a 50- to 100-fold shift in affinity of a subpopulation of receptors would not be detectable under the assay conditions used, but rather would be manifest as a reduction in the apparent Bₘₐₓ. Similar decreases in apparent Bₘₐₓ have been reported in saturation isotherms studying guanyl nucleotide regulation of [³H]agonist binding.
to G protein-coupled receptors, such as α- (Rouot et al., 1980) and β-adrenergic (Williams and Lefkowitz, 1977) receptors.

Many of our observations are consistent with findings in the β-adrenergic receptor system, where the receptor-G protein interactions have been extensively characterized (Raymond et al., 1990). The efficacies of guanyl nucleotides to regulate agonist binding to EDTA-washed membranes were enhanced by MgCl₂ in both the CORT (Fig. III.4) and β-adrenergic (O'Donnell et al., 1984) receptor systems. This phenomenon may be related to the ability of Mg²⁺ to promote the formation of the ternary complex (agonist-receptor-G protein), which increases the guanyl nucleotide sensitivity of [³H]CORT binding. The modulation of [³H]CORT binding by guanine nucleotides was also temperature-sensitive, in a manner similar to the β-adrenergic system (O'Donnell et al., 1984). In addition, treatment of the membranes with 100 μM dithiothreitol resulted in a slight reduction of the equilibrium binding of [³H]CORT, but a much greater reduction in the efficacy of GTP-γS as an inhibitor of [³H]CORT binding. Similarly, in the β-adrenergic system, the oxidation state of cysteine residues appears to be important not only for ligand binding, but also in maintaining the receptor in a conformation required for activation of G proteins (Fraser, 1989; Boege et al., 1991). It is noteworthy that a 10- to 50-fold higher concentration of dithiothreitol is routinely included in assays of intracellular adrenal steroid receptors, suggesting that the steroid recognition sites on the membrane-bound and intracellular receptors may differ significantly.
Little is known about the interaction of small, hydrophobic ligands, such as steroids, with membrane-bound receptors. It has been suggested that these compounds would be unlikely to bind to receptor proteins in the lipid milieu of plasma membranes. However, agonist binding has been characterized (Devane et al., 1988), an effector mechanism determined (Howlett et al., 1986), and a receptor cloned and expressed (Matsuda et al., 1990) for at least one other class of lipophilic compounds, the cannabinoids. Although cannabinoids do not inhibit \[^{3}H\]CORT binding (data not shown), there are similarities in the ligand-receptor interactions between the CORT and cannabinoid receptors systems. Equilibrium saturation studies in both receptor systems indicate that ligands bind with high specificity to a single population of recognition sites with subnanomolar affinity (Orchinik et al., 1991a; Devane et al., 1988). Ligand binding to both the CORT and cannabinoid receptors was enhanced by MgCl\(_2\), while guanyl nucleotides inhibited binding and induced a rapid dissociation of ligand from the receptors. The maximal inhibition of cannabinoid binding by guanyl nucleotides reported was also less than 100% (Devane et al., 1988), but the deduced molecular structure of the receptor indicates clearly that it belongs to the family of membrane-spanning receptors that couple to G proteins (Matsuda et al., 1990). The sensitivity of agonist binding to inhibition by nucleotides in different receptor systems may depend on the tightness of coupling between the receptor and G protein, the membrane preparation, cation and ligand concentration, and the relative abilities of various agonists to discriminate different affinity states of the
receptor.

There is precedent for the hypothesis that a membrane-bound steroid receptor may be coupled to G proteins. Progesterone induces maturation of *Xenopus* oocytes through mechanisms that are independent of classical intracellular receptors (Godeau et al., 1978), and progesterone binding sites on oocyte membranes have been described (Blondeau and Baulieu, 1984; Sadler and Maller, 1982). While progesterone inhibits cholera toxin-stimulated adenylate cyclase activity in oocyte membranes (Finidori-Lepicard et al., 1981), the inhibition of adenylate cyclase activity is not mediated by the pertussis toxin-sensitive G, (Sadler et al., 1984). Therefore, the mechanism of progesterone action in oocyte membranes cannot easily be explained by the conventional model of receptor-G protein interaction (Smith, 1989). However, the demonstration that the CORT receptor in *Taricha* neuronal membranes is sensitive to negative modulation by guanyl nucleotides is entirely consistent with the coupling of a steroid receptor to heterotrimeric G proteins in the brain.

The latency of electrophysiological responses to CORT is consistent with the time course of a receptor-G protein-effector mechanism of action. The excitability of *Taricha* hindbrain neurons is altered within 2 min of topical CORT application, and the response to CORT is reversed within 2-5 minutes following removal of CORT (Rose et al., 1991). Some neurons in the rat brain respond to corticosteroid administration with the same latency (7), which also would be consistent with a steroid receptor-G protein interaction. It is reasonable to
speculate that a membrane receptor for estradiol also couples to G proteins,
inasmuch as estradiol depolarizes some neurons in the rat hypothalamus within 3-5 min of application, through a decrease in K⁺ conductance that is apparently mediated by cAMP (Nabekura et al., 1986; Minami et al., 1990). Therefore, in the mammalian brain as well, it is likely that some steroid hormones may effect rapid alterations in brain function through the use of receptor and transduction mechanisms similar those described in Taricha brains.
Fig. III.1. The effect of MgCl₂ on the specific binding of [³H]CORT to neuronal membranes. The membrane preparation was incubated with 0.75 nM [³H]CORT and increasing concentrations of MgCl₂. The addition of MgCl₂ enhanced [³H]CORT binding with EC₅₀ = 0.51 ± 0.17 mM. The hyperbolic fit shown was derived using least squares regression analysis with Graph Pad-In Plot (San Diego, CA).
Fig. III.1

$[^3]H \text{CORT}$ Specific Binding (Percent Increase)

MgCl$_2$ Concentration (mM)
Fig. III.2. Equilibrium saturation binding of [3H]CORT to neuronal membranes in the presence or absence of guanyl nucleotide. Neuronal membranes were incubated with increasing concentrations of [3H]CORT. Nonspecific binding (open squares) was defined as that occurring in the presence of 1 or 10 µM unlabeled CORT. Specific binding in the absence of guanyl nucleotide (open circles) was described by $K_d = 0.14 \pm 0.01$ nM and $B_{max} = 183 \pm 4$ fmol/mg protein (Hill coefficient = 1.08). In the presence of 100 µM GTP-γS (filled circles), the saturation isotherm was described by $K_d = 0.21 \pm 0.02$ nM and $B_{max} = 140 \pm 3$ fmol/mg protein (Hill coefficient = 1.12). In both cases, the data were best fit by a one-site model using Lundon I software and LIGAND software (Elsevier-Biosoft, Cambridge, UK). Inset, linear Scatchard-Rosenthal replot of saturation data.
Fig. III.2

[\text{\textsuperscript{3}H}\text{CORT} \text{Bound (dpm} \times 10^{-3})]

[\text{\textsuperscript{3}H}\text{CORT Concentration (nM)}]
Fig. III.3. Guanyl nucleotides inhibit the specific binding of [³H]CORT in *Taricha* brain membranes. Membranes were incubated with 0.75 nM [³H]CORT and increasing concentrations of the nucleotides shown. The titration curves were replicated three times with similar results. IC₅₀ estimates ± standard error were obtained for GTP-γS (43.2 ± 11 μM) and Gpp(NH)p (104 ± 65 μM) using EBDA software. The inhibition of [³H]CORT binding by GTP (not shown) varied between experiments, with maximal inhibition ranging from 20% to 35%.
Fig. III.3

[3H]CORT Specific Binding
(Percent Control)

log Nucleotide Concentration (Molar)

- ATP
- GDP
- GMP
- Gpp(NH)p
- GTP-γS
**Fig. III.4.** Effect of MgCl₂ on the inhibition of [³H]CORT binding by guanyl nucleotides. Membranes were incubated with 0.75 nM [³H]CORT and increasing concentrations of nucleotide, either without MgCl₂ added, or with 10 mM MgCl₂ added. The efficacy of both GTP-γS (left panel) and Gpp(NH)p (right panel) as inhibitors of [³H]CORT binding was greatly enhanced by MgCl₂.
Fig. III.4

[3H]CORT Specific Binding (Percent Control)

log Nucleotide Concentration (Molar)

- GTP-γS without MgCl₂
- GTP-γS with MgCl₂
- Gpp(NH)p without MgCl₂
- Gpp(NH)p with MgCl₂
Fig. III.5. Dissociation of specifically bound $[^3]$H]CORT from *Taricha* neuronal membranes. $[^3]$H]CORT (0.75 nM) was allowed to equilibrate with membranes for 2 hrs, at which time dissociation was initiated by the addition of 25 $\mu$l of unlabelled CORT or CORT plus 1 mM GTP-$\gamma$S. For CORT alone, $k_1$ was determined to be $0.014 \pm 0.0006$ min$^{-1}$. The addition of GTP-$\gamma$S plus CORT resulted in biphasic dissociation rates, with 22% of $[^3]$H]CORT dissociating with $k_1 = 0.515 \pm 0.134$ min$^{-1}$, and 78% dissociating with $k_1 = 0.015 \pm 0.0004$ min$^{-1}$, as determined by KINETIC software.
Fig. III.5

- [3H]CORT Specific Binding (dpm x 10^3)

- CORT
- CORT plus GTP-γS

Time (minutes)
Fig. III.6. Effect of disulfide bond reducing agent on the sensitivity of [³H]CORT binding to negative modulation by GTP-γS. Brains were homogenized, and membranes prepared and assayed as in Fig. III.3., or with 100 μM dithiothreitol added to each buffer. In the presence of dithiothreitol, the equilibrium binding of [³H]CORT was reduced by 13%. The highest concentration of GTP-γS inhibited the specific binding of [³H]CORT by 88% in control membranes, while GTP-γS inhibited only 32% of [³H]CORT specific binding to membranes in the presence of dithiothreitol.
Fig. III.6

- [H] CORT Specific Binding (fmol/mg protein)
- log GTP-γS Concentration (Molar)

- Control
- +100μM Dithiothreitol
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CHAPTER IV. STEROID MODULATION OF GABA \textsubscript{A} RECEPTORS IN AN AMPHIBIAN BRAIN

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Abstract

Compelling evidence indicates that certain steroids are potent modulators of the GABA<sub>A</sub> receptor/Cl<sup>-</sup>channel complex in the rat brain. The 3α-hydroxylated ring A reduced metabolites of progesterone and deoxycorticosterone are released in response to stress and alter ligand binding to GABA<sub>A</sub> receptors and potentiate GABA-stimulated Cl<sup>-</sup> flux. However, the physiological relevance of this nongenomically-mediated mechanism of steroid action is still unclear. We performed radioligand binding studies, using an amphibian, *Taricha granulosa*, to determine if steroid modulation of GABA<sub>A</sub> receptors has been highly conserved during vertebrate evolution. The binding parameters for the convulsant t-butyl bicyclophosphorothionate ([<sup>35</sup>S]TBPS) and the benzodiazepine [<sup>3</sup>H]flunitrazepam were similar in *Taricha* and mammalian brains. The allosteric regulation of [<sup>35</sup>S]TBPS and [<sup>3</sup>H]flunitrazepam binding by GABA were also similar between species. We also found that the rank order and absolute potencies of steroids to inhibit [<sup>35</sup>S]TBPS binding and enhance [<sup>3</sup>H]flunitrazepam binding to GABA<sub>A</sub> receptors were similar in *Tarichas* and rat brains. In autoradiographic studies, [<sup>3</sup>H]flunitrazepam binding sites were more uniformly distributed than [<sup>35</sup>S]TBPS binding sites, but [<sup>35</sup>S]TBPS binding sites in all brain regions were inhibited by 3α-OH-5α-pregnan-20-one. As in mammalian studies, corticosterone was far less potent in inhibiting [<sup>35</sup>S]TBPS binding or enhancing [<sup>3</sup>H]flunitrazepam binding. Corticosterone also had no effect on GABA-stimulated Cl<sup>-</sup> uptake into *Taricha*
synaptoneurosomes. Therefore, the recently described corticosteroid receptor in *Taricha* neuronal membranes does not appear to be associated with the GABA_A receptor. However, since GABA_A receptors are modulated by steroids in a very similar manner in the brains of mammals and a "primitive" vertebrate, this mechanism appears to have been highly conserved and therefore is likely to serve important roles in regulating vertebrate brain function.
Introduction

Seyle discovered 50 years ago that certain steroids have anti-convulsant and anesthetic properties (Seyle, 1941, 1942) in addition to their classical endocrine actions. More recent evidence indicates that a number of endogenous, neuroactive steroids can alter neuronal excitability, neurosecretion, neurochemical receptors, or behavior within seconds to minutes of administration (reviews: Duval, 1983; Schumacher, 1990; McEwen, 1991; Chadwick and Widdows, 1990; Costa and Paul, 1991; Moore and Orchinik, 1991; Paul and Purdy, 1992). In contrast to the classical actions of steroid hormones that are mediated through intracellular receptor-directed changes in protein synthesis (Yamamoto, 1985; Evans, 1988; Carson-Jurica et al., 1990), the actions of neuroactive steroids are presumed to occur through interaction with specific recognition sites on neuronal membranes.

The best characterized neuroactive steroids are the 3α-hydroxylated ring A reduced metabolites of progesterone and deoxycorticosterone, 3α-OH-5α-pregnan-20-one (3α-DHP) and 3α,21-OH-5α-pregnan-20-one (5α-THDOC), respectively (reviews: Lambert et al., 1987; Gee, 1988; Schumacher and McEwen, 1989; Harrison et al., 1989; Lan et al., 1991; Paul and Purdy, 1992). Compelling evidence indicates that these steroids alter neuronal excitability through direct modulation of the GABA_A (gamma-aminobutyric acid)/benzodiazepine receptor-chloride ionophore complex (Harrison and Simmonds, 1987; Majewska et al.,
1986; Callachan, 1987; Morrow et al., 1987; Gee et al., 1988; Peters et al., 1988; Turner et al., 1989; Lan et al., 1990; Im et al., 1990). These naturally occurring steroids are among the most potent GABA_Α receptor ligands known, capable of enhancing GABA-activated Cl⁻ conductance in nanomolar concentrations. Sensitivity to steroids in transiently expressed GABA_Α receptor subunits (Puia et al., 1990; Lan et al., 1990; Shingai et al., 1991; Hill-Venning et al., 1991; Woodward et al., 1992) strongly suggests that steroid recognition sites reside on GABA_Α receptors, however these steroid recognition sites have not been characterized in radioligand binding studies.

In the rat, concentrations of 3α-DHP and 5α-THDOC in the plasma and brain increase significantly in response to stress (Purdy et al., 1991). When administered, these steroids possess hypnotic and anxiolytic properties similar to the benzodiazepines and barbiturates (Crawley et al., 1986; Kavaliers and Wiebe, 1987; Mendelson et al., 1987; Bitran et al., 1991; Wieland et al., 1991). This has led researchers to speculate that these steroids may be endogenous ligands for GABA_Α receptors, mediating anxiolytic or sedative processes in response to stress (Paul and Purdy, 1992). But thus far, a physiological role for endogenous GABA_Α-active steroids has not been established (Delville et al., 1991; Costa and Paul, 1991). If steroid modulation of GABA_Α receptors has been highly conserved through vertebrate evolution, it would strengthen the conclusion that this mechanism of action for neuroactive steroids is likely to subserve important physiological roles. Therefore, we investigated the possibility that GABA_Α
receptors are modulated by 3α-hydroxylated, ring A reduced pregnane steroids in a "primitive", non-mammalian vertebrate brain.

As a second point of interest, male reproductive behavior is suppressed by stress or injection of corticosterone in an amphibian, *Taricha granulosa* (Moore and Miller, 1984; Moore and Orchinik, 1991). The response to corticosterone is rapid; behavioral changes are apparent within 8 minutes of intraperitoneal injection (Orchinik et al., 1991a), and the excitability of hindbrain neurons is altered within 2 minutes of corticosterone application (Rose et al., 1991). Since the behavioral response to corticosterone (CORT) is blocked by the GABA<sub>A</sub> receptor antagonist bicuculline (Boyd and Moore, 1990), this response might be mediated through a steroid recognition site on GABA<sub>A</sub> receptors. We have previously characterized a high-affinity corticosteroid receptor in neuronal membranes (Orchinik et al., 1991a; Orchinik et al., 1992); in this current study we examine the hypothesis that the membrane-bound corticosteroid recognition site is associated with the GABA<sub>A</sub> receptor complex.

Our results indicate that steroids modulate GABA<sub>A</sub> receptors in a primitive vertebrate brain in a manner very similar to mammalian brains. However, the pharmacological profile of the steroid recognition sites on GABA<sub>A</sub> receptors is distinct from the biochemical and behavioral profile of the corticosterone receptors in neuronal membranes.
Materials and Methods

Animals. Adult male *Taricha granulosa* (rough-skinned newts), mean weight 20 g, were collected from local ponds (Benton County, OR) and maintained in a laboratory tank in dechlorinated water at 17°C with approximately natural photoperiod. Animals were maintained and sacrificed in accordance with I.A.C.U.C. guidelines.

Radioligand Binding Assays. Neuronal (P2) membranes were prepared from whole *Taricha* brains (Orchinik et al., 1991a). Briefly, P2 pellets were subjected to 2 freeze/thaw cycles and a 4 hour preincubation in a large volume of cold, hypoosmotic buffer to remove endogenous GABA and steroids. The well washed P2 pellet was resuspended in assay buffer (25 mM HEPES, 0.5 mM EDTA salt, 200 mM NaCl, pH=7.45) to a protein concentration of 400-500 µg/ml. The binding of radiolabeled t-butylbicyclophosphorothionate (TBPS) or the central benzodiazepine ligand, flunitrazepam, to neuronal membranes was initiated by the addition of 100 µl [³H]flunitrazepam or [³⁵S]TBPS to 100 µl of the P2 membrane preparation and 100 µl of steroid, GABA, competing compound or buffer. Steroids were added to the membranes 15 minutes prior to the addition of radiolabeled compound. The membranes were incubated for 2 hours at 15°C (both radioligands reach equilibrium within 2 hours) and the reactions were terminated by rapid filtration and 9 ml rinse with ice cold buffer (25 mM Tris, 200 mM NaCl, pH=7.45) over Whatman GF-C filters, using a Brandel harvester.
Filters were soaked in 0.5% polyethylenimine prior to use in [35S]-TBPS binding studies. Radioactivity bound to the filters was quantified by standard liquid scintillation spectroscopy using 7 ml of Ecolume (ICN Biomedicals Inc) as scintillant. Non-specific binding was defined as that occurring in the presence of 100 μM picrotoxin ([35S]TBPS) or 10 μM diazepam ([3H]flunitrazepam). Protein concentration was determined by the Bio-Rad protein microassay method (Bio-Rad, Richmond CA), using bovine serum albumin as standard. For each experiment, the binding curves were performed in triplicate and the experiments were repeated at least twice, with similar results.

Estimates of $K_d$ and $B_{\text{max}}$ were obtained by non-linear regression analysis using LUNDON I Saturation Analysis Software (Lundon Software, Cleveland, OH) or LIGAND (Elsevier-Biosoft, Cambridge, UK). $EC_{50}$ and $IC_{50}$ estimates were obtained by least squares regression analysis using EBDA (Elsevier-Biosoft, Cambridge, UK) or Graph Pad-In Plot (San Diego, CA). Hyperbolic and sigmoidal curves were fit using Graph Pad-In Plot.

**Measurement of $^{36}$Cl$^+$ Uptake.** Synaptoneurosomes were prepared from whole *Taricha* brains as described (Schwartz et al., 1985) with minor modifications. Freshly removed brains were homogenized in 10 volumes (v/w) cold buffer (20 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM D-glucose; pH 7.4) using a glass Dounce (8 strokes) and filtered through 2 layers of nylon mesh (pore size: 160 μm). The filtrate was washed with 30 volumes of buffer and centrifuged (1000 x g, 15 min, 4°C) twice. The
final pellet was resuspended in 2.5 volumes (vol/original weight) buffer to a protein concentration of approximately 5 mg/ml.

$^{36}\text{Cl}^-$ uptake was determined as described by Allan and Harris (1986) with minor modifications. Aliquots of the membranes (150 μl) were pre-incubated at 22°C for 10 min. Uptake was initiated by the addition of 0.1 μCi $^{36}\text{Cl}^-$ (150 μl) with or without GABA, and gently vortexing. Bicuculline methiodide, picrotoxin, or steroids were added 10 min prior to the addition of $^{36}\text{Cl}^-$. Influx was terminated by the addition of 3 ml cold buffer containing 100 μM picrotoxin, followed by rapid filtration under partial vacuum over glass-fiber filters on a single manifold harvester, and additional wash with 6 ml cold buffer. Whatman GF-C filters were presoaked in 0.1% bovine serum albumin. The radioactivity bound to filters was quantified by standard liquid scintillation spectrometry. The amount of $^{36}\text{Cl}^-$ bound to filters in the absence of tissue was subtracted from the total binding in of each tubes. GABA-stimulated $^{36}\text{Cl}^-$ influx was determined to be maximal between 5 - 15 seconds at 22°C, so incubations were carried out for 12 seconds. All determinations were performed in triplicate.

Receptor Autoradiography. Thaw mounted 25-μm brain sections, cut in the transverse plane, were dried in a desiccator overnight under partial vacuum at -5°C and stored at -80°C until use. To determine the distribution of $[^{35}\text{S}]$TBPS binding sites, slides were pre-incubated for 30 min in cold assay buffer (25 mM HEPES, 200 mM NaCl, 0.5 mM EDTA; pH = 7.45), then incubated for 2 hours with 15 nM $[^{35}\text{S}]$TBPS at 15°C. Nonspecific binding was determined in alternate
sections by the addition of 200 μM picrotoxin. To assess potential regional
differences in sensitivity of [35S]TBPS binding to modulation by steroids, sections
were incubated with 15 nM [35S]TBPS and 10 μM 3α-DHP or 100 μM CORT.
To visualize benzodiazepine binding sites, sections were incubated with 5 nM
[3H]flunitrazepam (2 hours, 15°C), in the presence or absence of 20 μM
diazepam to determine nonspecific binding. For both radioligands, the slides
were rinsed twice (2 x 1-min) in ice cold buffer, dipped in ice cold dH2O, and
dried under cool air. [35S]TBPS treated slides were apposed to XAR-2 X-OMAT
film (Kodak) and [3H]flunitrazepam treated slides apposed to Ultrafilm [3H]-
sensitive film (Reichert-Jung) for 3 wks. Sections were then stained for
histology. The autoradiograms were analyzed by computer-assisted densitometry
(DUMAS). Specific binding was determined by subtracting nonspecific binding
from the total binding sections. Due to the difficulties in making comparisons
between GABA\textsubscript{A} receptor ligands with differing optimal binding conditions, the
results are presented in a semi-quantitative form reflecting the relative distribution
of binding sites for each of the ligands. The mean optical density values for
specific binding in each region were grouped into one of 4 ranks: + Detectable;
++ Low density; +++ Moderate density; ++++ High density.

**Materials.** [3H]Flunitrazepam (103.1 Ci/mmol) and [35S]TBPS (87.5 -
111.5 Ci/mmol) and 36Cl\textsuperscript{-} (14.97 mCi/g) were obtained from New England
Nuclear (Boston, MA). Steroids were obtained from Steraloids (Wilton, NH) or
Sigma (St. Louis, MO). Other chemicals were obtained from Sigma or Research
Biochemicals Incorporated (Natick, MA).
Results

The convulsant $^{35}$S]TBPS, bound to a single population of recognition sites in Taricha neuronal membranes (Fig. IV.1A and B) with a $K_d = 17.1 \pm 1.3$ nM and $B_{max} = 901 \pm 32$ fmol/mg protein (Hill coefficient = 0.998). The binding of $^{35}$S]TBPS was inhibited in a concentration-dependent manner by GABA ($IC_{50} = 1.41 \pm 0.12 \mu M$; Fig. IV.2). In most experiments, bicuculline methiodide enhanced $^{35}$S]TBPS binding by approximately 10%, relative to control tubes (data not shown), presumably due to residual endogenous GABA despite the extensive washing in tissue preparation.

The binding of 2.5 nM $^{35}$S]TBPS was potently inhibited by the steroids 3α-DHP and 5α-THDOC in titration experiments (Fig. IV.3; Table IV.1). When GABA was excluded from the incubation buffer, the potency of 3α-DHP to inhibit $^{35}$S]TBPS binding was decreased more than 3-fold ($IC_{50} = 47.2 \pm 9.8$ nM). In contrast, 100 μM CORT inhibited only a fraction of $^{35}$S]TBPS specific binding, in the presence or absence of GABA. CORT was devoid of inhibitory activity in the presence of bicuculline.

The equilibrium saturation binding of $^3$H]flunitrazepam to Taricha neuronal membranes (Fig. IV.4) was described by a one site model with $K_d = 4.03 \pm 0.55$ nM and $B_{max} = 2830 \pm 83$ fmol/mg protein (Hill coefficient = 0.965). The binding of $^3$H]flunitrazepam was enhanced by GABA ($EC_{50} = 1.95 \pm 0.15 \mu M$) and by the barbiturate sodium pentobarbital ($EC_{50} = 228 \pm 35 \mu M$;
The steroids 3α-DHP and 5α-THDOC enhanced [3H]flunitrazepam binding (Fig. IV.6) with EC₅₀ values = 116 ± 50 nM and 225 ± 96 nM, respectively. In contrast, CORT had little or no effect on basal (Fig. IV.6) or barbiturate-stimulated [3H]flunitrazepam binding (data not shown). The addition of 3 µM GABA did not enhance the potencies of either 3α-DHP or CORT as modulators of [3H]flunitrazepam binding.

GABA stimulated 36Cl⁻ flux into Taricha brain synaptoneurosomes (Fig. IV.7), with an estimated EC₅₀ = 5.8 ± 2.5 μM, similar to the EC₅₀ for GABA-mediated inhibition of [³⁵S]TBPS binding. The basal level of 36Cl⁻ uptake was 62.9 nmol/mg protein/12 seconds. Of this, 10.9 nmol/mg protein/12 seconds was blocked by the addition of 200 µM picrotoxin or bicuculline methiodide, GABAₐ receptor antagonists. The addition of CORT (1 nM - 1 µM) produced no discernable effect on either GABA-stimulated or GABA-independent 36Cl⁻ influx (Fig. IV.8). Because of the large quantity of tissue required for 36Cl⁻ flux studies, dose response curves for other steroids were not performed.

To determine if neuroanatomical differences in distribution might underlie the differences in the densities of GABAₐ receptors defined with [³⁵S]TBPS versus [³H]flunitrazepam in membrane preparations, and to detect potential differences in the regional sensitivity to steroids, we performed receptor autoradiography (Fig. IV.9). [³⁵S]TBPS and [³H]flunitrazepam binding sites were found in all brain regions, but [³H]flunitrazepam binding sites were more...
uniformly distributed than the $[^{35}S]$TBPS binding sites (Table IV.2). Convulsant sites were most enriched in the midbrain and dorsal thalamus, while moderate to high densities of benzodiazepine binding sites were found throughout the telencephalon, midbrain and hindbrain. The greatest discrepancy between TBPS and benzodiazepine binding sites was found in the telencephalon, where $[^{35}S]$TBPS binding sites were sparse, but $[^{3}H]$flunitrazepam sites were abundant.

Receptor autoradiography indicated that $[^{35}S]$TBPS binding was inhibited in every brain region by 10 μM 3α-DHP. There were no marked regional differences in the sensitivity of $[^{35}S]$TBPS binding to steroid; 3α-DHP inhibited between 80 - 92% of $[^{35}S]$TBPS specific binding in each of the 13 brain regions examined. There were no brain regions with any apparent sensitivity of $[^{35}S]$TBPS binding to modulation by CORT. However, in contrast to 3α-DHP, 100 μM CORT inhibited less than 10% of $[^{35}S]$TBPS specific binding in every brain region examined.
GABA_\alpha receptors in Taricha brains are allosterically modulated by ligands, including steroids, in a manner very similar to that described for the rat brain. The convulsant \[^{35}S\]TBPS bound with an affinity (K_d = 17 nM) similar to the K_d reported for dialyzed mammalian brain membranes (Squires et al., 1983). As in the rat brain, TBPS binding was inhibited by low micromolar concentrations of GABA. The benzodiazepine flunitrazepam also bound to Taricha brain membranes with an affinity (K_d = 4 nM) similar to the rat brain (Stephenson, 1988; Knapp et al., 1990), and as in mammals, the binding of \[^{3}H\]flunitrazepam was enhanced by both GABA and barbiturates. Significantly, both \[^{35}S\]TBPS and \[^{3}H\]flunitrazepam binding were potently modulated by 3\alpha-hydroxylated 5\alpha-reduced steroids, as in the rat brain (Majewska et al., 1986) and the potency of steroids to inhibit \[^{35}S\]TBPS binding was enhanced by GABA (Gee et al., 1987). The rank order and absolute potencies of a series of steroids to modulate \[^{35}S\]TBPS binding were in similar in Taricha and rat brains (Table IV.1). These studies suggest that the recognition sites for steroids on GABA_\alpha receptors have been highly conserved through vertebrate evolution and thus portend physiologically important functions.

This conclusion is supported by the findings that 3\alpha-hydroxylated reduced pregnane steroids produce anesthesia in amphibians (Mok and Krieger, 1990), and indeed, in all vertebrate groups (Oliver et al., 1991). This steroid-induced
anesthesia appears to be mediated by GABAₐ receptors, inasmuch as picrotoxin antagonizes the anesthetic effect in fish (Oliver et al., 1991). Interestingly, non-vertebrate chordates and many invertebrate phyla possess GABAₐ-like receptors that are insensitive to pregnanolone (3α-OH-5β-pregnane-20-one)-induced anesthesia, suggesting that the steroid recognition site evolved early in chordate evolution (Oliver et al., 1991). The benzodiazepine binding site on the GABAₐ receptor appears to have evolved later phylogenetically than the steroid site (Nielsen et al., 1978; Hebebrand et al., 1987).

The physiological and behavioral relevance of steroid modulation of GABAₐ receptors in Taricha is as yet unknown. However, significant concentrations of 3α-DHP accumulate in amphibian brains following injection of the parent compound progesterone (Mok and Krieger, 1990), indicating that the required converting enzymes are present in amphibians as well as mammals. Preliminary studies suggest that, as in mammals, the 3α-hydroxylated ring A-reduced steroids may modulate some behavioral responses to stress in Taricha (Orchinik and Moore, 1989). But in contrast to corticosterone, which suppresses male behavior during stress, injection of 5α-THDOC can produce a short-latency facilitation of reproductive behavior in mildly stressed males (Orchinik and Moore, 1989).

A second major objective was to determine whether there is a high-affinity interaction of corticosterone with GABAₐ receptors. Most studies have found that the 3α-hydroxylated, ring A-reduced structure is required for high-affinity
modulation of ligand binding or GABA-evoked Cl⁻ conductance (Harrison et al., 1987; Gee et al., 1988; Morrow et al., 1990; Purdy et al., 1990; Im et al., 1990; Lan et al., 1991). However, there have been reports showing a modest corticosterone-induced enhancement of [³⁵S]TBPS binding in the absence of GABA (Sutanto et al., 1989), as well as biphasic effects of corticosterone on [³⁵S]TBPS binding (Majewska, 1987) and GABA-induced contractions of guinea pig ileum (Ong et al., 1987). Also, species differences might exist in the structural requirements for steroid interaction with the GABAₐ receptor.

Our results indicated that corticosterone had very limited efficacy as a modulator of GABAₐ receptor function, as in mammalian brains. In the concentration range of 0.1 nM - 100 µM, with or without GABA, we detected no significant effects of corticosterone on [³⁵S]TBPS binding, [³H]flunitrazepam binding, or GABA-stimulated Cl⁻ flux in brain membranes. Corticosterone did not inhibit [³⁵S]TBPS binding to a regionally specific subpopulation of GABAₐ receptors in autoradiograms, including the hindbrain where populations of corticosterone-sensitive neurons are found (Rose et al., 1991).

Receptor autoradiographic studies also found that the distributions of [³⁵S]TBPS or [³H]flunitrazepam binding sites did not correspond to the distribution of membrane-bound [³H]corticosterone receptors (Orchinik et al., 1991a). In contrast to the GABAₐ receptor ligands, [³H]corticosterone binding sites were found in high density in the neuropil surrounding the preoptic area, hypothalamus, and amygdala.
Further, the behavioral responses to injection of GABA<sub>α</sub>-active steroids (intraperitoneal and intracerebroventricular) differed from the responses to corticosterone. In addition, none of the steroidal and nonsteroidal GABA<sub>α</sub> receptor ligands tested were potent competitors for [³H]CORT binding sites (Orchinik et al., 1991a; Orchinik et al., 1991b). Given the potency of corticosterone in behavioral and electrophysiological studies, the high-affinity binding of [³H]CORT to neuronal membranes (K<sub>d</sub> ≤ 0.5 nM; Orchinik et al., 1991a; Orchinik et al., 1992), and the limited potency of corticosterone to modulate any of the parameters of GABA<sub>α</sub> receptor function we assayed, we conclude that the rapid behavioral responses to corticosterone are not due to direct modulation of GABA<sub>α</sub> receptors. This conclusion is further supported by our recent findings that [³H]corticosterone binding to neuronal membranes is sensitive to modulation by guanyl nucleotides—strong evidence that the corticosteroid receptor on neuronal membranes is coupled to G proteins (Orchinik et al., 1992).

Numerous discrepancies in correspondence between the distribution of GABA<sub>α</sub> receptors labeled with the convulsant TBPS, benzodiazepines and ligands specific for the GABA binding site have been reported (Unnerstall et al., 1981; McCabe and Wamsley, 1986; Wamsley et al., 1986; Schmitz et al., 1988; Olsen et al., 1990; Burt and Kamatchi, 1991). In well-washed Taricha brain membranes, the density of [³H]flunitrazepam binding sites was 3-fold higher than [³⁵S]TBPS binding sites (B<sub>max</sub> = 2830 fmol/mg protein vs. 901 fmol/mg protein,
respectively). In autoradiograms, [³H]flunitrazepam binding sites were rather uniformly distributed in *Taricha* brains, whereas [³⁵S]TBPS sites had a restricted distribution, being highly concentrated in the midbrain and barely detectable in much of the telencephalon.

This lack of correspondence could result, in part, from regional variation in endogenous GABA levels, inasmuch as GABA enhances benzodiazepine binding but inhibits TBPS binding. However, there is a widespread distribution of GABAergic neurons and fibers in the brain of a closely related urodele amphibian, *Triturus cristatus* (Franzoni and Morino, 1989). The distribution of GABA immunoreactivity largely parallels the distribution of [³H]flunitrazepam binding sites in *Taricha* brains: intensive GABA immunoreactivity in the olfactory bulb, dorsal and medial pallium, striatum, dorsal thalamus, tectum, midbrain tegmentum, cerebellum and the lateral acoustic area—all areas with moderate to heavy [³H]flunitrazepam binding. Given the widespread distribution of GABA in the urodele brain, it is difficult to attribute the low density of [³⁵S]TBPS binding sites in the telencephalon relative to the midbrain to inhibition by GABA alone. In another primitive vertebrate, the eel, [³H]flunitrazepam and [³⁵S]TBPS binding sites are also differentially distributed, but [³⁵S]TBPS binding sites are more abundant and more evenly distributed, while benzodiazepine receptors are enriched in the midbrain (Corda et al., 1989).

A more likely explanation is that the differences in distribution reflect GABA receptor heterogeneity, a phenomenon that has been documented in
mammals (reviews: Sieghart, 1989; Seeburg et al., 1990; Burt and Kamatchi, 1991; Olsen and Tobin, 1990) and non-mammalian vertebrates (Schmitz et al., 1988). \[^{35}S\]TBPS and \[^{3}H\]flunitrazepam probably label different subpopulations of GABA\(_A\) receptors, although amphibians lack the full complement of GABA\(_A\) receptor isoforms found in mammals. A single receptor subunit is photoaffinity labeled with \[^{3}H\]flunitrazepam in many fish and amphibians, whereas 2 bands are labeled in reptiles and birds, and multiple receptor subunits are labeled in some mammalian species (Hebebrand et al., 1987; Deng et al., 1991). This increasing diversity of flunitrazepam-binding molecules probably reflects the appearance of multiple forms of the \(\alpha\) subunit (Burt and Kamatchi, 1991).

If steroid sensitivity resides on the \(\alpha\)-subunit of GABA\(_A\) receptors (Lan et al., 1991), and amphibians possess few \(\alpha\)-subunit subtypes, one might expect few regional differences in the steroid sensitivity of GABA\(_A\) receptors in *Taricha* brains. The relatively uniform inhibition \[^{35}S\]TBPS binding by 3\(\alpha\)-OH-DHP in autoradiograms suggests that all the TBPS binding sites are functionally coupled to steroid recognition sites in *Taricha* brains. Similarly, 3\(\alpha\)-DHP inhibited \[^{35}S\]TBPS binding in autoradiographic sections of rat brains, although modest differences in potency existed between brain regions (Gee et al., 1988). However, a more recent study found striking differences in the potency of 3\(\alpha\)-hydroxylated ring A-reduced steroids (especially 5\(\beta\)-THDOC) to inhibit \[^{35}S\]TBPS binding in the frontal cortex versus the spinal cord of rats, as well as a different rank order potency of steroids in the two regions (Gee and Lan, 1991). This
favors the existence of a heterogeneous population of steroid recognition sites
coupled to GABA<sub>A</sub> receptors. At present, it is not clear whether this
heterogeneity reflects a multiplicity of steroid recognition sites on a single
GABA<sub>A</sub> receptor, multiple affinity states of the receptor, or different populations
of GABA<sub>A</sub> receptors having differing sensitivities to steroids (Morrow et al.,
1990; Lan et al., 1990; Puia et al., 1990; Shingai et al., 1991; Vincini et al.,
1991; Woodward et al., 1992). There were no striking differences in the efficacy
of either 3α-DHP or corticosterone to inhibit [<sup>35</sup>S]TBPS binding from
telencephalon to the caudal hindbrain to suggest the presence of multiple steroid
recognition sites on Taricha GABA<sub>A</sub> receptors. More detailed studies are
required to determine whether a heterogenous population of steroid recognition
sites resides on GABA<sub>A</sub> receptors in Taricha brains.

In conclusion, the allosteric modulation of GABA<sub>A</sub> receptor function by
steroids appears to be an ancient characteristic of vertebrate brains. Our studies
demonstrated that steroid modulation of GABA<sub>A</sub> receptors in Taricha brains is
remarkably similar to that in mammalian brains. This suggests that the
recognition sites for steroids on GABA<sub>A</sub> receptors have been highly conserved
during vertebrate evolution, making it likely that this mechanism subserves
important physiological roles. However, the pharmacological profiles for the
GABA<sub>A</sub> receptor and the high-affinity corticosteroid receptor in neuronal
membranes are inconsistent, suggesting that neuroactive steroids utilize multiple
non-genomic mechanisms to regulate brain function.
Thus, it appears that steroids can modulate neuronal activity through intracellular receptors that regulate long-term transcriptional events, G protein-coupled receptors that may regulate second messenger pathways, and direct modulation of GABA_A receptor function.
Table IV.1. Potencies of steroids as inhibitors of $[^{35}\text{S}]$TBPS binding. Brain membranes were incubated with 2.5 nM $[^{35}\text{S}]$TBPS, 1 µM GABA, and increasing concentrations of steroids. Steroids were dissolved in a concentrated stock solution of ethanol or ethanol:DMSO (2:1) and then diluted in buffer. The highest concentration of solvent used (maximum 0.3%) was added to control tubes. IC$_{50}$ values are reported as means ± SE from pooled data representing 3 curves. Inhibition is the maximal percent inhibition of basal $[^{35}\text{S}]$TBPS specific binding. Highest concentration of steroid used was 100 µM steroid. Also listed are IC$_{50}$ values reported for steroid inhibition of 2 nM $[^{35}\text{S}]$TBPS binding to rat cerebral cortex. Pregnenolone is 3β-OH-5-pregnen-20-one.
<table>
<thead>
<tr>
<th>Steroid</th>
<th>Current study of Taricha <em>IC₅₀</em> (nM)</th>
<th>Published values for rat <em>IC₅₀</em> (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition</td>
<td></td>
</tr>
<tr>
<td>3α-DHP</td>
<td>14.3 ± 8.9</td>
<td>100</td>
</tr>
<tr>
<td>5α-THDOC</td>
<td>86.9 ± 26</td>
<td>100</td>
</tr>
<tr>
<td>Pregnenolone SO₄</td>
<td>18,200 ± 6,500</td>
<td>74</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>37,600 ± 12,900</td>
<td>34</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>&gt;50,000</td>
<td>33</td>
</tr>
</tbody>
</table>

⁺ Gee et al. (1987)  
= Gee et al. (1989) Percent inhibition estimated from curve.

Table IV.1
Table IV.2. Distribution of $[^{35}S]$TBPS and $[^{3}H]$flunitrazepam specific binding sites. Multiple (4-6) densitometric determinations of relative optical density were made for total and non-specific binding in 13 brain regions for each brain. Non-specific $[^{3}H]$flunitrazepam binding was relatively uniform, but non-specific $[^{35}S]$TBPS binding varied across brain regions. After subtracting non-specific binding, the mean values for each region were grouped into one of 4 ranks:

+ Detectable; ++ Low density; +++ Moderate density;
+++ High density. The rankings were consistent if the brains regions were ranked for individuals or pooled for all animals (n=4 for $[^{35}S]$TBPS; n=2 for $[^{3}H]$flunitrazepam).
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>TBPS</th>
<th>Flunitrazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Telencephalon</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Striatum</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Medial Pallium</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Dorsal/lateral Pallium</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Basal Forebrain complex</td>
<td>++(^a)</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Diencephalon</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoptic Area</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Dorsal thalamus</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Ventral thalamus</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Midbrain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tectum</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Tegmentum</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Hindbrain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Tegmentum</td>
<td>+++</td>
<td>++(^b)</td>
</tr>
</tbody>
</table>

\(^a\) +++ in amygdala pars lateralis
\(^b\) ++++ in lateral acoustic area

Table IV.2
Fig. IV.1. Equilibrium saturation binding of $[^{35}\text{S}]$TBPS to neuronal membranes.

(A) Saturation isotherm. Membranes were incubated with one of 8 concentrations of $[^{35}\text{S}]$TBPS for 2 hrs at 15°C. Nonspecific binding was defined as that occurring in the presence of 100 μM picrotoxin. Specific binding was described by $K_d = 17.1 \pm 1.3$ nM and $B_{max} = 901 \pm 32$ fmol/mg protein (Hill coefficient $= 0.998$). The data were best fit by a one-site model using Lundon I software and LIGAND software (Elsevier-Biosoft, Cambridge, UK). (B) Scatchard-Rosenthal replot of saturation data.
Fig. IV.1
Fig. IV.2. Inhibition of $[^{35}\text{S}]$TBPS binding by GABA. Membranes were incubated with 2.5 nM $[^{35}\text{S}]$TBPS and increasing concentrations of GABA. The IC$_{50}$ (concentration that inhibited 50% of specific $[^{35}\text{S}]$TBPS binding) was estimated as 1.41 ± 0.12 μM. The data shown are pooled results from 3 curves.
Fig. IV.2

[35S]TBPS Specific Binding (dpm)

log GABA Concentration (M)

Fig. IV.2
Fig. IV.3. Steroid modulation of [35S]TBPS binding. The addition of steroids inhibited the specific binding of the convulsant [35S]TBPS. Increasing concentrations of various steroids were added to assay tubes 15 minutes prior to addition of 2.5 nM [35S]-TBPS. Results are reported as specific binding, percent of [35S]-TBPS bound in the absence of steroid and presence of 1 µM GABA. Non-specific binding was determined in the presence of 100 µM picrotoxin. The data shown are results pooled from 3 three competition curves.
Fig. IV.3

[35S]–TBPS Specific Binding
(Percent Control)

log Steroid Concentration (Molar)

-10 -9 -8 -7 -6 -5 -4

3α-OH-DHP
5α-THDOC
Pregnenolone–SO₄
CORT

96
Fig. IV.4. Equilibrium saturation binding of [³H]flunitrazepam to Taricha neuronal membranes. (A) Saturation isotherm. Membranes were incubated with one of 8 concentrations of [³H]flunitrazepam for 2 hrs at 15°C. Non-specific binding was determined in the presence of 10 µM diazepam. Specific binding data were fit with a one site model yielding estimates of $K_d = 4.03 \pm 0.55$ nM and $B_{max} = 2830 \pm 83$ fmol/mg protein (Hill coefficient = 0.965 (Lundon I; LIGAND). (B) Scatchard-Rosenthal replot of saturation data.
Fig. IV.4

**A**


**B**

Bound/Free x 10$^{-3}$ vs. Bound (pmol/mg protein)
Fig. IV.5. Effect of GABA and pentobarbital on $[^3]H$flunitrazepam specific binding. Membranes were incubated with 2 nM $[^3]H$flunitrazepam plus increasing concentrations of GABA or pentobarbital. Both GABA and pentobarbital enhanced $[^3]H$flunitrazepam binding with EC$_{50}$ values of 1.95 ± 0.15 µM and 228 ± 35 µM, respectively.
Fig. IV.5

[3H]Flunitrazepam Specific Binding
(Percent Control)

log Concentration (Molar)

GABA
Pentobarbital
Fig. IV.6. Modulation of [³H]flunitrazepam binding by steroids. Tissue was incubated with 2 nM [³H]-flunitrazepam and increasing concentrations of steroid. Non-specific binding was determined in the presence of 10 μM diazepam. Results are reported as [³H]flunitrazepam specific binding, means and standard errors, fmol/mg protein from a representative experiment performed in triplicate.
Fig. IV.6

[3H]Flunitrazepam Specific Binding (Percent Control)

Log Steroid Concentration (Molar)

- 3α-DHP
- 5α-THDOC
- CORT

Fig. IV.6
Fig. IV.7. GABA-stimulated $^{36}\text{Cl}^-$ uptake into *Taricha* synaptoneurosomes. $^{36}\text{Cl}^-$ uptake was initiated by the simultaneous addition of 0.1 $\mu$Ci $^{36}\text{Cl}^-$ and GABA. Bicuculline-sensitive $^{36}\text{Cl}^-$ uptake was defined as the difference between $^{36}\text{Cl}^-$ uptake in the presence of GABA and that in the presence of 200 $\mu$M bicuculline methiodide. Data are pooled results, reported as nmol $^{36}\text{Cl}^/$mg protein/12 seconds, means ± SE, for a representative experiment performed in triplicate. Basal $^{36}\text{Cl}^-$ uptake in the presence of bicuculline was 51.9 nmol/mg protein/12 seconds.
Fig. IV.7

Bicuculline-sensitive Cl⁻ Uptake (nmol/mg protein/12 sec)

log Concentration GABA Added (M)

Fig. IV.7
Fig. IV.8. Effect of CORT on $^{36}$Cl$^-$ uptake. Synaptoneurosomes were preincubated for 10 minutes with the concentration of CORT indicated plus. $^{36}$Cl$^-$ was added to tubes concurrently with either 3 $\mu$M GABA or 100 $\mu$M picrotoxin. Uptake was terminated after 12 seconds. The data are pooled results, presented as the percentage of $^{36}$Cl$^-$ uptake in the presence of picrotoxin and absence of steroid; means ± SE, for one experiment performed in triplicate.
Fig. IV.9. Representative receptor autoradiograms showing [35S]TBPS binding sites in *Taricha* midbrain. Alternate transverse sections were incubated with 15 nM [35S]TBPS +/- 200 μM picrotoxin. Total binding is shown in (A), nonspecific binding in (B), and pseudocolor-enhanced specific binding in (C). Binding sites were enriched in the tectum and midbrain tegmentum, but relatively sparse in the caudal hypothalamus.
CHAPTER V: CONCLUSIONS

Summary

The major findings in this thesis can be briefly summarized. A genuine, high-affinity receptor for corticosteroids, with a unique pharmacology, is present in neuronal membranes. These receptors are not allosterically linked to GABA_A receptors, but appear to utilize G proteins for signal transduction. A variety of studies indicate that these receptors may play a key role in mediating rapid behavioral responses to stress. Other steroid molecules, also stress-related, may alter brain function through a second, non-genomic mechanism of action--the modulation of GABA_A receptors.

A number of standard pharmacological criteria are applied in establishing the existence of genuine, physiologically relevant neuroreceptors. These include the demonstration of saturability and appropriate binding affinity. The kinetics of receptor occupancy should be consistent with physiological responses and receptor binding should be reversible. The kinetically derived dissociation constant should agree with the $K_d$ derived from equilibrium studies. Binding to the recognition site should be disrupted under conditions that denature proteins; heating the tissue and treatment with proteases should eliminate specific binding. The recognition site should exhibit pharmacological specificity. The distribution of receptors within the brain, and within the neuron, should make sense. Ultimately, one
should demonstrate a physiological or behavioral role for the receptor.

Chapter II addressed these criteria. The biochemical and neuroanatomical criteria were largely satisfied. Chapter II also provided strong correlational evidence supporting a behavioral role for this receptor. Recent electrophysiological data (Rose et al., 1991) further support the conclusion that [³H]corticosterone binding sites in *Taricha* brains represent functional, behaviorally relevant, steroid receptors in neuronal membranes.

The neuronal responses initiated by a vast array of membrane-bound receptors for neurochemical signals are mediated by one of three fundamental transduction mechanisms: a receptor may be an integral part of a ligand-gated ion channel, a transmembrane-regulated enzyme, or a transmembrane protein that couples to G protein. Based on our current understanding of the cycle of receptor-G protein-effector interaction, one can make predictions concerning the modulation of [³H]corticosterone binding if the receptor is a G protein-coupled receptor. Implicit in these predictions is the assumption that corticosterone acts as an agonist at the receptor.

Chapter III was a test of these predictions. If a receptor interacts with G proteins, Mg²⁺ should enhance agonist binding by promoting formation of the high-affinity ternary complex. Guanyl nucleotides, especially the nonhydrolyzable ones, should negatively modulate agonist binding by promoting disruption of the high-affinity ternary complex. The decreased affinity of the
receptor for agonist binding following exposure to nonhydrolyzable guanyl nucleotides should result from an increase in the rate of agonist dissociation from the receptor. Therefore, the shift in affinity state of the receptor should be reflected in both equilibrium saturation binding and kinetic studies. In general, one expects guanyl nucleotide effects on agonist binding to be sensitive to Mg$^{2+}$ concentrations. The results in Chapter III were entirely consistent with these predictions. We were unable to quantify the low affinity state of the receptor in saturation isotherms because of a technical problem--we lacked a radiolabeled receptor antagonist. Therefore, Chapter III strongly suggests that corticosterone has agonist properties at a receptor in neuronal membranes that utilizes G proteins for signal transduction.

In Chapter IV, we examined that possibility that these corticosteroid receptors, or a subpopulation of them, is an integral part of a ligand-gated ion channel, the GABA$_A$ receptor. In the mammalian brain there appear to be stringent structural requirements for steroid interaction with recognition sites on the GABA$_A$ receptor/ chloride ionophore complex. The 3α-hydroxylated ring A-reduced pregnane steroids are potent modulators of GABA$_A$ receptor function, while steroids which lack this structure, such as corticosterone, lack efficacy. Therefore, the objectives in Chapter IV were two-fold. First, to determine whether GABA$_A$ receptors in a primitive vertebrate brain are sensitive to modulation by 3α-hydroxylated ring A-reduced pregnane steroids in a manner
similar to mammalian brains. Second, to test the specific hypothesis that corticosterone is a potent modulator of GABA$_A$ receptors.

One would expect steroids to allosterically alter ligand binding in other species in a similar manner, if steroid recognition sites on GABA$_A$ receptors have been conserved. In membrane preparations, the binding of ligands to the convulsant site on or near the chloride channel (such as $[^{35}\text{S}]$TBPS) should be sensitive to negative modulation by reduced pregnane steroids. The sensitivity of $[^{35}\text{S}]$TBPS binding to inhibition by steroids should be enhanced by GABA. The potencies of steroids to non-competitively inhibit $[^{35}\text{S}]$TBPS binding should correlate with their potencies to enhance GABA-stimulated $^{36}\text{Cl}^-$ uptake. GABA$_A$ receptor-active steroids should also enhance the binding of central benzodiazepines, such as $[^{3}\text{H}]$flunitrazepam. The studies in Chapter IV indicate that GABA$_A$ receptors in Taricha brains are allosterically modulated by steroids in a manner very similar to the rat brain. The $3\alpha$-hydroxylated ring A-reduced pregnane steroids were potent modulators of $[^{35}\text{S}]$TBPS and $[^{3}\text{H}]$flunitrazepam binding, but corticosterone was devoid of activity at the GABA$_A$ receptor. Therefore, the steroid recognition site on GABA$_A$ receptors appears to have been highly conserved during vertebrate evolution, suggesting two things: corticosteroid receptors are not associated with GABA$_A$ receptors and modulation of GABA$_A$ receptors by pregnane steroids is likely to be important in the regulation of brain function in many species.
These studies are the first characterization of a high-affinity recognition site on brain membranes for any naturally-occurring steroid, and the first to satisfy the minimal pharmacological criteria expected for a genuine, membrane-bound steroid receptor. These studies provide the first direct evidence that steroid receptors may be coupled to G proteins in neuronal membranes. Also, these data are the first evidence that steroids can modulate ligand binding to GABA_A receptors in a non-mammalian species. Taken together, these studies make a strong case that non-genomically-mediated steroid actions in the brain subserve important physiological and behavioral functions.

**Speculation**

We have identified a corticosteroid receptor in *Xenopus* brain membranes with very similar binding characteristics. Several implications follow from this finding. Since anuran and urodele amphibians diverged evolutionarily approximately 350 million years ago, steroid regulation of neuronal function through membrane-bound G protein-coupled receptors appears to be an ancient characteristic of vertebrate brains. It also suggests that this mechanism was found among vertebrates on the main line towards mammalian evolution.

It is likely that similar receptor and transduction mechanisms mediate a number of rapid changes in neuronal activity induced by corticosteroids, as well as gonadal steroids, in all vertebrate brains. For example, the excitability of
certain hypothalamic neurons in the rat brain is rapidly altered by corticosteroids (Hua and Chen, 1989; Chen et al., 1991). These steroid-induced changes in membrane potential exhibit a pharmacology and response latency that is consistent with the corticosteroid receptor and electrophysiological responses characterized in Taricha brains. Also, the K⁺ conductance regulated by estradiol in rat hypothalamic neurons is dependent upon cAMP (Nabekura et al., 1986; Minami et al., 1990), suggesting that these effects of estradiol are mediated by a G protein-coupled receptor. There is also a cell-surface receptor for progesterone on Xenopus oocytes that regulates intracellular cAMP levels, although the mechanism is unclear (reviewed in Smith, 1989). In fish, cortisol rapidly regulates prolactin release, as well as intracellular Ca²⁺ and cAMP levels (Borski et al., 1991). I think it is likely that a conserved family of G protein-coupled steroid receptors exists in vertebrates.

Since the GABA₄ receptor belongs to a family of ligand-gated ion channels that share sequence homology and structural motifs, it is possible that steroids also modulate excitatory amino acid receptors, nicotinic cholinergic receptors, or glycine receptors. Recent studies suggest this may be the case (Smith et al., 1987; Wu et al., 1990; Wu et al., 1991).

The unique pharmacology of the membrane-bound corticosteroid receptors allow them to be distinguished from intracellular corticosteroid receptors. This is a tool which should facilitate further studies into the regulation of neuroendocrine
activity and behavior by neuroactive corticosteroids. Further, the specificity of the recognition site could facilitate development of therapeutic drugs with the potential to treat conditions ranging from emotional disorders, particularly clinical depression, to seizure susceptibility.

Why then haven’t high-affinity steroid receptors been characterized in the mammalian brain? One possibility, knowing that steroid receptors may be coupled to G proteins, is that Towle and Sze (1983) described genuine receptors, but their assay conditions isolated the recognition sites in a very low-affinity state. Another problem relates to the high non-specific binding of lipophilic steroids to neuronal membranes. Our studies were successful, in part, because we achieved 80% specific binding using [3H]corticosterone and Taricha brain membranes. However, we have had little success working with other steroids, including tritiated high-affinity GABA_α receptor-active steroids, because of problems with nonspecific binding. The differences between the ligands may be related to the relative tendencies of the steroids to partition into the lipid bilayer. 3α-DHP and 5α-THDOC are more saturated, being reduced in the 5 position, and therefore more lipophilic than corticosterone. In addition, corticosterone, with two hydroxyls, is more polar than 3α-DHP. Characterization of the high-affinity G protein-coupled receptors for the lipophilic cannabinoids was dependent upon the development of suitable ligands. An alternative to radioligand binding studies for identification of membrane-bound steroid receptors in mammalian
brains may be receptor cloning. This task will be less formidable if the receptors belong to the superfamily of G protein-coupled receptors, and when the effector mechanisms utilized by the receptor are identified, enabling expression cloning.

There is a unifying concept amidst the growing diversity of mechanisms utilized by steroids. Steroids may exemplify the concept that the complexity of signalling in the brain is achieved through the use of relatively few neurochemicals, but a diversity of receptor subtypes (Schofield et al., 1990). It appears to be the rule, rather than the exception, that neurotransmitters utilize multiple receptor subtypes. Acetylcholine, for example, binds to a ligand-gated ion channel as well as multiple subtypes of G protein-coupled receptors. Dopamine binds to at least five G protein-coupled receptor subtypes, but in addition, may activate receptors that are members of the intracellular steroid receptor family (Power et al., 1991a, 1991b). This provides a sense of symmetry--neurotransmitters may utilize classical steroid receptor mechanisms, while steroids may utilize classical neurotransmitter receptor mechanisms to regulate brain function.

Steroids appear to utilize a multitude of receptor and transduction mechanisms that are segregated temporally. Intracellular receptor-mediated changes in gene transcription may take hours or days, regulation of second messenger systems through G protein-coupled receptors occurs within minutes, steroid modulation of ionic conductance through ligand-gated or voltage-gated
(ffrench-Mullen and Spence, 1991) ion channels occurs within seconds. It is possible that the complex, often biphasic physiological responses to adrenal steroids during stress may be mediated by the activation of multiple receptor mechanisms with differing temporal characteristics. These responses might result from activation of different receptor subtypes in different regions, or from the activation of different receptors in the same cell. As an example, vitamin D metabolites bind to receptors belonging to the family of intracellular steroid receptors, but also rapidly activate a Ca$^{2+}$ conductance with a different rank order potency (Cancela et al., 1988; Farach-Carson et al., 1991). These genomic and non-genomic responses can be elicited in the same cell. Therefore, it is possible that a given steroid may elicit complex responses in a single neuron through the use of multiple receptor mechanisms.
BIBLIOGRAPHY


Birnbaumer, L. (1990b) Transduction of receptor signal into modulation of effector activity by G proteins: the first 20 years or so... FASEB J. 4: 3068-3078


Majewska, M.D. (1987a) Antagonist-type interaction of glucocorticoids with the GABA receptor-coupled chloride channel. Brain Res. 418: 377-382


Mok, W.M. and Krieger, N.R. (1990) Evidence that 5α-pregnan-3α-ol-20-one is the metabolite responsible for progesterone anesthesia. Brain Res. 533:42-45


