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Nuclear and Extranuclear Estrogen Receptor-Alpha in the Hippocampus

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ABSTRACT

Nuclear and extranuclear estrogen receptor-alpha in the hippocampus

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Estrogens are traditionally thought to act through a nuclear receptor-mediated mechanism to regulate gene transcription in target tissues. The hippocampus has been shown to be sensitive to estrogen; estrogen regulates GABAergic inhibition and spine density of dorsal CA1 pyramidal cells. However, very few neurons expressing nuclear estrogen receptors (ERs) have been detected in the hippocampus. I used double-label immunohistochemistry (Chapter 2) to quantify and map cells expressing nuclear ER-alpha (ERα) and/or cytoplasmic GABA in the hippocampus and determined that, in the dorsal CA1 region, nuclear ERα is expressed exclusively in inhibitory interneurons, but is limited to a small subset of those, while in the ventral hippocampus, ~50-60% of pyramidal cells express nuclear ERα as well. Rapid estrogen effects observed in the hippocampal CA1 suggest that estrogen may act via extranuclear ERs. In order to determine a potential role of extranuclear ERα in the dorsal hippocampus, I used serial electron microscopy (Chapter 3) to create 3-D reconstructions of inhibitory axons innervating somata of CA1 pyramidal cells and discovered that only a portion of boutons on a given axon contain ERα-IR and in those, approximately 10% of presynaptic vesicles contain extranuclear ERα. Vesicles containing ERα-IR are always clustered together within a bouton and are located significantly closer to the synapse 24 hours after estrogen treatment. In order to assess the scope of extranuclear ERα expression, I used double-label immunofluorescence and confocal microscopy (Chapter 4) to determine that approximately 32% of GABAergic varicosities in the CA1 pyramidal cell layer contain ERα-IR. Furthermore, of the two neurochemically distinct subpopulations of basket cells in CA1, only axonal varicosities arising from cholecystokinin basket cells contained ERα-IR. Addition analysis also revealed that approximately 50% of ERα-IR puncta colocalized with NPY-IR. The presence of ERα near inhibitory synapses and the sensitivity of ERα-IR vesicle clusters to estrogen suggest that estrogen acts directly

through non-nuclear ERα in a special subset of cholecystokinin boutons to regulate GABAergic inhibition of CA1 pyramidal cells. The results of these experiments increase our understanding of ERs in the hippocampus and lay the foundation for elucidating new mechanisms of estrogen action in the hippocampus.

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CHAPTER 1 - INTRODUCTION AND BACKGROUND

Estrogen is a steroid hormone well known for its role in female reproduction. At puberty, estrogen production is increased in the ovaries, and higher levels of estrogen begin circulating in the blood. This allows estrogen to reach target tissues and initiate the development of female post pubescent sexual characteristics. In adulthood, circulating levels of estrogen vary across the menstrual cycle, peaking at around day 14. This increase in estrogen stimulates the proliferation of the endometrium and is a critical step in preparing the uterus for the implantation of a fertilized egg. Estrogen levels continue to fluctuate in an adult female throughout her reproductive years.

Circulating estrogen reaches and affects many tissues throughout the body, not just those directly involved with reproduction. Tissues that are sensitive to estrogen include bone, smooth muscle, arterial endothelium, and brain. Estrogen is also an important hormone in males; aromatase converts circulating androgens into estrogen locally in the tissues in which it will be used. The responsiveness of a tissue to estrogen is determined by its expression of estrogen receptors.

While a large number of studies have examined the distribution and mechanisms of action through estrogen receptors in organ systems outside of the CNS and in select areas of the brain, such as the hypothalamus, relatively little was known about estrogen receptors (ERs) in non-reproductive brain regions, such as the hippocampus for many years. Estrogen affects both the structure and physiology of the hippocampus in animal models, and many of these effects are mediated through ERs. In order to elucidate the role of estrogen in modulating the function of the hippocampus and, consequently, the potential impact of estrogen on hippocampal-dependent brain processes, we must

understand the mechanisms of estrogen action in the hippocampus. A first step in understanding the mechanisms of estrogen action is knowledge of the distribution of ERs, sensitivity of ERs to estrogen, and cell types expressing ERs. Quantitative studies of ERs in the hippocampus were lacking when I began my studies, and this was an obstacle that had to be overcome before we could begin to understand how a variety of brain phenomena, such as epileptic seizure susceptibility, learning and memory, and mood disorders, might be affected by estrogen's actions in the hippocampus.

The experiments discussed in the following chapters provide data on ERs that constitute important building blocks in the foundation upon which to build our understanding of the mechanisms of estrogen action in the hippocampus. Interpretation of these data requires proper placement within the existing framework of what is known about ERs, estrogen effects, and hippocampal anatomy. The remaining sections of this chapter will review the studies relevant to these topics. Nuclear ERs, their mechanism of action, and early surveys of nuclear ERs in the brain will be discussed first, followed by a review of extranuclear ER studies in a variety of organ systems and experimental preparations. Effects of estrogen on the hippocampus, both structural and physiological, will then be discussed, as well as what was known about ER expression in the hippocampus when I began my experiments. Finally, selected hippocampal anatomy, such as that pertaining to interneurons and subcortical projections, will be reviewed. This background will facilitate the interpretation of the ER data in the following chapters and the formulation of a model for estrogen action through ERs in the hippocampus.

Nuclear estrogen receptors

In the late 1950s the presence of a receptor that mediated the action of the steroid hormone estrogen was first suggested (Jensen 1962). The prevailing theory of the time proposed that estrogen receptors present in target tissues were responsible for the specificity of estrogen to affect only certain organ systems and mediated the character of the tissue response. Nearly a decade later, Toft and Gorski (Toft and Gorski 1966) provided experimental evidence for the theoretical estrogen receptor (ER) when they isolated a protein that specifically bound 17β-estradiol and was present in the uterus, but not in blood serum or the small intestine. This fueled the search for the identification of estrogen receptors in other tissues that were known or believed to be sensitive to estrogen, including the brain. The brain was known to play a role in reproductive behavior, and so, was likely to be responsive to reproductive hormones. The initial search for estrogen receptors in the brain culminated in the publication of two studies that mapped the location of cells in the brain that accumulated ^{3}H-estradiol (Pfaff and Keiner 1973; Stumpf et al. 1975). This was the only method available at the time to identify cells expressing estrogen receptors. Cells that concentrated estradiol were observed throughout the brain, but areas important for reproduction, such as the hypothalamus, contained the highest density of cells that were intensely labeled by ^{3}H-estradiol. Brain regions in which few cells demonstrated ^{3}H-estradiol binding, such as the hippocampus, were interpreted to be relatively unresponsive to estrogen. It is important to note that the binding detection protocol was optimized for the identification of cells in the hypothalamus (Pfaff and Keiner 1973), an area of particular interest to the lab conducting

the study, and so, cells expressing lower levels of ERs in other brain regions may not have been detected. Additionally, this study identified cells by how much ^{3}H-estradiol binding was present over the nucleus relative to all extranuclear binding. At the time, it was thought that ERs translocated to the nucleus after being bound by ligand, so ^{3}H-estradiol bound ERs should be in the nucleus. The result was the reporting of only cells expressing ERs in the nucleus, the cellular compartment in which functional ERs, those capable of binding estradiol and initiating gene transcription, were expected to be located.

The next major advancement in characterizing ERs occurred when human (Green et al. 1986; Greene et al. 1986) and rat (Koike et al. 1987) estrogen receptors were cloned and sequenced. These studies determined the predicted molecular weight of ER at ~66 and ~67 kDa for the human and rat, respectively. Rat ER and human ER show a high degree of homology, 88%, with the ligand-binding domain being highly conserved at 96% homology, and the DNA-binding domain identical between the two ERs. Interestingly, Greene et al. (Greene et al. 1986) also detected a lower molecular weight protein that they suggested might be a degradation product of ER, which foreshadowed the discovery of a second ER.

A second ER, ERβ, was first identified in human ovaries and testis (Mosselman et al. 1996) and in rat ovaries and prostate (Kuiper et al. 1996). ERβ has a predicted molecular weight of ~54 kDa, and after its discovery, the higher molecular weight ER was renamed ERα to distinguish between the two. The DNA-binding domain of ERβ is highly conserved compared to ERα, 96% and 95% homology for the human and the rat, respectively (**Fig. 1**). However, the ligand-binding domain of ERβ is somewhat less

similar to that of ERα, 58% and 55% homology for the human and the rat, respectively. This difference in the ligand-binding domain does not affect the ability of either ER to bind 17β-estradiol, but is likely responsible for experimentally determined differences in the binding affinities of ERα and ERβ for various agonists and antagonists (Kuiper et al. 1996). Other portions of the receptor, such as the A/B domain, containing the activation-function-1 site, and the hinge region, which is important for the formation of ER dimers, are not conserved between the two forms of ER. Differences in these domains may account for the observation that the activation of transcription of a reporter construct by 17β-estradiol was higher for ERα compared to ERβ (Mosselman et al. 1996).

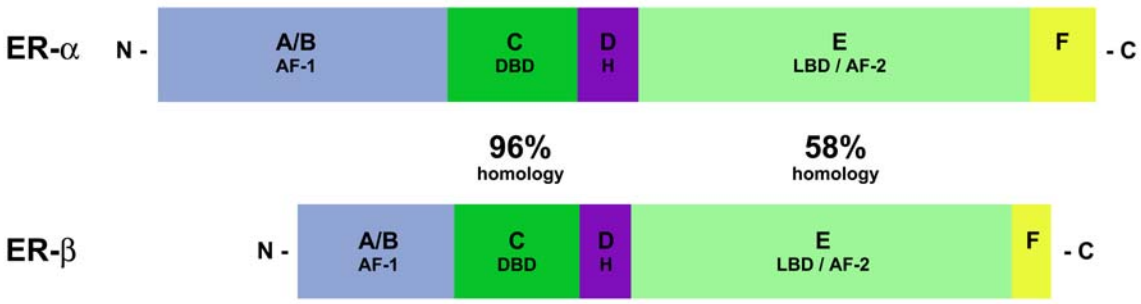

Figure 1. Estrogen receptor domains.

The functional domains of ERα (top) and ERβ (bottom) are shown. The DNA-binding domain (C) and the hinge region (D) are highly conserved between the two receptors. AF-1 - Activation function 1; DBD - DNA-binding domain; H - Hinge; LBD - Ligand-binding domain; AF-2 - Activation function 2.

The classical mechanism of action for ERs

The anatomical studies discussed in the previous section focus on the expression of nuclear ERs. This is not surprising given that the earliest identified function of ERs was the ability to act as a nuclear transcription factor (Jensen et al. 1968; Jensen and DeSombre 1972). The mechanisms of nuclear ER function are well known. Unbound monomeric ERs are present in the nucleus and held in an inactive state through association with heat shock protein 90 (HSP90). Estrogen binds to ERs causing the dissociation of HSP90 and dimerization with another ligand-bound ER. In cells that express both ERα and ERβ, both homodimers and heterodimers can form. After dimerization, three important areas are exposed and active: a DNA binding site contained within the ER DNA-binding domain, activation-function-1 (AF-1) site contained within the N-terminal region of the ER, and activation-function-2 (AF-2) site contained within the ligand-binding domain of the ER (**Fig. 1**). Transcription of genes that contain an estrogen response element (ERE) occurs when the ER dimer binds to DNA at the ERE and recruits transcriptional cofactors to its AF2 site. Differences in expression patterns of ERE genes between different cell types are due to variation in the cofactors present in each type of cell (Edwards 2000; Smith and O'Malley 2004).

While this mechanism seems relatively straightforward, many studies have revealed an increasingly complex interaction of ERs and genes that lack an ERE. ERs have been shown to regulate transcription of genes at other promoter sites, such as AP1 (Paech et al. 1997), through interactions between the AF-1 and AF-2 regions with Jun/Fos transcription factors that normally mediate transcription at these sites (Webb et

al. 1999). Interestingly, it is ligand-bound ER monomers that mediate this effect. Additionally, estrogen can modulate the expression of genes lacking EREs by phosphorylating various transcription factors, such as CREB and NFκB, through protein kinase pathways (Bjornstrom and Sjoberg 2005), an effect mediated by ERs (see discussion below). Also, recently, ER-mediated ligand-independent transcription has been demonstrated following the phosphorylation of ERs by growth factors (Bunone et al. 1996). Clearly, ERs and estrogen have the capacity to dramatically influence the expression of many proteins and thereby have profound effects on the function of cells expressing nuclear ERs.

Distribution of ERs in the brain

As hinted at by the types of tissue they were originally isolated from, ERα and ERβ show different patterns of distribution in many tissues including the brain. For example, in the ovary, high levels of mRNA have been detected for both ERα and ERβ. But, in the uterus, pituitary, and adrenal glands ERα mRNA is present with little or no ERβ mRNA, while in the lung and prostate ERβ mRNA predominates (Kuiper et al. 1996; Couse et al. 1997). Similar studies of ERα and ERβ mRNA in the central nervous system have found that both are present in many areas throughout the brain (Shughrue et al. 1997). However, there are regions that contain exclusively mRNA for only ERα or ERβ: cerebellar cortex (ERβ), dorsomedial tegmental area (ERα) and multiple areas in the hypothalamus, such as the paraventricular (ERβ), suprachiasmatic (ERβ), and ventromedial nuclei (ERα). The results of these mRNA experiments should be applied

cautiously, though, as the presence of mRNA does not necessarily guarantee functional ERα or ERβ protein. Immunohistochemistry has shown labeling for nuclear ERα but not ERβ in the lateral septum and labeling for nuclear ERβ but not ERα in the medial septum and the vertical limb of the diagonal band (Mitra et al. 2003). Shughrue et al. (Shughrue et al. 1997) reported mRNA for both ERα and ERβ in all 3 of these areas. Presumably, the differences in distribution and relative amounts of ERα and ERβ are important in determining the effect of estrogen in various brain areas, but currently, not enough is known about the functional role of each receptor type to understand how effects mediated through each might interact.

Rapid effects of estrogen

There are many effects of estrogen that occur within minutes of steroid exposure, much too rapidly to be mediated through a genomic mechanism involving gene transcription and protein synthesis. One of the earliest reports of a rapid effect of estrogen showed a dramatic decrease in the firing rate of some neurons in the preoptic area almost immediately following the direct application of estradiol *in vivo* (Kelly et al. 1976). Many studies demonstrating rapid effects of estrogen have followed in the hypothalamus (Minami et al. 1990; Qiu et al. 2003), cerebellum (Smith et al. 1988), pituitary cells (Bulayeva et al. 2005), and hippocampus (Teyler et al. 1980; Wong and Moss 1992; Gu et al. 1996; Rudick and Woolley 2003). These studies mostly report electrophysiological changes, and the specific effect of estrogen varies between cell type and brain region examined, just as the effects of nuclear ERs are tissue specific.

Estrogen has also been shown to rapidly activate intracellular signaling pathways. The mitogen-activated protein kinase (MAPK) pathway is activated by estradiol in as few as 15 minutes in many brain regions (Gu et al. 1996; Zhou et al. 1996; Singh et al. 1999; Wade and Dorsa 2003; Abraham et al. 2004) and non-neuronal cells (Ivanova et al. 2001). Additional pathways, such as phosphatidylinositol-3 kinase (PI3K), are also rapidly stimulated by estrogen (Simoncini et al. 2000). Intracellular signaling pathways, like MAPK and PI3K, are often activated by membrane receptors, such as metabotropic glutamate receptors (Berkeley and Levey 2003) and growth factor receptors (Zheng and Quirion 2004), suggesting that estrogen may also act through a membrane receptor in both neuronal and non-neuronal cell types.

Many of the studies reporting rapid estrogen effects also provide evidence for the existence of membrane ERs. Most reported rapid effects are stereospecific, meaning that treatment with 17β-estradiol but not 17α-estradiol is required to elicit the response, and many are blocked by ER antagonists, suggesting that many rapid effects are mediated through ERs (Minami et al. 1990; Wong and Moss 1992; Rudick and Woolley 2003; Abraham et al. 2004). Additionally, many rapid effects have been shown to occur following exposure to estradiol conjugated to BSA, a construct designed to prevent estradiol from passing through the cell membrane and limits its binding to membrane sites only (Beyer and Raab 1998; Kuroki et al. 2000; Christian and Morris 2002; Fatehi et al. 2005). Clearly, membrane ERs must exist if estradiol-BSA is excluded from the interior of the cell and elicits the same response as estradiol alone. However, extranuclear ER expression is not limited to cell membranes. Despite almost 30 years of studies

reporting rapid estrogen effects, the extent of extranuclear ER expression in the brain is only now being realized. The potential for estrogen to act through extranuclear ERs in the hippocampus has not been fully embraced, perhaps because ERs were first identified and are well understood as nuclear transcription factors, whereas, much less is known about how extranuclear ERs interact with cellular processes.

Extranuclear ERs

The concept of extranuclear ERs was introduced almost 30 years ago and roughly coincided with the first reports of rapid estrogen effects. Estrogen-binding sites, presumably ERs, were first reported on the surface of endometrial cells (Pietras and Szego 1977), and the observation of 17β-estradiol binding to the plasma membrane of neurons followed soon after (Towle and Sze 1983). Since then, extranuclear ER immunoreactivity (ER-IR), specifically ERα-IR, has been identified in dendrites and axons in hypothalamic neurons (Blaustein et al. 1992), neuronal processes in primary hippocampal cultures (Clarke et al. 2000), and dendrites, axons, and glia in CA1 and dentate gyrus (DG) of the dorsal hippocampus (Adams et al. 2001; Milner et al. 2001). Extranuclear ERβ-IR has also been reported in the brain and, specifically, in the hippocampus (Mitra et al. 2003; Kalita et al. 2005; Milner et al. 2005). Additionally, a third type of membrane-only ER, ER-X, has been proposed and shown to rapidly activate MAPK in cortical explants (Singh et al. 1999). It differs from ERα and ERβ in that it activates MAPK after binding either 17α- or 17β-estradiol, an effect not blocked by the ERα/β antagonist ICI 182,780 (Singh et al. 1999). However, ER-X has yet to be cloned,

so its relationship to ERα and ERβ is unknown. Furthermore, ER-X is expressed during development (Toran-Allerand 2004) and unlikely to play a role in the adult brain. Extranuclear ERs expressed in brain regions that, in the past, have been thought to be relatively insensitive to estrogen because they contain very few neurons expressing nuclear ERs may be responsible for rapid estrogen effects observed in those areas.

Mechanisms of extranuclear ERs

Through both nuclear and extranuclear ERs, estrogen has profound effects on cellular functions. The vast majority of what is known about the mechanism of ER action comes from *in vitro* studies using non-neuronal cells. This body of knowledge is important to consider when developing an understanding of the mechanisms of estrogen effects in the brain, especially regions that may not contain many neurons expressing nuclear ERs, such as the dorsal hippocampus.

In recent years, an increasing number of reports have shed light on the nature of extranuclear ERs and the cellular mechanisms with which they interact to bring about both rapid and genomic estrogen effects. One major question is whether or not ERs located at the membrane are derived from the same genes as nuclear ERs and just localized to a different cellular compartment. Transfection of cDNA for ERα or ERβ into Chinese hamster ovary (CHO) cells resulted in expression of functional membrane ERs that were able to activate the MAPK pathway (Razandi et al. 1999). Additionally, the many antibodies and antisera used to label extranuclear ERs were originally raised against various portions of the nuclear form of each ER, further suggesting that

extranuclear receptors are the same or a very similar protein as their nuclear counterparts. Perhaps the most compelling evidence, though, is the observation that no membrane ERs of either type are detected in endothelial cells from ERα/ERβ double-knockout mice, indicating that the endogenous membrane ERs normally expressed by these cells derive from the same genes as nuclear ERs (Razandi et al. 2004). Most studies in the brain (Blaustein et al. 1992; Adams et al. 2001; Milner et al. 2001) or in neuronal cell cultures (Clarke et al. 2000) have identified extranuclear ERα-IR, and one study that specifically labeled for both ERs detected only ERα and not ERβ at cell membranes (Beyer et al. 2003). Consistent with this finding, a different study determined that, in the hippocampus, ERβ localized to the cytosol fraction of homogenates, a fraction that does not include nuclei, mitochondria, vesicles, or membranes (Kalita et al. 2005). While Mitra et al. (Mitra et al. 2003) did report extranuclear ERβ-IR in the brain, it was present in only a few areas, and it is highly likely that the neurons assayed in Beyer et al. were from one of the many regions in which no extranuclear ERβ-IR was observed.

In the hippocampus, the overall distribution of extranuclear ERβ-IR has been reported to be similar to that of ERα-IR. A notable exception is in pyramidal cell somata and dendrites and in the CA3 str. lucidum, where ERβ-IR predominates (Milner et al. 2005). Milner et al. (2005) report the number of ERβ-IR ultrastructural profiles for every layer of each region of the hippocampus except for the str. pyramidale. This suggests that beyond ERβ-IR in pyramidal cell somata, extranuclear ERβ-IR was scarce in the cell body layer. The lack of extranuclear ERβ-IR in the pyramidal cell layer leads one to

conclude that extranuclear ERα may be the primary mediator of direct estrogen effects in this area.

Extranuclear and nuclear ERs most likely originate from the same genes, but the known sequences of nuclear ERs lack any known features of integral membrane proteins, such as a membrane targeting sequence or stretches of hydrophobic residues. How, then, do ERs get to and associate with the plasma membrane? Multiple *in vitro* studies have shown that membrane ERα is associated with caveolae (Kim et al. 1999; Chambliss et al. 2000; Deecher et al. 2003). Caveolae are specialized invaginations of the plasma membrane whose main structural protein is caveolin (Glenney and Soppet 1992). Multiple caveolin molecules bind together forming caveolin rafts (Sargiacomo et al. 1995) creating membrane-anchored scaffolding complexes for many intracellular signaling proteins, such as G-protein α-subunits, Ras, and Src tyrosine kinases (Li et al. 1995; Song et al. 1996). ERα has also been shown to associate with caveolin, and this interaction plays a key role in localizing ERα to the plasma membrane. A single point mutation in the ligand-binding domain of ERα resulted in approximately 60% decreases in ERα-caveolin-1 binding, quantity of ERα located at the membrane, and rapid activation of MAPK by estradiol when expressed in CHO cells (Razandi et al. 2003). This mutation did not have any effect on nuclear ERα expression and transcriptional function in these same cells, suggesting that the observed membrane ERα deficiencies were due to reduced ability to interact with caveolin-1 rather than impaired estradiol-binding or AF-2 function. More recent studies show that ERs destined for the plasma membrane undergo a posttranslational modification resulting in palmitoylation at

cysteine 447, a modification that promotes interaction with caveolin (Acconcia et al. 2005; Pedram et al. 2007). Razandi and associates have also shown that estradiol increases the association of ERα and caveolin at the plasma membrane and stimulates caveolin protein synthesis in vascular smooth muscle cells (Razandi et al. 2002), providing further evidence for the importance of caveolin in localizing ERα to the membrane.

Caveolin rafts create microenvironments in which components of multiple intracellular signaling pathways form interactive complexes, which can include ERα anchored to the plasma membrane via caveolin. Estradiol has been shown to promote the formation of protein complexes containing ERα, insulin-like growth factor receptor (IGF-1R), and Src-homology and collagen protein (Shc), and these complexes have been shown to be important for activation of MAPK by estradiol in MCF-7 breast cancer cells (Song et al. 2002; Song et al. 2004). The ER antagonist ICI 182,870 (ICI), which prevents the formation of ER dimers, blocks this effect, indicating that estrogen acting through ERα is a critical component. Other studies have shown that the rapid activation of MAPK, cAMP, and PI3K signaling pathways by estradiol occurs via membrane ERα interactions between epidermal growth factor receptor (EGFR) and G-proteins (Razandi et al. 2003; Razandi et al. 2004). Interestingly, this effect requires membrane ERs to dimerize and involves the ligand-binding domain and AF-2. Interactions between membrane ERs and signaling proteins have been shown to be important in other cells types, as well. The rapid production of nitric oxide induced by estradiol in endothelial cells occurs through the association of membrane ERα with $G\alpha_i$ protein subunits, an

interaction that is blocked by ICI (Wyckoff et al. 2001). Recently, a protein has been identified that provides a link between the membrane and genomic effects of ERs. Wong et al. (Wong et al. 2002) have shown that modulator of nongenomic activity of estrogen receptor (MNAR) modulates the interaction between membrane ERα and Src tyrosine kinases, resulting in MAPK activation and altered ER transcriptional activity in the nucleus. This finding is not surprising when one considers the myriad ways that intracellular signaling pathways regulate gene transcription. Given the numerous studies demonstrating the ability of estrogen to activate many of these pathways, it would be surprising if membrane ERs did not modulate nuclear ER activity or the activity of many other transcriptional factors.

Estrogen effects in the hippocampus

Despite the fact that it was deemed insensitive to estrogen by early studies that detected only a few estradiol-binding cells (Pfaff and Keiner 1973; Stumpf et al. 1975), the hippocampus has been shown to respond structurally and physiologically to estrogen, both in experimental paradigms and across the estrous cycle. These cellular responses may underlie the effects of estrogen observed in behavioral and cognitive processes known to involve the hippocampus. Epilepsy, memory, and depression are three areas in which the roles of estrogen have been studied extensively.

Approximately 3 million Americans suffer from epilepsy with the highest incidence of disease in children under 2 and in adults over 65 (Epilepsy Foundation 2007). It is thought that over 50% of epileptics suffer from temporal lobe epilepsy (TLE),

a form of epilepsy characterized by complex partial seizures originating in the temporal lobe, which includes the hippocampus. The structural pathology most often observed in TLE is hippocampal sclerosis, which results from neuronal death and gliosis (Blumcke et al. 2002; Sloviter et al. 2004; Thom et al. 2005). In fact, only 35% of people with TLE have sclerotic lesions outside the hippocampus (Kim et al. 1990; Blumcke et al. 2002). These findings suggest that the hippocampus is the most common brain region for epileptic foci.

Physicians observed a connection between epileptic seizures and stages of the menstrual cycle in female patients at least as early as the 19th century. Since then, the evolution of experimental techniques has advanced our understanding of the interaction between estrogen, seizures, and the hippocampus. Catamenial epilepsy is the general term for the form of epilepsy in which seizure frequency is increased in women at times of high estrogen during the menstrual cycle (Herkes et al. 1993; Herzog et al. 1997; Harden et al. 1999). Furthermore, in postmenopausal epileptic women, menopause significantly decreases the number of seizures in those with catamenial epilepsy and hormone replacement therapy (HRT; most commonly conjugated estrogens with or without progestin) increased the number seizures in the perimenopausal period, a time when estrogen levels fluctuate wildly (Harden et al. 1999). Experimentally, estrogen has been shown to decrease seizure threshold in the hippocampus (Terasawa and Timiras 1968), and estradiol facilitates kindling in the dorsal hippocampus (Buterbaugh and Hudson 1991). These observations reveal a link between estrogen and seizures in the hippocampus. Additionally, estradiol decreases the latency of seizure-related behaviors in rats treated with kainic acid, a compound that induces seizures known to involve the

hippocampus, but has no effect in rats treated with flurothyl, a compound that induces generalized seizures (Woolley 2000), providing further evidence for a role of estrogen in hippocampal seizures.

The effect of estrogen on hippocampal-dependent learning and memory has also been intensely studied. Many studies report that estradiol treatment enhances spatial memory and object memory in both young and aged ovariectomized rodents compared to controls treated with vehicle (Williams et al. 1990; Luine and Rodriguez 1994; Daniel et al. 1997; Luine et al. 1998; Gibbs 1999; Daniel and Dohanich 2001; Sandstrom and Williams 2001; Bowman et al. 2002; Frick et al. 2002; El-Bakri et al. 2004; Fernandez and Frick 2004; Li et al. 2004). Additionally, estradiol has been shown to enhance memory acutely both when administered 48 or 72 hours before or immediately after training (Packard and Teather 1997; Sandstrom and Williams 2001; Frye et al. 2005), and female rats perform better in avoidance tasks during proestrus, the few hours when circulating estrogen levels are highest during the short 4-5 day estrous cycle (Rhodes and Frye 2004). A recent study reports that spatial memory declines after ovariectomy, an effect that is blocked by estradiol replacement (Wallace et al. 2006). This finding indicates that estradiol treatment most likely preserves memory function rather than improving it, a result that potentially has important implications for HRT in humans. In addition, aged rats performed significantly better on a spatial memory task after receiving long-term estradiol treatment if estradiol administration began immediately or 3 months following ovariectomy, but not when treatment was delayed for 10 months (Gibbs 2000). This suggests a critical period following the loss of endogenous hormones, during which exogenous estradiol treatment must be started in order to preserve memory function.

A direct role for the hippocampus in estrogen's effects on learning and memory has also been demonstrated. Spatial memory is improved in rats following post-training administration of estradiol directly into the hippocampus (Packard and Teather 1997). Estradiol infused into the dorsal hippocampus results in increased latency time (enhanced performance) in an avoidance task (Frye and Rhodes 2002). Interestingly, infusion of estradiol conjugated to BSA also improves performance in this same task. Since it is unable to initiate a response mediated through nuclear ERs, the ability of estradiol-BSA to enhance memory suggests that this effect of estradiol may occur through a membrane ER. ERs in the dorsal hippocampus have been shown to mediate the effect of estradiol on place learning (Zurkovsky et al. 2006). Estradiol infusion directly into the hippocampus enhanced performance in an elevated T-maze task in which rats had to rely on fixed room cues to find a food reward. When the ER antagonist ICI 182,780 was present in the hippocampus before estradiol treatment was initiated, there was no improvement in place learning, indicating that ERs in the hippocampus mediate estrogen's effect on this form of learning.

The effect of estrogen on depression is another area of active investigation and many researchers make extensive use of animal models and behavioral tests, such as the forced swim test (FST), to assess depression-like behavior. Several studies report an anti-depressant effect of estradiol in rodents (Estrada-Camarena et al. 2003; Bekku and Yoshimura 2005; Walf and Frye 2005; Walf and Frye 2007), and one determined that the effect of estradiol on depression-like behavior persisted for 72 hours after cessation of treatment (Estrada-Camarena et al. 2003). Additionally, estradiol augments the action of antidepressants resulting in a therapeutic effect of suboptimal doses (Estrada-Camarena et

al. 2004). Sun and Alkon (2006) also show that the vulnerability of female rats to "depression" (depression-like behavior induced by stress) fluctuates across the estrous cycle, with animals being more likely to develop depression when estrogen levels are naturally low. Interestingly, learning and memory, as measured by a passive avoidance task, are also impaired after the rats develop depression (Sun and Alkon 2006).

Estradiol effects in animal models of post-partum depression have also been studied and reveal that a sudden drop in elevated levels of estradiol, similar to what occurs in the immediate post-partum period, results in increased depression-like behavior in rats (Galea et al. 2001; Stoffel and Craft 2004). Experiments using an animal model simulating menopause show that chronic estradiol treatment after ovariectomy decreases depression-like behavior in the FST (Bekku and Yoshimura 2005). This same study also reports a similar effect of antidepressants to decrease depression-like behavior in ovariectomized rats but not in intact animals, further suggesting that depression-like behavior develops as a result of the loss of endogenous estrogens.

Selective serotonin reuptake inhibitors (SSRIs) are one of the most common classes of pharmaceuticals prescribed and taken for depression. As the name suggests, SSRIs ameliorate depression by acutely inhibiting the reuptake of serotonin by presynaptic terminals, thereby increasing the quantity of serotonin in the synaptic cleft and eventually leading to downregulation of both pre- and postsynaptic serotonin receptors (Stahl 1998). An antidepressant effect of estrogen is further suggested by *in vitro* experiments that demonstrate a similar ability of estrogen agonists to decrease serotonin uptake by synaptosomes (Michel et al. 1987). Also, estradiol has also been shown to acutely inhibit serotonin reuptake, most likely through a non-nuclear

mechanism (Chang and Chang 1999). It is interesting to note that of the 19 steroid metabolites and analogs examined by Chang and Chang, including testosterone, progesterone, and diethylstilbestrol, only estradiol, estriol, and tamoxifen were capable of inhibiting serotonin reuptake.

Structure of the hippocampus

An understanding of the organization of the hippocampus is critical to the study of how estrogen affects its function. The hippocampus is a highly structured brain region, and its functional pathways are reflected in its spatial and anatomical organization. There are primarily two types of neurons in the hippocampus. Principal cells are glutamatergic excitatory neurons, which can be further divided into pyramidal cells and granule cells, and interneurons, which are primarily GABAergic inhibitory cells. Interneurons in the hippocampus display a high degree of heterogeneity (Freund and Buzsaki 1996). There are 5 subregions represented throughout the entire septotemporal axis of the hippocampus: CA1, CA2, CA3, dentate gyrus, and subiculum. The flow of information between these subregions is also highly organized. Incoming excitatory signals to the hippocampus enter through the perforant path and travel primarily to the dentate gyrus. The mossy fibers carry the signal from the dentate gyrus to CA3. Information can either leave the circuit at this point or continue on to CA1 via the Schaffer collaterals. At CA1, signals leave the circuit or continue to the subiculum and then leave.

Each of the subregions mentioned above is further organized into laminae. Specifically, the CA1 is comprised of 4 layers: stratum oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare (Anderson et al. 1971; Amaral and

Witter 1989). Each of these layers has distinct characteristics. Stratum (str.) pyramidale contains the cell bodies of all pyramidal cells in CA1 and also contains the cell bodies of some interneurons, specifically basket cells and chandelier cells (Freund and Buzsaki 1996). Str. oriens contains the axons and basal dendrites of pyramidal cells and the somata of some interneurons, many of which project to the str. lacunosum-moleculare to innervate the distal apical dendrites of pyramidal cells. Str. radiatum contains a few interneurons but is mostly occupied by the apical dendrites of the pyramidal cells and by the Schaffer collaterals. The Schaffer collaterals are the axonal projections from the CA3 pyramidal cells and are the primary excitatory input into the CA1 region (Ishizuka et al. 1990). The str. lacunosum-moleculare contains the most distal branches of the apical dendrites and many interneurons, some of which innervate other interneurons in the str. radiatum (Kunkel et al. 1988).

Structural effects of estrogen in the hippocampus

Estrogen has multiple effects on the structure of neurons in the hippocampus, particularly in the dorsal CA1 region. Dendritic spine density on CA1 pyramidal cells is increased by approximately 30% in estradiol-treated animals (Gould et al. 1990). This effect begins within 48 hours of estradiol treatment and maximal density is reached by 72 hours (Woolley and McEwen 1993). Spine density also fluctuates naturally across the estrous cycle with the highest density occurring during proestrus, when circulating estrogen is high (Woolley et al. 1990). Interestingly, progesterone has been shown to be important for the rapid decrease in spine density that occurs within the 24 hours after the estrogen peak in proestrus (Woolley and McEwen 1993).

In parallel with the increased spine density, the density of synapses in the CA1 stratum radiatum also increases by ~32%, suggesting that estrogen-induced spines form synapses with boutons (Woolley and McEwen 1992). Electron microscopy studies have shown an increase in the frequency of boutons that form synapses with multiple pyramidal cell spines in estradiol-treated animals (Woolley et al. 1996), and that multiple synapse boutons form synapses with more than one pyramidal cell, suggesting that estrogen causes a divergence of excitatory inputs into the CA1 (Yankova et al. 2001). These studies demonstrate that estrogen is a natural modulator of both neuron structure and connectivity in the hippocampus.

The expression of N-methyl-D-aspartate (NMDA) receptors, a type of glutamate receptor present at synapses on pyramidal cell spines, is also regulated by estrogen in the CA1. Estradiol increases agonist binding of NMDA receptors without affecting non-NMDA binding (Weiland 1992; Woolley et al. 1997; Cyr et al. 2000; Daniel and Dohanich 2001), and increases immunofluorescence labeling of NMDAR-1, the obligatory subunit of the NMDA receptor (Gazzaley et al. 1996). NMDAR-1 expression is also differentially affected by estrogen in young versus old rats (Adams et al. 2001). Daniel and Dohanich (2001) further report that working memory is enhanced in animals treated with estradiol, compared to controls, at the same time after treatment at which NMDA binding is increased. These data suggest the possibility that the spines formed in response to estrogen are enriched in NMDA receptors, a scenario that is supported by electrophysiological experiments discussed below.

Physiological effects of estrogen in the hippocampus

Estrogen regulates both excitatory and inhibitory synaptic input to CA1 pyramidal cells. One of the earliest studies recorded field potentials in the CA1 cell body layer and found increased excitability as early as 5 minutes after estradiol exposure (Teyler et al. 1980). Estradiol has also been reported to increase the amplitude of excitatory postsynaptic potentials (EPSPs) and excitatory postsynaptic currents (EPSCs) recorded in CA1 pyramidal cells within 2-3 minutes (Wong and Moss 1992; Rudick et al. 2003). This effect is activated by 17β-, but not 17α-estradiol (Wong and Moss 1992), and is blocked by 4OH-Tamoxifen, an ER antagonist (Rudick et al. 2003), indicating that it is ER-mediated. Additionally, the rapid timing of the estradiol effect suggests a non-nuclear ER mechanism.

Further studies demonstrate an estrogen effect on NMDA receptor inputs to pyramidal cells. CA1 pyramidal cells show an increased sensitivity to NMDA receptor-mediated synaptic input when spine density is higher following estradiol-treatment (Woolley et al. 1997; Rudick and Woolley 2001). Taken together with the anatomical studies discussed above, these findings suggest that the synapses formed by estrogen-induced spines are enriched in NMDA receptors. Interestingly, MK801, an NMDA receptor antagonist, blocks the increase in dendritic spine density following estradiol-treatment (Woolley and McEwen 1994), indicating the importance of NMDA receptors in estradiol-induced structural plasticity in the hippocampus.

Inhibition and estrogen-induced spines

Experimental evidence suggests that synaptic inhibition plays a critical role in the formation of spines following estradiol-treatment. In primary cultures of hippocampal neurons, estradiol increased spine density by ~50% (Murphy and Segal 1996) over a time course similar to that observed *in vivo*. Levels of glutamic acid decarboxylase (GAD), the rate limiting enzyme in GABA synthesis, and the amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) were decreased in these cultures 24 hours after estradiol-treatment (Murphy et al. 1998) suggesting that reduced inhibition is important for the formation of spines. Murphy et al. also showed that treatment of cultures with an inhibitor of GABA synthesis, mercaptopropionic acid (MA), resulted in an increase in spine density similar in magnitude to estrogen, and treatment with both MA and estradiol together did not increase spine density above that observed by MA alone, indicating that the effect of estrogen could occur through the same disinhibition mechanism. In the dorsal CA1 *in vivo*, estradiol also decreases the number of GAD65-IR interneurons in the strata radiatum and oriens and reduces pyramidal cell inhibition 24 hours after treatment (Rudick and Woolley 2001), an effect that is mediated by ERs (Rudick et al. 2003). This is the same time when spine density is beginning to increase, suggesting that estradiol-induced spine density changes are also occurring through a disinhibition mechanism *in vivo*. This conclusion led to the hypothesis that ERs are likely to expressed in inhibitory interneurons where they would be in position to modulate inhibition.

Estrogen receptor expression in the hippocampus

As stated before, binding studies conducted in the early 1970's detected very few estradiol-concentrating cells in the dorsal hippocampus. However, as the number of studies reporting estrogen effects increased, the search for ERs in the hippocampus intensified. Loy et al. (Loy et al. 1988) also reported few ^{3}H-estradiol-concentrating cells in the hippocampus, but further hypothesized that they were interneurons rather than pyramidal cells based on their location. Multiple studies (Simerly et al. 1990; Shughrue et al. 1997; Laflamme et al. 1998) have detected ER mRNA signals throughout the hippocampus, mostly over the CA1, CA2, and CA3 cell body layers, which is contradictory to the ^{3}H-estradiol binding studies. However, as discussed earlier, ER protein is not always observed where mRNA signals are detected. Early immunohistochemical studies (DonCarlos et al. 1991; Weiland et al. 1997) reported ERα nuclear labeling of scattered cells in the hippocampus, most of which were located outside of the cell body layer, supporting Loy's earlier conclusion that ERs are expressed in interneurons. ERβ studies have found no nuclear ERβ-IR in the dorsal hippocampus (Blurton-Jones and Tuszynski 2002; Mitra et al. 2003; Kalita et al. 2005) suggesting that estrogen effects that are mediated by nuclear ERs are mediated solely through ERα in this area.

Understanding of the true extent of ER expression in the hippocampus continues to progress as techniques and technology develop and improve. A more recent binding study using 17α-iodovinyl-11β-methoxyestradiol (^{125}I-estradiol), a compound that has similar binding affinity for both ERα and ERβ and high specific binding activity in rats

in vivo, revealed dramatically more estradiol-concentrating cells throughout the hippocampus than previously detected with any method (Shughrue and Merchenthaler 2000). Additionally, extranuclear ERα-IR has been observed in the CA1 (Milner et al. 2001; Adams et al. 2002) the presence of which is predicted by the rapid effects of estrogen discussed earlier. While knowledge of the existence of ERs has increased dramatically since the first binding studies, there was still much basic information, such as the number and neurochemical nature of cells expressing ERs, which remained to be determined before we could begin understand the mechanisms of estrogen action in the hippocampus.

Interneurons in the hippocampus

Interneurons comprise only about 10% of the neurons in the hippocampus, but their inhibitory influence is critical for the function of the hippocampus and the proper excitatory activity of almost half a million pyramidal cells in the rat and over 1.5 million pyramidal cells in the human (Boss et al. 1987; West and Gundersen 1990; West et al. 1991). Even though almost all interneurons are GABAergic (Freund and Buzsaki 1996), they are an extremely diverse group of neurons that can be divided into subpopulations based on various morphological, physiological, and neurochemical properties. Immunohistochemistry is the easiest and most common method used to divide interneurons into subpopulations, and common neurochemical markers are calcium-binding proteins, such as parvalbumin (PV) and calbindin (CB), and neuropeptides, such as cholecystokinin (CCK) and neuropeptide-Y (NPY). The extent and location of an

interneuron's axonal arbor and dendritic tree are also used for categorization because they indicate distinct input and output sources.

Basket cells in the CA1 are an example of a subpopulation of inhibitory interneurons that are defined by their anatomical structure and neurochemical expression. Studies have shown that certain interneurons form networks of boutons surrounding pyramidal cell somata, hence the designation "basket cells", and provide inhibition through multiple axosomatic synapses. Basket cells can be further subdivided based on PV (Kosaka et al. 1987) or CCK (Nunzi et al. 1985) immunolabeling, suggesting that, although they both perisomatically inhibit the same cells, they are functionally different. Many studies have examined structural and functional characteristics of PV and CCK basket cells, and collectively, the results suggest that PV basket cells are responsible for maintaining rhythmic pyramidal cell activity, while CCK basket cells provide fine-tuning of pyramidal cell output (Freund 2003); discussed in detail in Chapter 4).

Subcortical projections to the hippocampus

Multiple subcortical brain regions project to the hippocampus and target specific subpopulations of interneurons. The septohippocampal pathway contains both GABAergic and cholinergic afferents, with the later having the most interesting potential effects. Both PV and CCK basket cells express cholinergic receptors, but they express different types and activation has different effects: activation of M_2 muscarinic receptors on PV axon terminals (Hajos et al. 1998) results in decreased GABA release (Freund and Gulyas 1997), while activation of α-7 nicotinic receptors on CCK somata (Freedman et

al. 1993) is believed to increase excitation of CCK basket cells (File et al. 1998; Freund 2003). Thus, acetylcholine released into the CA1 by septohippocampal projections would decrease inhibition on pyramidal cells from PV basket cell boutons, but increase inhibition from CCK basket cell boutons. The purpose of this differential effect on perisomatic inhibition is unknown, but may be related to the different functional roles of the two types of basket cells as mentioned above.

Another important subcortical pathway to the hippocampus is the serotonergic projections from the median raphe nucleus. Studies have shown that 5-HT_3 serotonin receptors are expressed on CCK interneurons, but are absent in PV cells (Freund et al. 1990; Morales and Bloom 1997) indicating a differential effect of serotonin on their respective contributions to pyramidal cell inhibition. Additionally, serotonergic afferents from the median raphe specifically innervate CCK and calretinin (CR)-containing interneurons in the CA1 (Miettinen et al. 1992), further suggesting that pyramidal cell inhibition mediated through CCK basket cells is sensitive to input from the median raphe, but PV basket cell inhibition is not. Systemic depletion of serotonin has been shown to decrease the density of spine synapses in the CA1 radiatum by 28% (Matsukawa et al. 1997), demonstrating a role for serotonin in the maintenance of the same hippocampal structures that are also affected by estrogen.

It is also important to note that some neurons in the brain regions giving rise to the subcortical projections discussed above express nuclear ERα. A portion of the serotonergic neurons in the median raphe express nuclear ERα (Leranth et al. 1999), but whether or not these cells send axons to the hippocampus is unknown. Additionally,

nuclear ERα is expressed in cholinergic neurons in the medial septum (Shughrue and Merchenthaler 2000), but their contribution to the septohippocampal pathway is also undetermined. Estrogen could potentially modulate hippocampal function via subcortical inputs either through nuclear ERs in neurons projecting to the hippocampus or through nuclear ERs in neurons that synapse on neurons that send axons to the hippocampus. Placement of estradiol locally into the median raphe (Prange-Kiel et al. 2004) or the medial septum (Lam and Leranth 2003) resulted in greater spine synapse density compared to controls in the CA1, indicating the ability of estrogen to act indirectly through subcortical pathways to regulate structural plasticity in the hippocampus. However, estradiol treatment increases pyramidal cell spine density in the dorsal CA1 of serotonin-depleted animals (Alves et al. 2002), and the destruction of cholinergic neurons in the medial septum only partially decreases the effect of estrogen on pyramidal cell disinhibition (Rudick et al. 2003), suggesting that estrogen acts locally within the hippocampus and the magnitude of effect may be modified by estrogen acting through nuclear ERα in subcortical brain regions projecting to the hippocampus.

NPY in the hippocampus

As mentioned above, interneurons in the hippocampus are often classified into groups based on their neurochemical content, and one neuronal marker is NPY. A subpopulation of interneurons in the hippocampus expresses NPY (Chronwall et al. 1985; de Quidt and Emson 1986), and, conversely, NPY is most often found in GABAergic neurons in the hippocampus (Hendry et al. 1984). NPY has been shown to inhibit

presynaptic glutamate release from Schaffer collateral terminals (Colmers et al. 1988), resulting in a reduction of excitatory transmission in the CA1. At the same time, however, NPY has no effect on synaptically mediated inhibition (Klapstein and Colmers 1993), revealing the ability to selectively modulate feedforward excitatory transmission. It is not surprising, then, that a role for NPY in seizure modulation has been demonstrated (Redrobe et al. 1999; Vezzani et al. 1999).

Anatomical studies have revealed the expression of multiple NPY-receptor subtypes throughout the hippocampus (Redrobe et al. 1999). However, which structures they are located in, besides Schaffer collateral terminals, are unknown. It is also interesting to note that NPY immunoreactivity has been observed in excitatory boutons in the hippocampal CA1 (Milner and Veznedaroglu 1992), indicating that NPY can be expressed in glutamatergic neurons, as well, adding another nuance to the role of NPY in the hippocampus.

Toward a greater understanding of estrogen receptors in the hippocampus

The above review highlights how little was known about ERs and how ERs mediate the effects of estrogen in the hippocampus when I began my studies. Nuclear ERα-IR was observed in relatively few cells scattered throughout all layers of the hippocampus, and previous studies suggested that ERα-IR cells were inhibitory interneurons (DonCarlos et al. 1991; Weiland et al. 1997). The actual neurochemical nature of ERα-IR cells was not known. Whether or not nuclear ERα expression was limited to interneurons or if nuclear ERα was expressed by a specific subpopulation of

interneurons was also undetermined. The first experiment I completed, presented in Chapter 2, was designed to address these gaps in our knowledge about nuclear ERα and interneurons in the hippocampus.

Consistent with previous anatomical studies, I observed a paucity of cell nuclei labeled with ERα-IR in the dorsal CA1, and it seemed unlikely that so few cells could mediate the many and varied effects of estrogen in the hippocampus (as reviewed in a previous section). The rapid estrogen effects reported in the dorsal hippocampus suggested the presence of extranuclear ER, and indeed, extranuclear ERα-IR had been observed in the hippocampus (Milner et al. 2001). During the data collection phase of my first experiment, I noticed what appeared to be punctate ERα-IR labeling in my tissue. Since estradiol decreases inhibition to CA1 pyramidal cells 24 hours after treatment (Rudick and Woolley 2001), and inhibition to CA1 pyramidal cell somata is provided primarily by synapses formed with perisomatic inhibitory boutons from basket cell axons, I hypothesized that some of the punctate ERα-IR in the cell body layer was labeling ERα in perisomatic inhibitory boutons. If ERα was present in those boutons, it would be poised to mediate estradiol-induced disinhibition of pyramidal cells directly at synapses.

Milner et al. (2001) focused on extranuclear ERα expression in the stratum radiatum and only briefly mentioned observing extranuclear ERα-IR in inhibitory boutons in the pyramidal cell layer. Nothing was known about ERα expression in perisomatic inhibitory boutons. In order to understand how extranuclear ERs mediate the effects of estrogen in the hippocampus, it is necessary to know where and in what structures extranuclear ERs are expressed. I designed the experiment discussed in

Chapter 3 to identify and quantify the structures labeled by ERα-IR in perisomatic inhibitory boutons and to determine the effect, if any, of estradiol. The experiment presented in Chapter 4 quantifies the extent of extranuclear ERα-IR in GABAergic structures in the dorsal CA1 pyramidal cell layer and identifies the type of basket cell that gives rise to axonal varicosities containing ERα. The results of these studies provide the foundation for understanding where and in what neuron types ERα mediates the effects of estrogen and serve as a launching point for the formulation of hypotheses of the mechanisms of estrogen action in the hippocampus.

CHAPTER 2 - QUANTITATIVE ANALYSIS OF ERα AND GAD COLOCALIZATION IN THE HIPPOCAMPUS OF THE ADULT FEMALE RAT

This chapter has been previously published – (Hart et al. 2001)

ABSTRACT

Despite many effects of estrogen on the structure and function of hippocampal circuitry, there has been little evidence that hippocampal principal cells express nuclear estrogen receptors. In the hippocampus, the α form of the nuclear estrogen receptor (ERα) previously has been localized to sparsely distributed cells with the morphological characteristics of inhibitory interneurons. Because inhibitory neurons have been suggested to be involved in producing estrogen's effects on hippocampal principal cells, quantitative description of ERα expression in γ-aminobutyric acid (GABA)ergic (inhibitory) and non-GABAergic cells of the hippocampus is a key step in understanding the mechanism(s) of estrogen action on hippocampal circuitry. We used single and double label immunohistochemistry for ERα and glutamic acid decarboxylase (GAD, a marker of GABAergic neurons) to determine the numbers and distributions of hippocampal GABAergic and non-GABAergic neurons that express ERα in the adult female rat. We found many more ERα expressing cells in the hippocampus than any previous study and observed distinct dorsal versus ventral differences in hippocampal ERα expression. In the dorsal hippocampus, most ERα positive cells were also GAD positive; however ERα was expressed in only a small subset of all GAD positive cells. The greatest concentration of GAD neurons that express ERα was found at the border

between str. radiatum and str. lacunosum-moleculare. In the ventral hippocampus, we found a very high number of ERα positive cells, the vast majority of which were not immunoreactive for GAD and are likely to be pyramidal cells. These findings suggest that ERα can mediate effects of estrogen primarily in GABAergic neurons in the dorsal hippocampus and in both GABAergic and non-GABAergic neurons in the ventral hippocampus.

INTRODUCTION

Estrogen has a wide range of effects on the structure and function of hippocampal circuitry, particularly in the CA1 region. Estrogen has been shown to regulate the density of dendritic spines (Gould et al. 1990; Woolley and McEwen 1993; McEwen et al. 1999) and spine synapses (Woolley and McEwen 1992; Woolley et al. 1996; Leranth et al. 2000) on CA1 pyramidal cells. Estrogen has also been shown to regulate N-methyl-D-aspartate (NMDA) receptor binding (Weiland 1992; Woolley et al. 1997) and immunoreactivity (Gazzaley et al. 1996) in CA1, as well as the sensitivity of CA1 pyramidal cells to NMDA receptor mediated synaptic input (Wong and Moss 1992; Woolley et al. 1997). Both long-term potentiation (Warren et al. 1995; Cordoba Montoya and Carrer 1997; Good et al. 1999) and long-term depression (Good et al. 1999; Desmond et al. 2000) in CA1 also are regulated by estrogen.

Despite the many effects of estrogen in the CA1 region of the hippocampus, the vast majority of evidence indicates that these cells do not express nuclear estrogen receptors (ERs). Early studies of ER containing cells used ^{3}H-estradiol for *in vivo*

autoradiography and reported very sparse labeling in dorsal subregions of the hippocampus with slightly greater labeling in ventral subregions (Pfaff and Keiner 1973) (Stumpf et al., 1975). Loy et al. (1988) also reported a small number of ^{3}H-estradiol concentrating cells in the hippocampus and, based on location, suggested that most were interneurons. *In situ* hybridization studies (Simerly et al. 1990; Shughrue et al. 1997; Laflamme et al. 1998; Wehrenberg et al. 2001) have generally detected low levels of ER mRNA throughout the hippocampus with signals found mostly over the cell body layers of CA1, CA2, CA3, and the dentate gyrus. Immunohistochemical analyses (DonCarlos et al. 1991; Weiland et al. 1997) have revealed scattered cells expressing the α form of the nuclear ER (ERα) in the dorsal hippocampus, with a slight increase in the ventral hippocampus. Weiland et al. (Weiland et al. 1997) concluded that, based on the distribution and location of ERα immunoreactive cells in the hippocampus, most are interneurons. In contrast to the studies above, a recent *in vivo* autoradiographic analysis (Shughrue and Merchenthaler 2000), which used a novel ^{125}I-labeled ER ligand, reported a much higher number of estrogen concentrating cells than suggested by any previous studies. Additionally, some ^{125}I-estrogen concentrating cells were located in the dorsal CA2/CA3 pyramidal cell layer and many were located in the ventral hippocampus.

Experiments on cultured hippocampal neurons suggest that estrogen may act on hippocampal pyramidal cells through an indirect mechanism that involves direct action on γ-aminobutyric acid (GABA)ergic interneurons (Murphy et al. 1998). Recent analysis of estrogen effects *in vivo* have confirmed that estrogen regulates GABAergic inhibitory synaptic input to hippocampal CA1 pyramidal cells (Rudick and Woolley 2001). The

possibility that estrogen acts directly on inhibitory interneurons in the hippocampus is consistent with reports that ERα is expressed primarily in interneurons. However, the previous identification of ERα expressing cells as interneurons has been based solely on their sparse distribution and location outside of principal cell layers. Thus, it is currently unknown how many ERα-expressing cells in the hippocampus are, in fact, GABAergic, or how many and what populations of GABAergic neurons express ERα. This information will be important for determining the mechanism(s) of estrogen action in the hippocampus.

In order to address these questions, we have used single- and double-label immunohistochemistry for ERα and glutamic acid decarboxylase (GAD, the rate limiting enzyme in GABA synthesis and a marker for GABAergic neurons) to determine the numbers and distributions of: 1) hippocampal GABAergic neurons that express ERα and, 2) ERα expressing neurons that are not GABAergic. Here we report a much greater number of ERα expressing cells in the hippocampus than has been found in any previous studies and we find distinct differences in ERα expression between the dorsal and ventral hippocampus.

METHODS

Animal Treatment

Ten adult female Sprague Dawley rats (250g) were maintained on a 12-hour light/dark cycle with free access to food and water. Animal procedures were performed in accordance with institutional guidelines. All animals were ovariectomized under

Metofane anesthesia and then on the 3rd and 4th days after surgery, half the animals received injections (s.c.) of 17β-estradiol benzoate (E; 10μg in 100μl of sesame oil vehicle) and the other half received oil vehicle (O) at the same times. Forty-eight hours after the second injection, all animals were deeply anesthetized with Nembutal (80mg/kg) and perfused with 180ml of 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (PB). Brains were removed, blocked to contain the hippocampus, and postfixed overnight at 4°C. Following postfixation, brains were rinsed, cryoprotected with 30% sucrose in PB, and then cut into 40μm coronal sections on a freezing microtome. Every section between –2.8 and –5.8 mm from bregma was collected and placed consecutively into one of the 8 groups below.

Immunohistochemistry

Sections were placed consecutively into each of the following groups so that every 8th section (8 sections per brain) was labeled as follows:

Single labeling:

1) ERα with MC-20 (0.5 μg/ml, Santa Cruz Biotechnology)
2) ERα with 6F11 (1:70, Novocastra)
3) GAD 65/67 with G5163 (1:10,000, Sigma)
4) GAD 65 with MAB351 (0.2 μg/ml, Chemicon)
5) ERα (MC-20) and counter-stained with cresyl violet

Double labeling:

6) ERα (MC-20) and GAD 65/67 (G5163)

7) ERα (MC-20) and GAD 65 (MAB351)

8) Control sections for ERα and GAD double labeling (see below)

These antibodies and antisera have differing characteristics. MC-20 is a polyclonal antisera raised against the c-terminus of ERα and is not cross-reactive with ERβ (Azcoitia et al. 1999). 6F11 is a monoclonal antibody raised against the full length of ERα and is specific for ERα only (Bevitt et al. 1997). MAB351 is a monoclonal antibody, specific for GAD 65, and G5163 is a polyclonal antisera which recognizes a c-terminal amino acid sequence common to both GAD 65 and GAD 67.

ERα was visualized with 3,3'-diaminobenzidine (DAB, Sigma), resulting in a brown precipitate primarily localized to the nuclei of cells, and GAD was visualized with Vector SG (Vector Labs), which produced a blue/gray precipitate present in the cell body, proximal dendrites, and axons of cells. For double labeled sections, processing for ERα was completed and visualized before immunohistochemistry for GAD was begun. Sections to be singly labeled for GAD were stored without any processing in 10% sucrose in PB at 4^{o}C until after the other sections had been processed for ERα, so that all ERα and GAD processing each occurred simultaneously.

Immunohistochemical processing was as follows: sections were first incubated in 1% sodium borohydride in PB for 10 min. After rinsing, sections were incubated in hydrogen peroxide in 0.1M Tris buffer, pH 7.4 (TB) (0.05% for 30 min., 0.1% for 60 min., and 0.05% for 30 min.). Sections were then rinsed and incubated for 1 hour in 3% bovine serum albumin (BSA), 0.3% dimethyl sulfoxide (DMSO), and 5% normal goat

serum (MC-20, G5163) or 5% normal horse serum (6F11, MAB351) in 0.5M Tris buffered saline, pH 7.4 (TBS). For sections double labeled with MC-20 and G5163, Avidin Solution (100 μl/ml) from an Avidin/Biotin Blocking Kit (Vector Labs) was also added to this step during the processing for G5163 (GAD 65/67). After multiple rinses, tissue was incubated for 48 hours at 4^{o}C in primary antisera or antibody with 2% BSA, 0.3% DMSO, 1% normal goat serum (MC-20, G5163) or 1% normal horse serum (6F11, MAB351) in 0.5M TBS. Biotin Solution (53 μl/ml) from an Avidin/Biotin Blocking Kit was also added in this step during processing with G5163 in double labeled tissue. Following incubation, tissue was rinsed thoroughly. For secondary antibody incubation, sections were incubated for 3 hours at room temperature with biotinylated anti-rabbit IgG (MC-20 and G5163 sections, 1:800, Vector Labs) or biotinylated anti-mouse IgG (6F11 and MAB351 sections, 1:800, Vector Labs) with 2% BSA, 0.3% DMSO, and 1% normal goat serum (MC-20, G5163) or 1% normal horse serum and 2% normal rat serum (6F11, MAB351) in 0.5M TBS. After rinsing, sections were incubated with avidin-biotin HRP complex (1:100, Vectastain Elite ABC kit, Vector Labs) for 3 hours at room temperature. For visualization of ERα labeling, tissue was preincubated for 20 min. in a solution of DAB dissolved in TB, pH 7.6 (0.25 mg/ml). Hydrogen peroxide was added to the solution (0.1%) and the DAB reaction was allowed to continue for 25 min. (MC-20) or 45 min. (6F11). For visualization of GAD, tissue was incubated in Vector SG according to the manufacturer's directions. The reaction was stopped after 10 min. (G5163 for GAD 65/67) or 15 min. (MAB351 for GAD 65). Sections were mounted in order on subbed slides, allowed to air dry, and then placed in a 50^{o}C oven overnight. One group

of sections labeled with MC-20 was counter-stained in 0.5% cresyl violet solution. All tissue was dehydrated in graded alcohols, cleared with xylene, and coverslipped under Permount.

Immunohistochemical Controls

Separate control experiments were done to determine nonspecific labeling of the primary and secondary antisera and antibodies. No labeled nuclei were observed in tissue processed with MC-20 that had been preadsorped with blocking peptide (5:1 SC-542P to MC-20, Santa Cruz Biotechnology). In previous single labeling studies, sections processed with the omission of each primary antibody or antisera showed no non-specific secondary antibody labeling of structures visualized with primaries. As part of this experiment, some sections processed for MC-20 (group #8 above) were further processed as for GAD double labeling but with the omission of G5163 or MAB351. The resulting tissue showed labeling that was not different from tissue processed for MC-20 alone. This indicates that the GAD staining in double-labeled tissue is due to specific labeling by G5163 and MAB351 and not an artifact of the double labeling process.

Quantification of ERα and GAD labeled cells

Slides were coded prior to cell counting, so the experimenter was blind to the treatment condition of each brain. The general pattern and quantity of ERα immunolabeling was similar for MC-20 and 6F11 tissue, but the labeling in 6F11 slices was less intense. For this reason, sections labeled with MC-20 were used for quantitative

analysis. Additionally, more GAD positive cells were detected in tissue labeled for GAD 65/67 compared to tissue labeled for GAD 65, while axonal labeling was similar. This is consistent with a report that some hippocampal interneurons express only the 67kD isoform of GAD (Stone et al. 1999). Since one of the goals of this study was to estimate the quantity and distribution of GABAergic cells that express ERα, we used sections labeled with the GAD 65/67 antisera for quantitative analysis in order to include the maximum number of GAD positive cells.

Estimation of numbers of ERα positive and GAD positive cells was accomplished by quantitative analysis of tissue singly labeled for ERα or GAD 65/67 using the optical disector with fractionator method (Sterio 1984; Gundersen et al. 1988; West et al. 1991). Tissue was visualized using an Olympus BX60 microscope equipped with a Dage DC330 camera and ImagePro Plus software. Briefly, a grid consisting of squares 353 μm/side was randomly superimposed over a low magnification image of every 8^{th} slice of the sectioned brain. Each grid square contained an identically placed counting frame that was $1/16^{th}$ the area of the square. When any part of the hippocampus was contained within the counting frame, the number of labeled cells contained within the frame was counted. Counts were made using a 100x oil objective starting 3 μm from the surface of the slice, through a depth of 13 μm. The total number of cells was calculated using the following formula:

$$\#cells = \Sigma Q^{-} (t/h)(1/asf)(1/ssf)$$

Q^{-} = total number of cells counted

t = section thickness

h = height of the disector

asf = area sampling fraction

ssf = section sampling fraction

With this method, the height of the disector is the depth of the section through which cells are counted (10μm). The area sampling fraction is the proportion of the area of the grid square that is contained within the counting frame (1/16). The section sampling fraction is the proportion of the entire block of hippocampus that was counted (1/8). Sufficient numbers of cells were counted using the optical disector to obtain data for each hippocampal subregion separately. The boundaries between the subiculum, CA1, CA2, and CA3 subregions were determined on the basis of differences in the size and spacing of cells in the pyramidal cell layer. Sparse distribution of ERα labeled neurons in the dorsal hippocampus precluded obtaining quantitative data for each layer using the optical disector.

Percentages of the total number of neuron-like cells in the pyramidal cell layer that were ERα positive were obtained semi-quantitatively from tissue singly labeled for ERα (MC-20) and counter-stained with cresyl violet. All cells with neuron-like morphology and all ERα positive cells were counted in randomly selected portions of the

pyramidal cell layer of 2-4 sections per brain to estimate the percentage of total neurons that express ERα.

Quantification of ERα and GAD colocalization in double-labeled tissue was done by directly counting all labeled cells in every 8th slice of the sectioned hippocampus. This approach allowed us to quantify the proportions of double and single labeled cells, as well as to obtain information about the distribution of cells between layers of hippocampal subregions. Using a 100x oil objective, cells were identified as either ERα positive (ERα+), GAD positive (GAD+), or both ERα+ and GAD+. The percentage of total GAD positive cells that were also ERα positive (%GAD+/ERα+) and the percentage of total ERα+ cells that were GAD negative (%ERα+/GAD-) were calculated for each layer within each subregion of the hippocampus. In the dorsal CA2 and CA3 subregions, it was difficult to accurately distinguish a boundary between str. radiatum and str. lacunosum-moleculare. Therefore, data from the portions of the CA2 and CA3 subregions between str. pyramidale and the hippocampal fissure are represented by the str. radiatum layer. We validated our approach to quantifying double labeled cells by comparing the numbers of ERα+ and GAD+ cells per subregion obtained by the optical factionator and direct counting in a subset of sections. The two methods produced numbers of double labeled cells that were within 10% of each other.

For all analyses, we divided the hippocampus into three rostral to caudal levels: rostral (-2.8 to -3.9mm from bregma; 12% of total hippocampal volume), middle (-3.9 to -4.6mm from bregma; 20% of total hippocampal volume), and caudal (-4.6 to -5.8mm from bregma; 43% of total hippocampal volume). We did not quantify the very most

rostral or caudal levels (6% and 19% of total hippocampal volume, respectively). There were no significant quantitative differences between O and E treated animals (ANOVA with repeated measures, $p > 0.05$), so data from the two groups were combined. All data in tables are presented as mean ± SEM. Figures were made using Adobe Photoshop software.

RESULTS

Overview of ERα labeling

ERα labeled cells were present in all subregions and layers of the hippocampus at all rostral to caudal levels. Quantitative analysis using the optical disector with fractionator method resulted in an average of 104,102 ± 6,252 ERα+ cells in the hippocampus of each brain, many more ERα+ cells than previously reported (Weiland et al. 1997). In general, ERα immunoreactivity was localized to the nuclei of neuron-like cells; we saw no evidence of ERα labeling in cells with glia-like morphology. In the amygdala and hypothalamus, we observed cytoplasmic labeling of some neuron-like cells in addition to the more typical nuclear staining. Additionally, in the hippocampus, some ERα+ cells in the rostral portion of the ventral dentate gyrus showed intense labeling of the cell body and proximal dendrites. Although ERα labeling was slightly more intense in E treated than O treated animals and sections from E treated animals initially appeared to have more ERα labeled nuclei than sections from O treated animals, quantitative analysis revealed that there were no significant differences in number of ERα+ cells between the two groups ($p > 0.05$). As described below, ERα labeling increased dramatically in a dorsal to ventral direction, and was greatest in ventral portions of CA2, CA3 and the subiculum. The total numbers of ERα+ cells for each subregion of the hippocampus are shown in **Table 1**.

Overview of GAD labeling

GAD+ cells were also present in all subregions and layers at all rostral to caudal levels of the hippocampus, with a pattern of distribution consistent with previous reports (Woodson et al. 1989; Houser and Esclapez 1994). Quantitative analysis with the optical disector/fractionator yielded an average of 223,046 ± 15,766 GAD+ cells in the hippocampus of each brain. GAD immunoreactivity was observed in the proximal dendrites, cell body, and axons of labeled cells. GAD+ cells showed a high degree of variability in size and shape.

Dorsal subregions showed similar patterns of GAD immunoreactivity throughout the rostral to caudal extent of the hippocampus. GAD+ cells were concentrated in str. oriens and pyramidal cell layers of CA1, CA2, and CA3, whereas GAD+ cells in the subiculum were more evenly distributed between layers. In CA1 and the subiculum, there was a concentration of GAD+ cells at the border between str. radiatum and str. lacunosum-moleculare. In the dorsal dentate gyrus, the majority of GAD+ cells were located in the subgranular zone and the hilus; few GAD+ cells were observed in the molecular layers. In the ventral CA2 and CA3 subregions, the vast majority of GAD+ cells were located in str. oriens and pyramidal cell layers. Fewer GAD+ cells were observed in the ventral subiculum than ventral CA3. In the ventral dentate gyrus, particularly at the middle level, many GAD+ cells were observed and these were evenly distributed across layers. The total numbers of GAD+ cells for each subregion of the hippocampus are shown in **Table 1**.

		ERα+ (# of cells)	GAD+ (# of cells)
Rostral			
	Subiculum	787 ± 138	5261 ± 739
	CA1	1056 ± 246	7085 ± 751
	CA2		2544 ± 205
		2918 ± 522	
	CA3		9293 ± 830
	Dentate gyrus	1786 ± 333	10,330 ± 754
Middle Dorsal			
	Subiculum	1075 ± 127	8774 ± 975
	CA1	1363 ± 263	7738 ± 676
	CA2		1997 ± 350
		5395 ± 906	
	CA3		9715 ± 909
	DG	730 ± 173	8237 ± 773
Ventral			
	CA2/CA3	47,155 ± 4785	27,667 ± 4214
	Dentate gyrus	2592 ± 687	5818 ± 1506
Caudal Dorsal			
	Subiculum	614 ± 87	11,712 ± 1397
	CA1	2189 ± 327	11,693 ± 1629
	Dentate gyrus	2131 ± 137	9677 ± 913
Ventral			
	Subiculum	13,728 ± 1293	21,485 ±2941
	CA1	1843 ± 449	8774 ± 1170
	CA2	9274 ± 1353	12,614 ± 1348
	CA3	7603 ± 885	24,461 ± 2500
	Dentate gyrus	2515 ± 396	14,304 ± 3443

Table 1. Estimated number of ERα+ cells or GAD+ cells in the hippocampus by level and subregion.
Data are presented as mean ± SEM.

Overview of ERα and GAD colocalization

Cells expressing both GAD and ERα were found in all subregions and layers of the hippocampus at all rostral to caudal levels. As in singly labeled tissue, ERα immunostaining was localized to the nucleus of labeled cells while GAD labeling was present in the cell body, dendrites, and axons. Double labeled cells exhibited variable intensity of staining for both ERα and GAD. Most ERα+ cells in dendritic layers were GAD+. However, neither location of the soma nor morphological features, such as size or shape, distinguished a particular subset of GAD+ cells (Freund and Buzsaki 1996; Parra et al. 1998) that expressed ERα. In order to describe the distribution of double labeled cells between layers and subregions, the proportion of all GAD labeled cells that were also labeled for ERα (%GAD+/ERα+) was calculated for each subregion and layer. Additionally, to describe ERα expression in non-GABAergic cells, the proportion of ERα labeled cells that were not immunoreactive for GAD (%ERα+/GAD-) was also calculated for each subregion and layer. These data are expressed as percentages in Tables 2-4. The SEM was quite high for some %ERα+/GAD- values due to the fact that some brains had no ERα+/GAD- cells in layers that on average had very low numbers of ERα+ neurons. This is particularly clear in the molecular layers of the dentate gyrus and str. oriens of the rostral CA2.

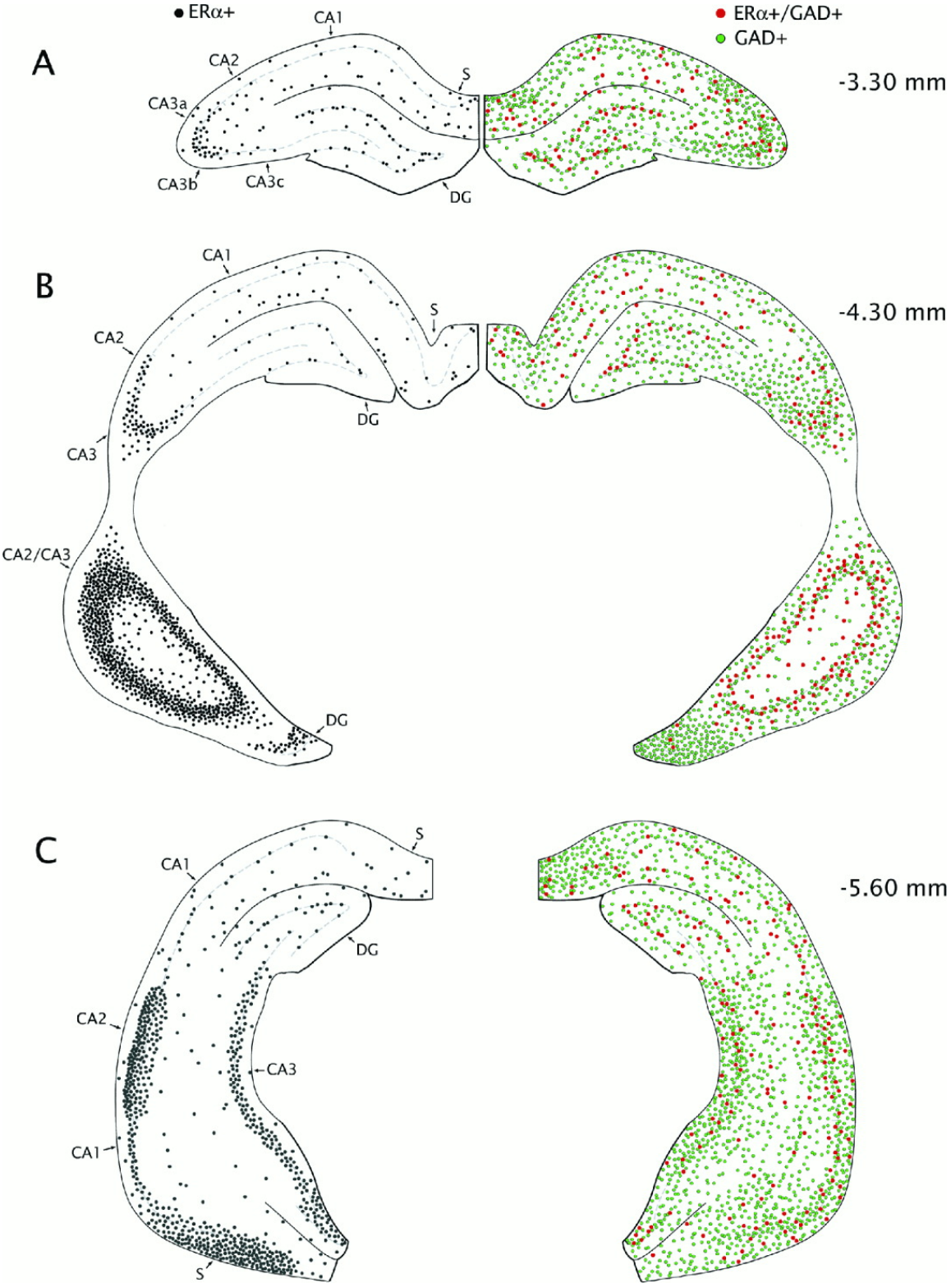
● ERα+
● ERα+/GAD+
● GAD+
A
CA1
CA2
CA3a
CA3b
CA3c
S
DG
-3.30 mm
B
CA1
CA2
CA3
CA2/CA3
S
DG
-4.30 mm
C
CA1
CA2
CA3
CA1
S
DG
S
-5.60 mm

Figure 2. Templates of ERα+, GAD+, and ERα+/GAD+ cells.

Templates showing the distributions of estrogen receptor (ER)α + (black dots, left side), glutamic acid decarboxylase (GAD)+ (green dots, right side), and ERα+/GAD+ (red dots, right side) cells at the rostral (**A**), middle (**B**), and caudal (**C**) levels of the hippocampus. Distance from bregma is indicated to the right of each template. Each dot represents one cell per 20 μm depth of section. Each template depicts summarized information for the rostral, middle, or caudal level. Drawings are modified from Paxinos and Watson (1998).

Distribution of ERα and GAD in the rostral hippocampus (-2.8 to -3.9mm from bregma)

Pattern of ERα labeling

The rostral hippocampus contained a relatively sparse distribution of ERα+ cells, most of which were labeled for GAD and thus appear to be GABAergic neurons. However, even though ERα+ nuclei were found primarily in GAD+ cells, double labeled cells represent only a small subset of all GAD+ cells in the rostral hippocampus (**Fig. 2A**). Quantitative analysis with the optical disector/fractionator method revealed a total of 6,547 ± 1,240 ERα+ cells in the rostral hippocampus. In the subiculum and CA1, ERα+ cells were located primarily in dendritic layers. **Figure 3** shows single labeling for ERα in the rostral CA1. Examination of cresyl violet counter-stained tissue indicated that fewer than 1% of cells in the CA1 pyramidal cell layer and fewer than 2% of cells in the subicular pyramidal cell layer were ERα+. In CA2 and CA3, ERα+ cells were also observed in the dendritic layers, however, the majority were concentrated in the pyramidal cell layer, particularly in CA3a and CA3b; few ERα+ cells were observed in CA3c. Approximately 30% of neuron-like cells in the rostral CA3a and CA3b pyramidal cell layer were ERα+. In the rostral dentate gyrus, most ERα+ cells were located in the subgranular zone, with a slightly higher concentration along the border of the suprapyramidal compared to the infrapyramidal blade of the granule cell layer.

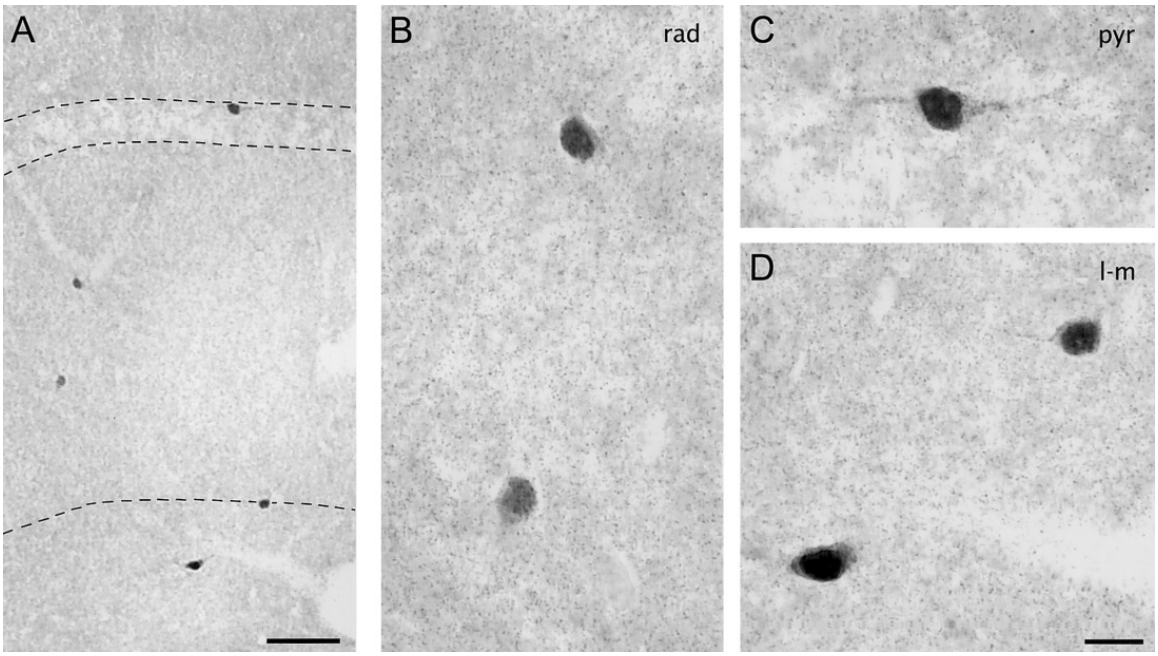

Figure 3. Single labeling for estrogen receptor (ER)α in dorsal CA1.

A: View of dorsal CA1 at low magnification showing ERα+ nuclei in all layers except the str. oriens. The two upper dashed lines indicate the boundary of the pyramidal cell layer and the lower dashed line is the border between the str. radiatum and str. lacunosum-moleculare. B-D: High magnification views of the same cells shown in panel A. **B:** ERα+ nuclei in the str. radiatum (rad). **C:** ERα+ nucleus in the pyramidal cell layer (pyr). **D:** ERα+ nuclei in the str. lacunosum-moleculare (l-m); the upper labeled nucleus is on the border with the str. radiatum and the other is deeper within the layer. Scale bar = 50 μm in A, 10 μm in D (applies to B,C,D).

Co-localization of ERα and GAD

Quantitative analysis of ERα/GAD co-labeling showed that GAD+ cells account for most of the ERα expressed in the rostral hippocampus, but only a subset of GAD+ cells in any subregion express ERα. **Figure 4** shows double-labeling for ERα and GAD in the rostral CA1. Percentages of GAD+/ERα+ cells in the rostral hippocampus are broken down by subregion and layer in **Table 2**. The greatest proportions of GAD+ cells that co-express ERα were found along the border between the str. radiatum and str. lacunosum-moleculare in the subiculum and CA1, and on the border between the hilus and suprapyramidal blade of the dentate gyrus granule cell layer. In each of these areas, more than 30% of GAD+ cells are also ERα+. Between 13-19% of GAD+ cells in the str. radiatum of subiculum, CA1, CA2 and CA3 are ERα+. Generally about 10% of GAD cells in the pyramidal cell layers express ERα. The percentage of GAD+/ERα+ cells in the str. oriens was only 5-6%. This low percentage is due to both a high density of GAD+ cells and a particularly low density of ERα+ cells in this layer. In the dentate gyrus molecular layers, percentages of GAD+/ERα+ cells were also very low, but there were very few GAD+ or ERα+ cells in these layers.

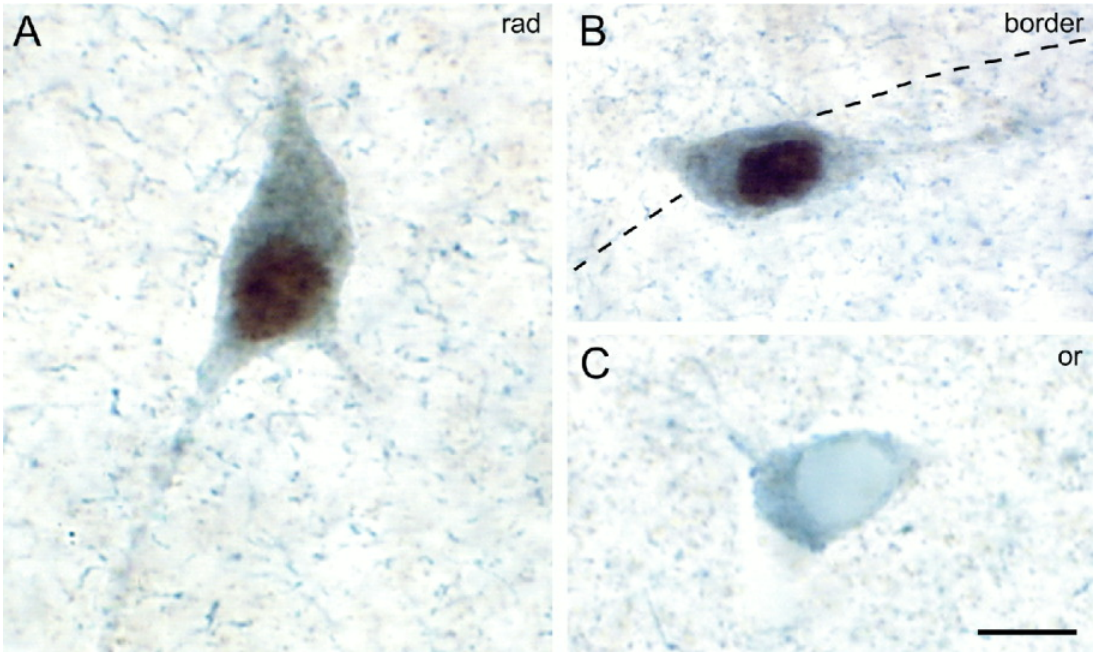

Figure 4. Double labeling for estrogen receptor alpha (ERα) and gultamic acid decarboxylase (GAD) in dorsal CA1.

A: Cell double labeled for ERα (dark brown nucleus) and GAD (blue cytoplasm) in the str. radiatum (rad). **B:** Cell immunoreactive for ERα (dark brown nucleus) and GAD (blue cytoplasm) on the border between the str. radiatum and str. lacunosum-moleculare. Dashed line indicates the border between the two layers. **C:** Cell immunoreactive for GAD (blue cytoplasm) in the str. oriens (or). Note the clear, unlabeled nucleus indicating that this cell is ERα-. Scale bar = 10 μm.

Subregion	Layer	%GAD+/ERα+	%ERα+/GAD-
Subiculum			
	oriens	6.5 ± 1.5	8.9 ± 6.4
	pyramidale	9.7 ± 1.9	11.6 ± 7.2
	radiatum	13.9 ± 1.8	7.1 ± 4.7
	border	31.1 ± 2.2	3.2 ± 2.7
	lacunosum-moleculare	12.1 ± 2.2	8.8 ± 7.7
CA1			
	oriens	5.4 ± 0.5	6.7 ± 5.5
	pyramidale	10.3 ± 1.1	10.0 ± 6.7
	radiatum	14.1 ± 1.1	8.3 ± 5.0
	border	31.9 ± 1.6	1.9 ± 1.6
	lacunosum-moleculare	10.5 ± 2.2	15.4 ± 7.7
CA2			
	oriens	5.4 ± 1.8	20.0 ± 20.0
	pyramidale	13.8 ± 3.1	46.0 ± 9.8
	radiatum	18.8 ± 3.2	7.8 ± 4.8
CA3			
	oriens	6.2 ± 1.3	11.2 ± 5.0
	pyramidale	9.8 ± 2.2	76.9 ± 5.1
	lucidum	12.6 ± 1.7	8.8 ± 3.8
	radiatum	13.2 ± 1.3	4.4 ± 3.1
Dentate gyrus			
	molecular-suprapyramidal	5.1 ± 1.0	22.1 ± 10.9
	gcl-suprapyramidal	31.3 ± 0.9	5.4 ± 3.6
	hilus	7.5 ± 0.9	7.3 ± 4.0
	gcl-infrapyramidal	22.8 ± 1.9	4.7 ± 3.7
	molecular-infrapyramidal	3.2 ± 1.8	12.0 ± 12.0

Table 2. Percentages of GAD+/ERα+ and ERα+/GAD- cells in the rostral hippocampus by subregion.

%GAD+/ERα+ is the percentage of all GAD+ cells that are also immunoreactive for ERα. %ERα+/GAD- is the percentage of all ERα+ cells that are not GAD+. Data are presented as mean ± SEM. Border – border region between str. radiatum and str. lacunosum-moleculare, gcl – granule cell layer.

ERα labeling in GAD- cells

In contrast to CA1 and the subiculum, we detected ERα labeling of GAD- cells in the pyramidal cell layers of CA2 and CA3. Percentages of ERα+/GAD- cells in the rostral hippocampus are broken down by subregion and layer in **Table 2**. Forty-six percent of ERα+ cells in the CA2 pyramidal cell layer and 77% in the CA3 pyramidal cell layer were GAD-, compared to only 8% in all other layers and subregions combined. Based on their morphology, ERα+/GAD- cells in the CA3 pyramidal cell layer are likely to be pyramidal cells. Comparison of the %ERα+/GAD- cells in the CA3 pyramidal cell layer with the estimation from counter-stained tissue that 30% of all neuron-like cells in the CA3 pyramidal cell layer are ERα+ indicates that approximately 23% of CA3 pyramidal cells in the rostral hippocampus express ERα.

Distribution of ERα and GAD in the middle hippocampus (-3.9 to -4.6mm from bregma)

Pattern of ERα labeling

The dorsal and ventral portions of the middle hippocampus showed distinctly different patterns of ERα immunoreactivity. ERα labeling in the dorsal middle hippocampus was similar to that in the rostral hippocampus, except that there were slightly more labeled cells in the CA2 pyramidal cell layer and slightly fewer in the subgranular zone of the dentate gyrus (**Fig. 2B**). Quantitative analysis with the optical disector/fractionator revealed 8,563 ± 1,469 ERα+ cells in the dorsal middle hippocampus. In contrast, the CA2/CA3 pyramidal cells layers of ventral middle sections

showed substantially more ERα labeling than any other area of the hippocampus (**Fig. 2B**). Quantitative analysis with the optical disector/fractionator revealed 49,747 ± 5,472 ERα+ cells in the ventral middle hippocampus. Examination of cresyl violet counter-stained tissue showed that 50-60% of neuron-like cells in the ventral CA2/CA3 pyramidal cell layer were ERα+. **Figure 5A and 5C** show single labeling for ERα in the ventral CA2/CA3 pyramidal cell layer; figure **5B** shows that no labeled cells were observed in tissue treated with preadsorbed antisera. Greater concentrations of ERα+ cells in ventral CA2/CA3 were found in more rostral sections and decreased caudally. Additionally, a substantial number of ERα+ cells were located in the ventral dentate gyrus, the majority of which were located in the hilus.

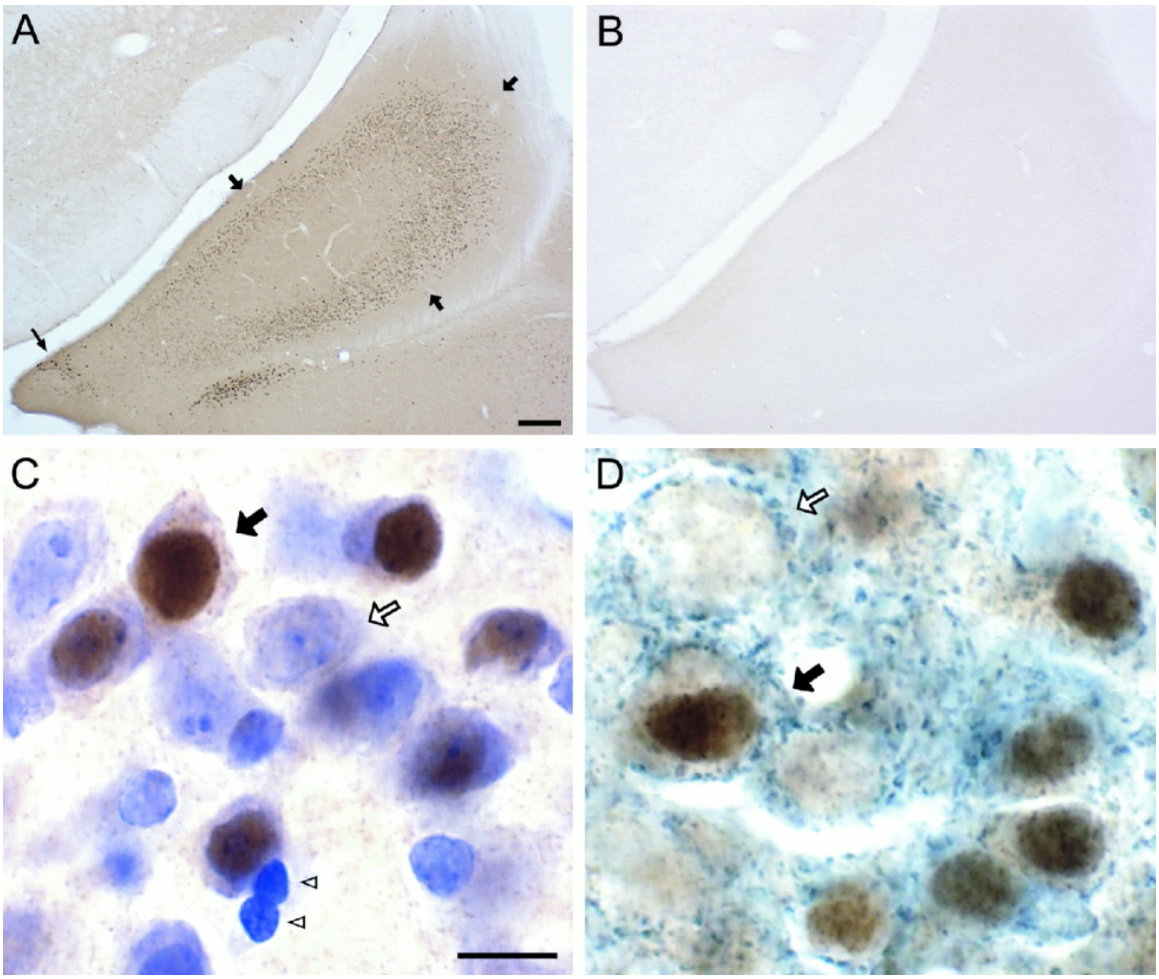

Figure 5. Estrogen receptor (ER)α and glutamic acid decarboxylase (GAD) immunoreactivity in the ventral CA2/CA3.

A: Low magnification view of ERα-immunoreactive cells in the ventral CA2/CA3 from tissue processed with MC-20. Many ERα+ cells are visible in CA2/CA3 and are concentrated in the cell body layer (wide arrows). Note there is also a high intensity of ERα labeling in the dentate gyrus (thin arrow). **B:** Low magnification view of approximately the same area shown in A from tissue processed with MC-20 preadsorbed with blocking peptide. No ERα labeling is detected. **C:** High magnification view of the

ventral CA3 pyramidal cell layer from tissue labeled with MC-20 and counterstained with cresyl violet. ERα+ nuclei are detected in 50-60% of neuron-like cells in this layer. **D:** High magnification view of the ventral CA3 pyramidal cell layer from tissue double labeled for both ERα (MC-20) and GAD 65/67. Note that cells with ERα+ nuclei are surrounded by GAD+ puncta (blue) but are not GAD+ themselves (cytoplasm is not blue). In C and D, a solid, wide arrow indicates a representative neuron-like cell that is ERα+; an open, wide arrow indicates a neuron-like cell that is ERα-; open arrowheads indicate glia-like cells, which are not immunoreactive for ERα. Scale bar = 200 μm in A (applies to A,B), 10 μm in C (applies to C,D).

Co-localization of ERα and GAD

In the dorsal middle hippocampus, the highest %GAD+/ERα+ cells were again located on the border between str. radiatum and str. lacunosum-moleculare of subiculum and CA1, as well as in the subgranular zone adjacent to the suprapyramidal blade of the granule cell layer. Percentages of GAD+/ERα+ and %ERα+/GAD- cells in the dorsal middle hippocampus are broken down by subregion and layer in **Table 3**. As before, low percentages of double labeled cells were found in the str. oriens. In the dentate gyrus, the %GAD+/ERα+ cells associated with the granule cell layers were higher than other areas of the dentate at this level, but lower than in the rostral hippocampus. The molecular layers of the dentate gyrus had the lowest percentages of GAD+/ERα+ cells, reflecting very low ERα expression in these areas.

In the ventral middle hippocampus, cells labeled for both ERα and GAD were concentrated in the pyramidal cell layer and dispersed throughout the str. oriens. Our analysis revealed that 25% of the of GAD+ cells in the CA2/CA3 pyramidal cell layer were ERα+, whereas only 9% of GAD+ cells in the str. oriens were double labeled for ERα.

Subregion	Layer	%GAD+/ERα+	%ERα+/GAD-
Dorsal			
Subiculum			
	oriens	5.4 ± 1.1	17.8 ± 10.9
	pyramidale	7.4 ± 1.0	18.2 ± 9.5
	radiatum	15.1 ± 1.2	16.9 ± 10.2
	border	29.9 ± 2.1	9.5 ± 6.6
	lacunosum-moleculare	9.9 ± 2.0	20.5 ± 10.4
CA1			
	oriens	5.4 ± 0.7	19.6 ± 10.3
	pyramidale	10.5 ± 1.4	19.8 ± 9.6
	radiatum	13.2 ± 1.3	17.9 ± 10.7
	border	30.4 ± 2.4	18.6 ± 10.1
	lacunosum-moleculare	9.1 ± 2.2	6.9 ± 2.5
CA2			
	oriens	6.7 ± 2.8	20.0 ± 9.8
	pyramidale	13.9 ± 3.1	70.2 ± 8.1
	radiatum	16.2 ± 3.5	16.2 ± 10.8
CA3			
	oriens	4.6 ± 1.0	44.9 ± 16.5
	pyramidale	9.9 ± 2.3	84.9 ± 5.8
	lucidum	10.3 ± 1.4	21.0 ± 10.2
	radiatum	12.0 ± 1.8	16.6 ± 9.1
Dentate gyrus			
	molecular-suprapyramidal	1.6 ± 0.7	27.1 ± 11.3
	gcl-suprapyramidal	24.0 ± 2.1	15.6 ± 8.6
	hilus	6.4 ± 0.8	19.2 ± 9.2
	gcl-infrapyramidal	15.2 ± 2.4	17.4 ± 10.3
	molecular-infrapyramidal	0.3 ± 0.3	40.0 ± 22.3
Ventral			
CA2/CA3			
	oriens	9.0 ± 2.1	54.1 ± 10.5
	pyramidale	25.0 ± 5.6	93.9 ± 1.4

Table 3. Percentages of GAD+/ERα+ and ERα+/GAD- cells in the middle hippocampus by subregion.

%GAD+/ERα+ is the percentage of all GAD+ cells that are also immunoreactive for ERα. %ERα+/GAD- is the percentage of all ERα+ cells that are not GAD+. Data are presented as mean ± SEM. Border – border region between str. radiatum and str. lacunosum-moleculare, gcl – granule cell layer.

ERα labeling in GAD- cells

ERα+/GAD- labeling in the CA2 and CA3 pyramidal cell layers was slightly greater in the dorsal middle than rostral hippocampus, whereas fewer ERα+/GAD+ cells were observed in the dendritic layers of CA2 and CA3 in the middle sections. This change represents the beginning of a dorsal to ventral shift in ERα labeling from being roughly equal between dendritic and pyramidal cell layers to a predominance of ERα+ labeling in pyramidal cell layers. This shift is due primarily to an increase in ERα+/GAD- cells. Percentages of ERα+/GAD- cells in the dorsal middle hippocampus are broken down by subregion and layer in **Table 3**. In dorsal middle sections, comparison of %ERα+/GAD- and % total neuron-like cells that are ERα positive (from counter-stained tissue) indicated that approximately 30% of putative CA2 and CA3 pyramidal cells were labeled for ERα.

ERα immunoreactivity in the ventral portion of the middle hippocampus was strikingly different from that in the dorsal subregions (**Fig. 2B**). The vast majority, 93.9%, of ERα+ nuclei in the ventral CA2/CA3 were found in GAD- cells in the pyramidal cell layer. **Figure 5D** shows examples of ERα+/GAD- cells in the ventral CA3 pyramidal cell layer. Considered in conjunction with the approximately 47,000 ERα+ cells in CA2/CA3, these data reveal the presence of an enormous number of non-inhibitory cells that express ERα in the ventral hippocampus.

Distribution of ERα and GAD in the caudal hippocampus (-4.6 to -5.8mm from bregma)

Pattern of ERα labeling

The dorsal to ventral gradient of increased ERα labeling continued in sections from the caudal hippocampus. The most striking features of the caudal hippocampus were robust expression of ERα in the CA2 and CA3 pyramidal cell layers and in the ventral subiculum (**Fig. 2C**). Quantitative analysis with the optical disector/fractionator revealed a total of 39,898 ± 4,927 ERα positive cells in the caudal hippocampus. Eighty-eight percent of all ERα+ nuclei in the caudal hippocampus were located in the pyramidal cell layers of CA2, CA3, and the ventral subiculum. ERα+ nuclei in these subregions tended to be concentrated along the basal side of the pyramidal cell layer, particularly in CA2. Examination of cresyl violet counter-stained tissue indicated that approximately 35% of cells in the CA2, 15% of cells in the CA3 and 20% of cells in the subicular pyramidal cell layer were ERα+.

Co-localization of ERα and GAD

The same trends in distribution of GAD+/ERα+ cells described for the rostral and dorsal middle hippocampus were also evident in the dorsal caudal hippocampus. Percentages of GAD+/ERα+ cells in the caudal hippocampus are broken down by subregion and layer in **Table 4**. In dorsal subregions, %GAD+/ERα+ cells were greatest at the border between the str. radiatum and str. lacunosum-moleculare and in the suprapyramidal subgranular zone. However in each case, values were somewhat lower

than in these same areas in the rostral and middle hippocampus. Again, str. oriens generally showed low percentages of GAD+/ERα+ cells.

In the ventral caudal hippocampus, the greatest %GAD+/ERα+ cells was seen in the CA2 pyramidal cell layer, where nearly 20% of GAD+ cells were also ERα+. Numbers of double labeled cells were similar in the caudal CA3 as in middle sections. However, because numbers of GAD+ cells were higher in caudal than middle CA3, values for %GAD+/ERα+ were slightly lower at the caudal level.

Subregion	Layer	%GAD+/ERα+	%ERα+/GAD-
Dorsal			
Subiculum			
	oriens	5.1 ± 1.1	17.2 ± 10.6
	pyramidale	6.7 ± 0.8	16.4 ± 9.7
	radiatum	12.2 ± 1.5	21.3 ± 10.7
	border	26.9 ± 2.1	8.2 ± 5.1
	lacunosum-moleculare	7.9 ± 2.3	27.0 ± 15.2
CA1			
	oriens	7.6 ± 0.5	21.4 ± 9.2
	pyramidale	11.7 ± 1.7	45.8 ± 7.9
	radiatum	13.2 ± 1.4	17.2 ± 7.9
	border	25.1 ± 2.0	10.0 ± 7.9
	lacunosum-moleculare	7.2 ± 1.4	17.0 ± 10.8
Ventral			
Subiculum			
	oriens	8.0 ± 0.9	93.2 ± 1.4
	pyramidale	9.8 ± 1.1	94.4 ± 0.8
	radiatum	18.7 ± 1.2	18.0 ± 5.9
	border	21.0 ± 1.9	4.7 ± 2.0
	lacunosum-moleculare	14.3 ± 0.9	8.6 ± 4.0
CA1			
	oriens	8.5 ± 2.2	50.4 ± 16.1
	pyramidale	14.8 ± 3.0	83.9 ± 3.5
	radiatum	13.9 ± 1.7	27.0 ± 10.7
	border	21.8 ± 2.2	4.5 ± 3.1
	lacunosum-moleculare	6.6 ± 1.9	14.3 ± 10.6
CA2			
	oriens	5.6 ± 1.7	55.7 ± 9.1
	pyramidale	19.9 ± 5.7	91.4 ± 2.6
	radiatum	17.2 ± 1.5	17.1 ± 7.2
CA3			
	oriens	9.9 ± 2.7	38.9 ± 11.9
	pyramidale	11.0 ± 2.7	89.7 ± 1.9
	radiatum	12.5 ± 2.0	18.7 ± 8.5
Dentate gyrus			
	molecular-suprapyramidal	2.5 ± 0.5	22.6 ± 14.5
	gcl-suprapyramidal	21.1 ± 2.9	15.4 ± 9.5
	hilus	7.4 ± 0.9	25.6 ± 10.6
	gcl-infrapyramidal	13.4 ± 3.1	30.3 ± 10.9
	molecular-infrapyramidal	0.8 ± 0.5	18.8 ± 11.5

Table 4. Percentages of GAD+/ERα+ and ERα+/GAD- cells in the caudal hippocampus by subregion.

%GAD+/ERα+ is the percentage of all GAD+ cells that are also immunoreactive for ERα. %ERα+/GAD- is the percentage of all ERα+ cells that are not GAD+. Data are presented as mean ± SEM. Border – border region between str. radiatum and str. lacunosum-moleculare, gcl – granule cell layer.

ERα labeling in GAD- cells

Many ERα+ nuclei were observed in the caudal hippocampus, and the vast majority of these were in GAD- cells. Percentages of ERα+/GAD- cells in the caudal hippocampus are broken down by subregion and layer in **Table 4**. ERα+ nuclei were concentrated in the CA2 and CA3 pyramidal cell layers and ventral subiculum, where values for %ERα+/GAD- were 91.4%, 89.7% and 94.4%, respectively. In conjunction with over 35,000 ERα+ nuclei estimated in the ventral portion of the caudal hippocampus, these data suggest a very large number of ERα+ pyramidal cells in the ventral caudal hippocampus.

DISCUSSION

Two principal findings emerged from our analysis of ERα and GAD colocalization in the hippocampus. First, our study revealed many more ERα labeled cells in the hippocampus than any previous studies (DonCarlos et al. 1991; Shughrue et al. 1997; Weiland et al. 1997; Laflamme et al. 1998). For example, we detected approximately 7 times more ERα labeled cells in the dorsal hippocampus and 1000 times more ERα labeled cells in the ventral hippocampus than Weiland et al. (1997). Second, we observed distinct dorsal versus ventral patterns of hippocampal ERα expression. At each rostral to caudal level of the dorsal hippocampus, ERα was expressed primarily in a small subset of GAD+ cells. Based on their GAD immunoreactivity, as well as their scarcity, location in dendritic layers and morphology, these cells are likely to be

GABAergic interneurons. Though no distinct subpopulation of GABA neurons (Freund and Buzsaki 1996; Parra et al. 1998) that express ERα could be identified, we did observe a concentration of double labeled cells at the border between str. radiatum and str. lacunosum-moleculare. In addition to ERα expression in GABAergic cells in dendritic layers, some ERα labeling was also observed in GAD- cells in the CA2 and CA3 pyramidal cell layers of the dorsal hippocampus. ERα labeling in GAD- cells increased dramatically in the ventral portions of middle and caudal hippocampal sections. Calculations based on total numbers of ERα+ cells and %ERα+/GAD- cells suggest upwards of 70,000 non-GABAergic cells in the ventral hippocampus express ERα. Given the numbers of labeled cells, their locations and lack of immunoreactivity for GAD, the vast majority of these are certainly subicular, CA2, and CA3 pyramidal cells. In parallel with the ventral increase in ERα labeling of GAD- cells in the pyramidal cell layers, ERα labeling in GAD+ cells in the dendritic layers decreased slightly. Together, these trends result in a dorsal to ventral shift in toward increased ERα expression in pyramidal cells.

We are confident about the specificity of labeling for ERα in this study because we saw approximately the same patterns of labeling using two different antibodies, MC-20, a polyclonal antisera raised against the C-terminus of ERα and 6F11, a monoclonal antibody raised against the full length of ERα. Further, preadsorbtion of MC-20 with its blocking peptide resulted in no ERα labeling. It is possible that differences in the sensitivity of MC-20 and 6F11 versus H222 (DonCarlos et al. 1991) and AS409 (Weiland

et al. 1997) account for the differences in quantity of ERα labeling between ours and previous studies.

Interestingly, a recent *in vivo* autoradiographic analysis of estrogen binding sites showed high levels of estrogen binding in the dorsal CA2/CA3 and multiple regions of the ventral hippocampus (Shughrue and Merchenthaler 2000). High estrogen binding was surprising given the low signal intensity for both ERα and ERβ mRNA detected with *in situ* hybridization in the same study and previously (Simerly et al. 1990; Shughrue et al. 1997; Laflamme et al. 1998). The ER ligand used by Shughrue and Merchenthaler (2000) was 17α-iodovinyl-11β-methoxyestradiol (^{125}I-estrogen), which has high affinity for both known forms of the nuclear ER, ERα and ERβ. Use of this probe for estrogen concentrating cells represented an important advance over previous autoradiographic studies, which used ^{3}H-estradiol and reported low estrogen binding (Pfaff and Keiner 1973; Stumpf et al. 1975; Loy et al. 1988). In our tissue labeled with antisera to ERα, we observed that ERα+ cells in brain regions identified in the ^{3}H-estradiol studies as locations of ERs, such as the hypothalamus and the amygdala, contained cells that were more intensely stained than most of the ERα+ cells in the hippocampus. This suggests that the level of ERα expressed by many hippocampal ERα+ cells was below the threshold for detection in these earlier studies. In contrast, the pattern of ERα expression detected in our study is virtually identical to that of estrogen-concentrating cells labeled with ^{125}I-estrogen (Shughrue and Merchenthaler 2000). Thus, ERα may account for much of the hippocampal ^{125}I-estrogen binding. Furthermore, our findings suggest that

most of the ^{125}I-estrogen-concentrating cells in the ventral hippocampus and in the dorsal CA2 and CA3 subregions are likely to be pyramidal cells.

Differences in patterns of ERα immunoreactivity suggest different roles for estrogen in regulation of function in dorsal versus ventral hippocampus. This possibility is consistent with well-documented dorsal/ventral differences in the distributions of neurotransmitters and chemical markers, such as choline acetyltransferase, serotonin, GABA, calretinin, and nitric oxide synthase (Hortnagl et al. 1991; Nomura et al. 1997) and in regulation of behaviors such as spatial learning (Moser et al. 1993; Hock and Bunsey 1998; Vann et al. 2000). Additionally, estrogen has been shown to differentially affect the dorsal versus ventral hippocampus in terms of seizure susceptibility (Buterbaugh and Hudson 1991). Based on our findings, estrogen is likely to directly affect a relatively small number of GABAergic interneurons in the dorsal hippocampus, while it can directly affect both some GABAergic and many non-GABAergic neurons in the ventral hippocampus.

A growing body of evidence suggests that estrogen may produce structural and functional changes in hippocampal pyramidal cells, at least in part, by acting directly on GABAergic interneurons. Estrogen has been shown to regulate hippocampal GAD mRNA levels (Weiland 1992) and GAD 65 (but not GAD 67) immunoreactivity (Rudick and Woolley 2001). Additionally, experiments on cultured hippocampal neurons (Murphy et al. 1998) have demonstrated that estrogen acts first on GABAergic interneurons to suppress GAD and GABA levels, which results in a transient disinhibition of pyramidal cells. This transient disinhibition is required for subsequent

changes in pyramidal cell dendritic spine density *in vitro*. Consistent with a similar mechanism *in vivo*, estrogen treatment also has been shown to transiently disinhibit CA1 pyramidal cells in adult animals (Rudick and Woolley 2001).

The observation that ERα is expressed in GABAergic interneurons throughout all subregions and all levels of the hippocampus is consistent with the suggestion that estrogen acts directly on GABAergic interneurons to regulate the activity of hippocampal pyramidal cells. Since a single inhibitory interneuron forms synapses with a few thousand pyramidal cells (Buhl et al. 1994; Sik et al. 1995; Freund and Buzsaki 1996), even the small number of GAD+/ERα+ cells we observed could dramatically affect the function of many hippocampal pyramidal cells. Further, we saw the greatest concentration of double-labeled cells at the border between str. radiatum and str. lacunosum-moleculare. These cells may be particularly well-suited to influence many pyramidal cells, as some interneurons at the str. radiatum/str. lacunosum-moleculare border project to other interneurons (Kunkel et al. 1988; Lacaille and Schwartzkroin 1988). Since most hippocampal interneurons are inhibitory (Freund and Buzsaki 1996), interneuron to interneuron connections provide the possibility of multiplicative increases in the number of pyramidal cells under the inhibitory influence of a few interneurons.

In summary, our data reveal that ERα expression is much more prevalent in the hippocampus than previously appreciated. Hippocampal ERα is expressed in both GABAergic and non-GABAergic neurons. In the dorsal hippocampus, ERα expression in GABAergic neurons predominates. More complete characterization of ERα+ GABAergic interneurons may elucidate the pathway through which estrogen affects

hippocampal pyramidal cells indirectly through direct action on GABAergic interneurons. In the ventral hippocampus, where numbers of ERα expressing neurons are particularly high, ERα expression in non-GABAergic neurons predominates. This finding strongly suggests that estrogen can regulate hippocampal function through direct effects on pyramidal cells, in addition to effects mediated indirectly by inhibitory interneurons.

CHAPTER 3 - ERα-IR IN PERISOMATIC INHIBITORY BOUTONS IN THE DORSAL CA1 PYRAMIDAL CELL LAYER OF THE HIPPOCAMPUS

ABSTRACT

The classical view of estrogen action is through nuclear receptor transcription factors. Interestingly, many estrogen effects, both structural and physiological, have been observed in the hippocampus, a brain area that contains relatively few cells expressing nuclear ERs. Some of these effects occur within minutes of estrogen exposure, too fast to occur via a classical nuclear ER mechanism, suggesting that some estrogen effects in the hippocampus are mediated through membrane ERs. Extranuclear ERα-IR is observed in many structures in the hippocampus, including excitatory and inhibitory boutons, dendrites, and glia. Estrogen has been shown to regulate inhibitory synaptic transmission in the dorsal CA1 of the hippocampus, an effect that may, at least partially, be mediated through extranuclear ERα receptors. In order to investigate whether ERα located in inhibitory boutons could play a role in estrogen's effects on inhibitory synapses, I used electron microscopy to serially reconstruct ERα immunoreactive and non-immunoreactive inhibitory axon segments in the CA1 pyramidal cell layer of hippocampi from animals 24 hours after treatment with either estradiol or oil vehicle. Analysis of reconstructed axon segments revealed that extranuclear ERα-IR labels ~10% of the presynaptic vesicles in a subset of perisomatic inhibitory boutons on any given axon and that ERα-IR vesicles are always clustered together within a bouton. In animals treated with estradiol, ERα-IR vesicle clusters are located significantly closer to synapses

compared to oil controls, while the overall distribution of non-ERα-IR vesicles remains unaffected. Quantitative analysis indicates that those perisomatic boutons containing ERα-IR are not structurally different from non-ERα-IR boutons. The identification of a subpopulation of vesicles in inhibitory boutons that express ERα and are sensitive to estradiol reveals a novel action of estrogen in the brain and suggests that estrogen may act directly at inhibitory synapses to regulate synaptic transmission in the hippocampus.

INTRODUCTION

As discussed in Chapter 1, the structure and physiology of the hippocampus are sensitive to estradiol. Many effects of estradiol, such as changes in spine and synapse density in the dorsal CA1, are widespread and directly involve ~ 420,000 pyramidal cells in the CA1 alone (Boss et al. 1987). As discussed in the previous chapter, there are relatively few GABAergic interneurons that express nuclear ERα in the dorsal hippocampus. In fact, ERα+ interneurons account for less than 0.3% of the cells in the dorsal CA1. It seems unlikely that so few interneurons expressing nuclear ERα would be sufficient to mediate the many effects of estrogen observed in the dorsal CA1.

This thought caused me to reconsider an observation I made while analyzing the tissue in the previous experiment. While counting ERα+ nuclei, I noticed the presence of numerous puncta that appeared to be ERα-IR. The puncta were located in all regions of the hippocampus and in all sections examined. Many puncta were visible in the cell body layer, and the appearance of rings of puncta surrounding unlabeled pyramidal cells led

me to hypothesize that the puncta were extranuclear ERα-IR labeling perisomatic inhibitory boutons.

The presence of extranuclear ERα in perisomatic inhibitory boutons could potentially be very important in determining the mechanism of action for estradiol-induced disinhibition. Inhibition to dorsal CA1 pyramidal cells is significantly decreased 24 hours following estradiol treatment (Rudick and Woolley 2001; Rudick et al. 2003). The primary source of inhibition recorded in CA1 pyramidal cells comes from perisomatic inhibitory boutons that form synapses on the somata. If ERα was located within these boutons it could potentially mediate the effect of estradiol directly at the synapse. Extranuclear ERα-IR in the dorsal hippocampus has been reported previously (Milner et al. 2001). However, information about ERα in perisomatic inhibitory boutons was lacking.

In order to determine if perisomatic inhibitory boutons contained ERα-IR, I processed tissue for ERα immunohistochemistry and then used serial electron microscopy to identify ultrastructural ERα labeling. I created three-dimensional reconstructions and then used them to quantify ERα-IR structures within boutons. Additionally, I assessed the effect of estradiol on ERα-IR structures 24 hours following estradiol treatment. This point was chosen because it is the time of maximal disinhibition.

METHODS

Animals

Eight adult female Sprague-Dawley rats (~250 g) were maintained on a 12-hour light/dark cycle with free access to food and water. Animals were ovariectomized under ketamine (85 mg/kg) / xlyazine (13 mg/kg, i.p.) anesthesia and 3 days later, injected (s.c.) with 10 μg 17β-estradiol benzoate or oil vehicle as previously described (Rudick and Woolley 2001). Twenty-four hours after injection, animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and gravity perfused for 30 minutes with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (PB). All animal procedures were approved by the Northwestern University Animal Care and Use Committee.

Immunocytochemistry and EM processing

Immunostaining for ERα was as described in Chapter 2. Briefly, brains were removed, blocked to contain the hippocampus, postfixed overnight at 4^{o}C, cryoprotected with 30% sucrose, and cut using a freezing microtome (Leica Instruments) into coronal sections (100 μm) spanning the rostro-caudal extent of the dorsal hippocampus. Alternating sections were labeled with rabbit polyclonal MC-20 (0.5 μg/ml, Santa Cruz Biotechnology) or mouse monoclonal 6F11 (1:50, Novocastra) followed by species-specific biotinylated IgG (1:800, Vector laboratories), and visualized with diaminobenzidine (Sigma) using an ABC kit (Vector). Some sections were then dehydrated, cleared, and coverslipped under Eukitt for brightfield microscopy.

The remaining sections were processed for EM. Tissue was fixed with 1% osmium tetroxide in 0.2M PB for 45 minutes. After several rinsing steps, sections were dehydrated in graded alcohols, soaked in propylene oxide, and then placed in a mixture of 50% Eponate resin (Ted Pella) and 50% propylene oxide overnight at room temperature. Sections were then transferred into 75% resin and 25% propylene oxide for 24 hours at room temperature. Sections were finally transferred into 100% resin for 2 two-hour periods, flat embedded between two sheets of Aklar (Ted Pella), and placed in a 60^{o}C oven overnight. Portions of dorsal CA1 containing the pyramidal cell layer were cut out of the flat embedded sections and attached with resin to the end of a BEEM capsule before sectioning on an ultramicrotome (Leica Instruments). For each brain, 200–230 serial thin sections (~70 nm thick) were collected on formvar-coated slot grids and then stained with uranyl acetate and lead citrate (Ted Pella). As a control, thin sections were also prepared from tissue blocks that had been processed for ERα-IR but with the primary antiserum or antibody (MC-20 or 6F11) omitted. Immunoreactivity was absent in these sections, and no DAB reaction product was observed in any structures. Brains were coded prior to sectioning, so that the experimenter was blind to treatment condition during all phases of image collection, three-dimensional reconstruction, and analysis.

Three-dimensional EM reconstruction and analysis

Five areas in the dorsal CA1 pyramidal cell layer, each containing an ERα-IR perisomatic bouton, were identified in a section near the middle of each set of serial thin sections. Negatives of these 5 areas were taken at 10,000X on a JEOL 100CX electron

microscope from the middle section and from 40–50 serial sections on both sides (5 stacks of 80-100 serial negatives per brain). Negatives were scanned at 1600 dpi and aligned with sEM Align software (http://synapses.mcg.edu). The plasma membrane, synaptic densities, and mitochondria were traced and reconstructed using IGL Trace software (http://synapses.mcg.edu). The location of each vesicle in a bouton was also marked. Measurements of volume, synaptic area, and numbers of vesicles for each bouton were generated using IGL Trace. The distance between each vesicle and synapse in a bouton was measured using Reconstruct software (http://synapses.mcg.edu) and then converted into a measurement of relative distance. A relative distance value of 1.0 represents the farthest distance that a vesicle could be from the synapse. For boutons with more than one synapse, the synapse nearest to the ERα-IR vesicle cluster was used for the relative distance measurement.

Cumulative histograms of vesicle relative distances were generated for oil and estradiol treatment groups and compared using the Kolmogorov-Smirnoff test (D_{max} = 0.163 for $p<0.01$, $n=100$). The mean relative distance of ERα-IR vesicles, and means for measures of basic structural characteristics of boutons as well as fluorescence and co-localization were calculated for each brain, and treatment groups were compared using Student's t-test (unpaired, two-tailed, n=4) using SPSS software.

All figures were prepared with Photoshop (Adobe). Graphs and histograms were plotted using SigmaPlot (SPSS). Bouton reconstruction files were imported into and rendered with 3D StudioMax (Autodesk).

RESULTS

First, I examined the sections mounted for light microscopy for non-nuclear ERα-IR. Non-nuclear ERα labeling was present in all subregions and layers of the rostral hippocampus (**Fig. 6**). Many small, puncta were ubiquitous, especially in the str. pyramidale (**Fig. 6A**) and were occasionally observed to form rings around unlabeled pyramidal cells. No labeled puncta were observed in tissue processed with the primary antisera omitted (**Fig. 6B**). Western blots confirmed that MC-20 recognized a single protein band at ~67 kDa (**Fig. 6C**), the predicted size of ERα (Greene et al. 1986; Koike et al. 1987)

Second, I examined the tissue pre-embedded for EM immunocytochemistry. ERα-IR profiles were numerous in the pyramidal cell layer and proximal stratum radiatum. Labeled structures included boutons, axons, dendrites, somata, and glia. The distribution of ERα-IR was qualitatively similar to that reported by Milner et al. (2001). No immunoreactivity was observed in tissue processed with the primary antisera omitted.

I then investigated which structures within perisomatic inhibitory boutons contain ERα-IR (**Fig. 7**). Inhibitory boutons were recognized as those that formed synapses with symmetric pre- and postsynaptic densities, over 95% of which in the dorsal CA1 cell body layer are GABA-IR (Ledoux and Woolley 2005). Dense core vesicles were frequently observed within these same boutons. Electron imaging of tissue labeled for ERα using either the MC-20 antiserum (**Fig. 7A-C**) or the 6F11 antibody (**Fig. 7D**) revealed that ERα-IR within inhibitory boutons was most often found on presynaptic vesicles that appeared to be clustered. Occasionally, I also observed labeling on patches

of the plasma membrane near ERα-IR vesicles. Small clumps of ERα-IR were also present within pyramidal cell bodies, excitatory boutons, dendrites, and glia. The pattern of immunoreactivity was identical in tissue labeled with 6F11 compared to MC-20.

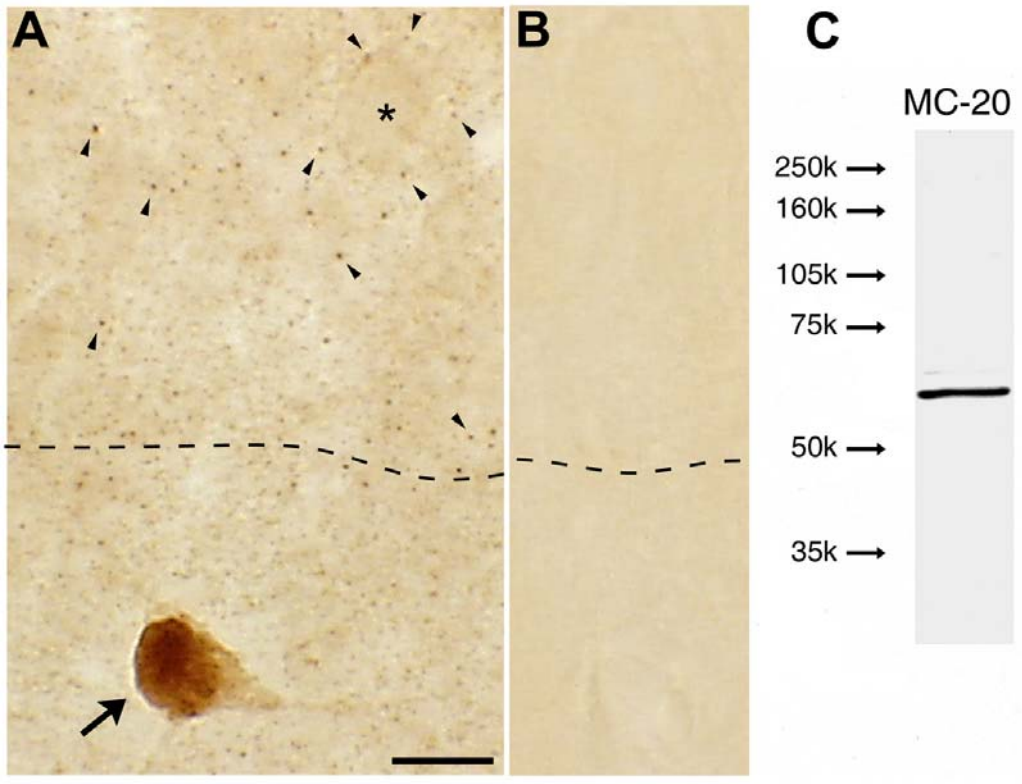

Figure 6. Punctate ERα-IR labeling in the dorsal CA1 cell body layer.

(**A**) High magnification view of ERα-IR puncta visualized with brightfield microscopy. ERα-IR puncta (small dark dots, arrowheads) are numerous and sometimes appear to ring unlabeled somata (asterisk). Dashed line delineates the boundary of the cell body layer and stratum radiatum. A cell with interneuron-like morphology located in the stratum radiatum contains a nucleus intensely labeled with ERα-IR (arrow). (**B**) High magnification view of the same area from tissue processed with primary antiserum omitted. No labeling is observed. (**C**) Western blot showing a single band at ~67 kDa labeled with MC-20, the antiserum we used for quantification of EM ERα-IR. Scale bar = 10 μm and applies to **A** and **B**.

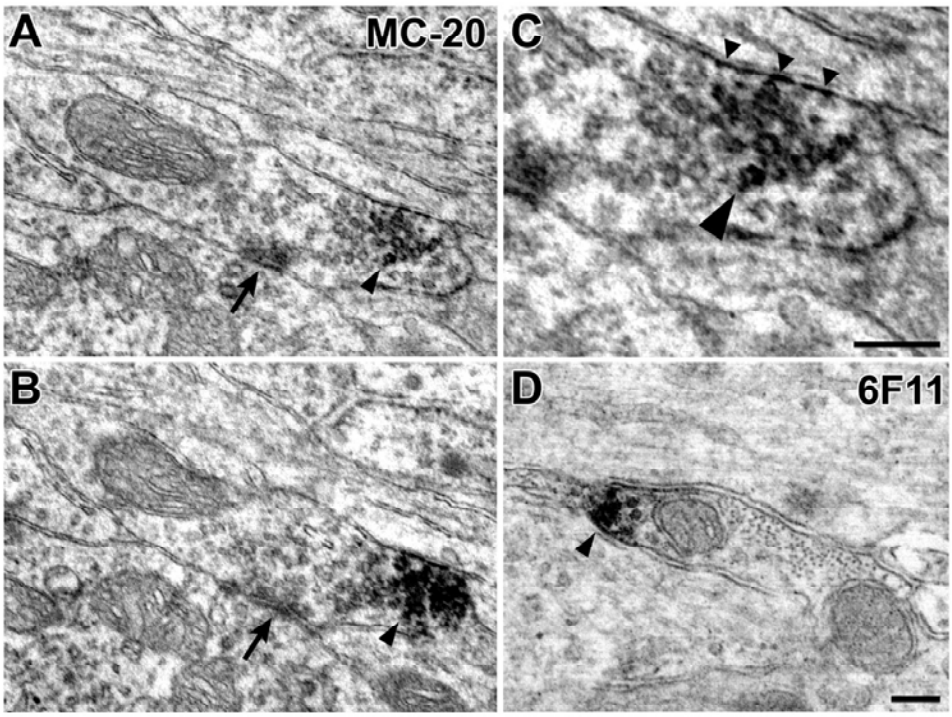

Figure 7. Ultrastructural localization of ERα-IR in perisomatic inhibitory boutons. (**A**, **B**) Two serial thin sections showing MC-20 labeling for ERα on a portion of vesicles (arrowhead) in a bouton forming a symmetric synapse (arrow) with a CA1 pyramidal cell soma. Note that ERα-IR vesicles are clustered. (**C**) Higher magnification of the same section as **A** shows ERα-IR associated with vesicles (arrowhead) and nearby on the bouton plasma membrane (small triangles). (**D**) ERα labeling with 6F11 is qualitatively similar to labeling with MC-20, with ERα-IR associated with vesicles and the bouton plasma membrane (arrowhead). Scale bars = 200 nm (bar in **D** also applies to **A** and **B**).

Next, we used serial section EM to generate three-dimensional reconstructions of axon segments containing ERα-IR vesicles to address three questions: 1) Does a single axon contain boutons both with and without ERα-IR vesicles? 2) Are boutons with ERα-IR vesicles structurally different from those without labeled vesicles? 3) Does estrogen treatment affect ERα-IR vesicles? We reconstructed axon segments containing ERα-IR in tissue from each of 8 rats, 4 OVX+O and 4 OVX+E (3-7 segments per rat). A total of 48 perisomatic inhibitory boutons from ERα-IR-containing axon segments were completely reconstructed and analyzed as were 32 neighboring inhibitory boutons that synapsed with the same CA1 pyramidal cells. Examination of reconstructed boutons confirmed that, when present, ERα-IR vesicles were invariably clustered (**Fig. 8 and 9**). Additionally, we found that clusters of labeled vesicles occur in only a subset of boutons on any single axon (**Fig. 8**). Fully reconstructed boutons on the same axon segment as ERα-IR boutons were never observed to contain any ERα-IR structures. Thirty-three of the 80 boutons that we reconstructed contained ERα-IR vesicle clusters (~ 41%). In boutons containing labeled clusters, ERα-IR vesicles accounted for 10.7±0.8% of all presynaptic vesicles. The number of vesicles per cluster did not differ between OVX+O and OVX+E boutons. Interestingly, patches of ERα-IR on the bouton plasma membrane were strongly associated with the presence of ERα-IR vesicles; we found only 1 bouton that lacked labeled vesicles and showed any detectable ERα-IR at the plasma membrane. None of the basic structural features of individual boutons: volume, presynaptic density area, single vs. multiple synapses, presence of mitochondria, total vesicle number or

vesicle density, was different between boutons containing ERα-IR clusters and those that did not, and none of these parameters were affected by estrogen treatment (**Table 5**).

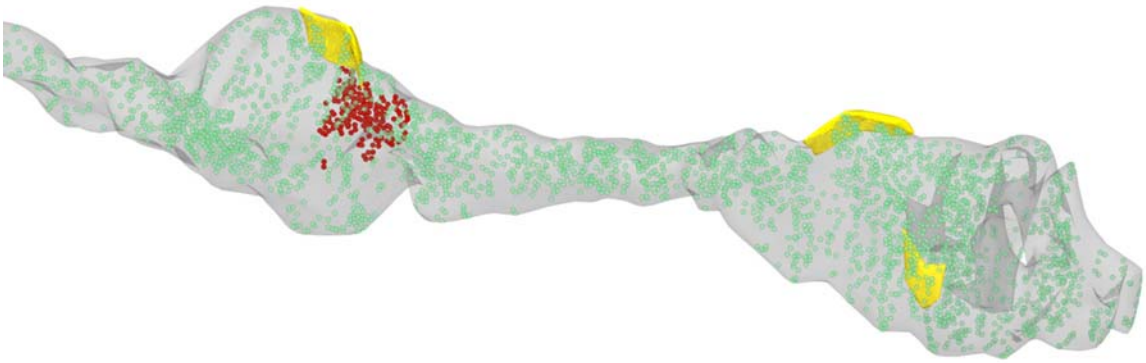

Figure 8. Three-dimensional reconstruction of an axon segment containing an ERα-IR vesicle cluster.

An axon segment reconstructed from 90 serial sections shows 2 boutons that form symmetric synapses (yellow) with a CA1 pyramidal cell body. Only one of the boutons contains a cluster of ERα-IR vesicles (red). This axon is from an animal treated with estradiol; note the ERα-IR vesicle cluster is near the synapse (non-ERα-IR vesicles – green, bouton plasma membrane – gray).

Bouton structural parameter	Oil		Estradiol	
	ERα+ (n=4)	ERα- (n=4)	ERα+ (n=4)	ERα- (n=4)
Volume (μm^3)	0.44 ±0.11	0.45 ±0.08	0.49 ±0.06	0.46 ±0.07
Presynaptic density area (μm^2)	0.08 ±0.02	0.09 ±0.02	0.08 ±0.01	0.09 ±0.01
Boutons with multiple synapses (%)	56 ±21	64 ±8	51 ±11	63 ±5
Boutons containing mitochondria (%)	100 ±0	89 ±7	88 ±7	87 ±4
Total number of vesicles	1891 ±144	2245 ±144	2325 ±324	2950 ±729
Vesicle density (#/μm^3)	4882 ±685	5676 ±764	5104 ±611	6298 ±780

Table 5. Quantification of structural characteristics of reconstructed perisomatic inhibitory boutons in the dorsal CA1 cell body layer.

The mean (± s.e.m.) for each parameter is shown both for boutons with (ERα+) and without (ERα-) ERα-IR vesicle clusters from oil- and estradiol-treated animals. Neither ERα-IR content nor estrogen treatment significantly affected any parameter measured.

Although the basic structural features of ERα-IR-containing boutons did not differ between OVX+O and OVX+E animals, plotting the location of presynaptic vesicles within each bouton revealed a striking effect of estrogen to shift the location of ERα-IR vesicle clusters closer to synapses (**Fig. 9A,B**). Because axonal boutons vary widely by size, we determined the proximity of presynaptic vesicles to synapses using a relative distance measurement. For each bouton, the distance between each presynaptic vesicle and the nearest synapse was measured and converted to a value between 0 and 1.0, with 1.0 being the maximum distance that a vesicle could be from the synapse. Comparison of histograms of relative distances in the 33 completely reconstructed boutons that contained ERα-IR (**Fig, 9C,D,** filled bars) showed that labeled vesicle clusters were located significantly closer to the nearest synapse in OVX+E boutons compared to OVX+O (n=13 OVX+O, n=20 OVX+E; K-S test, $p<0.01$; **Fig. 9E**). Comparison of the mean relative distances on a per animal basis confirmed that estrogen decreased the distance between ERα-IR vesicles and the nearest synapse by approximately one-half (n=4; unpaired, two-tailed, Student's t-test, $p=0.03$; **Fig. 9F**). Initial inspection of the histograms showing relative distances of unlabeled vesicles in OVX+O and OVX+E boutons (**Fig. 9C,D,** open bars) suggested that they also might be affected by estrogen. The cumulative histogram of relative distances of all vesicles in ERα+ boutons shows that estradiol treatment shifted all vesicles in the boutons significantly closer to synapses (**Fig. 10**). However, the cumulative histogram of the relative distances of the unlabeled vesicles in these boutons does not reveal a significant

shift (K-S test, $p>0.20$), indicating that the effect of estrogen to shift the location of presynaptic vesicles is specific for those that are ERα-IR.

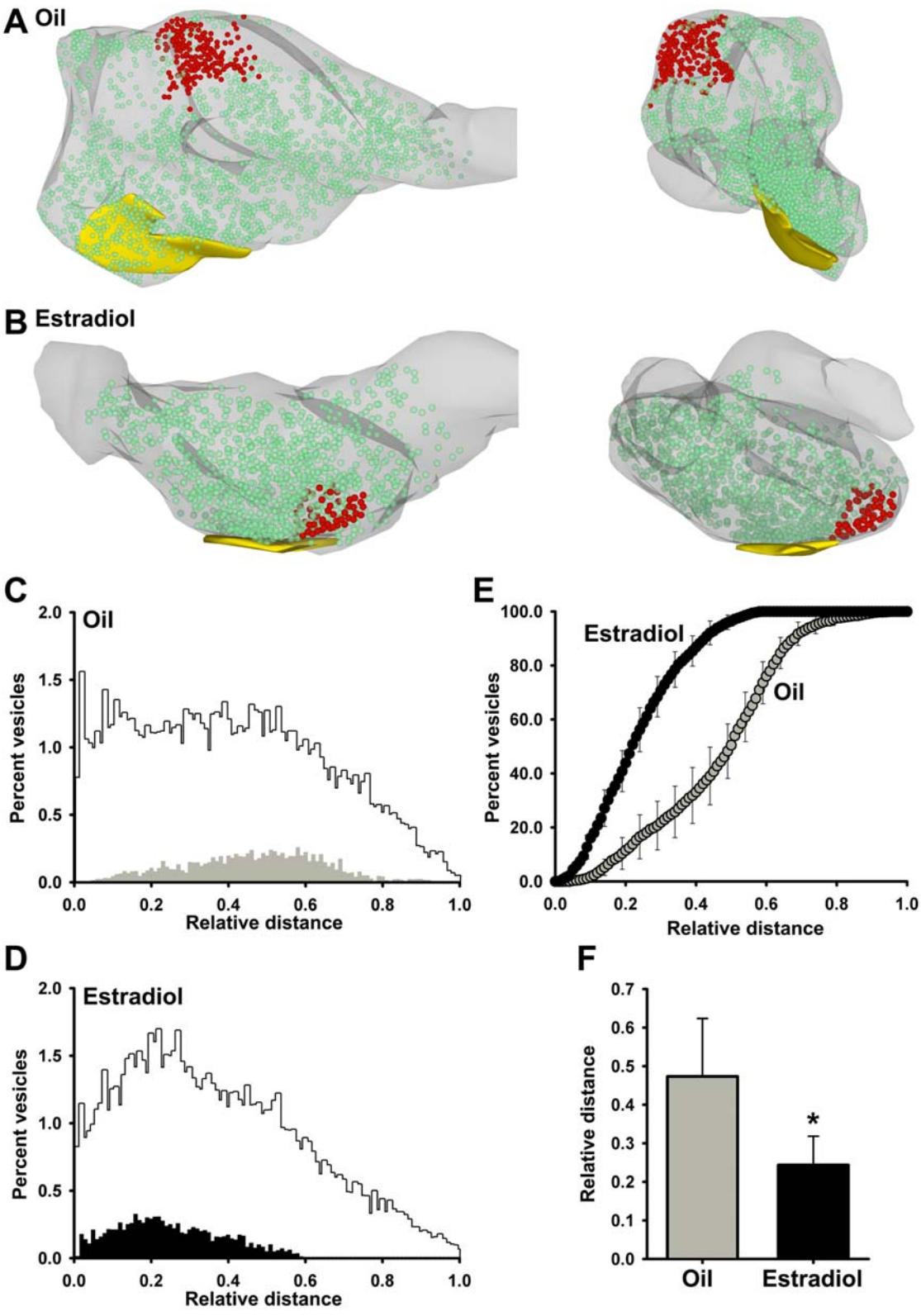
A Oil
B Estradiol
C
Oil
Percent vesicles
Relative distance
D
Estradiol
Percent vesicles
Relative distance
E
Estradiol
Oil
Percent vesicles
Relative distance
F
Relative distance
*
Oil
Estradiol

Figure 9. Three-dimensional reconstructions and vesicle distances in perisomatic inhibitory boutons containing ERα-IR vesicle clusters.

(**A, B**) Two views (rotated by 90^{o}) of reconstructed boutons from an oil- (**A**) and an estradiol-treated (**B**) animal showing that ERα-IR vesicles (red) are clustered and are located closer to the synapse (yellow) following estrogen treatment (non-ERα-IR vesicles – green, bouton plasma membrane – gray). (**C, D**) Histograms of relative distance from the synapse of vesicles in reconstructed boutons containing ERα-IR clusters are shown for oil- (**C**) and estradiol-treated (**D**) animals. Distributions of ERα-IR vesicles are represented with filled bars (oil-gray, estradiol-black), while the distribution of non-ERα-IR vesicles are represented in open bars. (**E**) Cumulative histograms of the data in **C** and **D** show that ERα-IR vesicles are located significantly closer to the synapse following estrogen treatment (K-S test, $p<0.01$). (**F**) Comparison of the mean relative distances on a per animal basis shows that estradiol reduced the distance between the synapse and ERα-IR vesicle clusters by approximately half (unpaired, two-tailed, Student's t-test, * indicates $p=0.03$, $n=4$). Error bars are s.e.m.

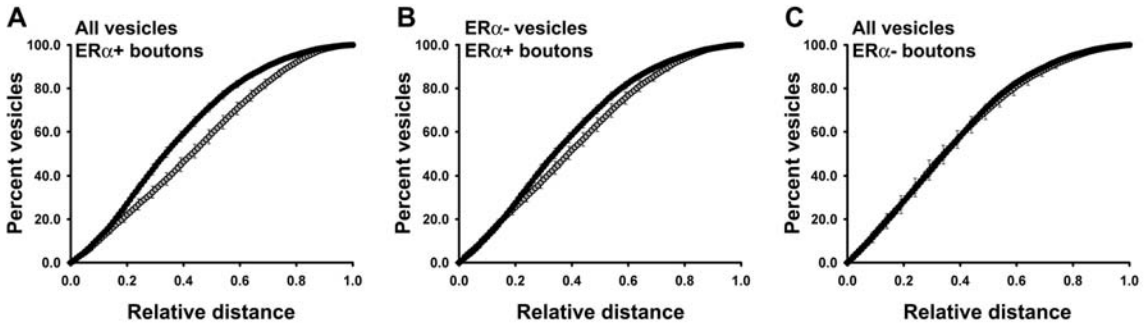

Figure 10. Cumulative histograms of the distribution of vesicles within perisomatic inhibitory boutons with ERα-IR clusters and those without.

(**A**) The cumulative histogram of the relative distances from the synapse of all vesicles (both ERα-IR and non-ERα-IR) in boutons with ERα-IR clusters, shows that estrogen treatment results in an overall shift of all vesicles closer to synapses (K-S test, $p<0.05$, oil-gray, estradiol-black). (**B**) Examination of the distributions of non-ERα-IR vesicles only in these same boutons (white bars in **Fig. 9 C,D**) reveal a non-significant shift closer to the synapse in response to estrogen. However, in boutons without ERα-IR clusters (**C**), the relative distances from synapses are identical between oil- and estradiol-treated animals, suggesting that estrogen effects are not limited to ERα-IR vesicles. Error bars are SEM.

DISCUSSION

I have used serial EM and three-dimensional reconstructions to show that clusters of presynaptic vesicles in perisomatic inhibitory boutons in the hippocampal CA1 cell body layer are immunoreactive for ERα, and that these clusters are located closer to synapses following estradiol treatment. We also found that only a subset of boutons on any given axon contain ERα-IR clusters. Estrogen did not affect boutons structural characteristics, such as volume, presynaptic density area, single vs. multiple synapses, presence of mitochondria, total vesicle number or vesicle density.

Specificity of ERα labeling

I am confident that the ERα-IR analyzed in this study is labeling extranuclear ERα for many reasons. First, we observed similar patterns of immunoreactivity in tissue labeled with MC-20, a polyclonal antiserum raised against the C-terminus of ERα, and in tissue labeled with 6F11, a monoclonal antibody raised against the full length of ERα. Both MC-20 and 6F11 have been shown to specifically label for ERα and not ERβ (Bevitt et al. 1997; Azcoitia et al. 1999). Second, omission of the primary antibody or antiserum and preadsorbtion of MC-20 with its blocking peptide eliminated labeling (Chapter 2) indicating that ERα-IR is due to specific labeling by the antibody or antiserum. Third, using Western blots, we confirmed that MC-20 recognizes a discrete protein band at ~67 kD (**Fig. 6C**), the molecular weight of ERα. Finally, other ERα antibodies and antisera not used in this study show similar patterns of labeling. Tissue processed with AS409, a polyclonal antiserum raised against near full-length rat ERα,

ER21, a monoclonal antibody raised against the N-terminus of human ERα, H222, a monoclonal antibody raised against the ligand-binding region of human ERα, or 6F11 all produced similar patterns of extranuclear ERα-IR (Milner et al. 2001). Since they are raised against different portions of ERα from different species and show similar patterns immunoreactivity, it is highly likely that MC-20, 6F11, AS409, ER21, and H222 are all labeling ERα. Furthermore, H222 and MC-20 have been used to label ERα expressed in CHO cells transfected with cDNA for mouse ERα (Razandi et al. 2004). All of this strongly suggests that the ERα-IR we analyzed in this study is labeling extranuclear ERα present in the cell body layer of the hippocampus.

Proportion of ERα-IR boutons on an axon

An estimate of the percentage of boutons containing ERα-IR clusters on any given axon can be obtained through deductive reasoning. Axon segments were reconstructed starting with a bouton that contained ERα-IR and then one or more boutons on either side, none of which were found to contain ERα-IR. Since the non-ERα-IR boutons were randomly sampled from either side of the ERα-IR bouton, this suggests that a maximum of one-half of the boutons on any given axon contain ERα-IR clusters (every other bouton). The percentage of boutons that contained ERα-IR clusters (~ 41%) we calculated in the results is somewhat similar to this prediction, but is likely tainted by sampling bias. The areas chosen for imaging were selected because they contained an ERα-IR bouton, and the neighboring boutons were chosen for reconstruction because they did not contain any ERα-IR.

ERα-IR vesicles

ERα-IR vesicles may contain GABA and undergo exocytosis to release neurotransmitter. Labeled vesicles in perisomatic boutons were small clear vesicles that were morphologically indistinguishable from unlabeled ones. Also, we did occasionally observe ERα-IR associated with presynaptic densities, suggesting fusion at synaptic active zones. If ERα-IR labeled vesicles function to release neurotransmitter, then our results indicate a specialized subpopulation of GABAergic vesicles within individual presynaptic boutons that is regulated independently of the others, i.e., by estrogen. If this is the case, then the simplest interpretation of the estrogen-induced mobilization of labeled vesicles is that it reflects their movement toward synaptic release sites.

This effect of estrogen to mobilize ERα-IR vesicle clusters toward synapses is distinct from a previous report that the same 24-hour estrogen treatment used here decreases the number of vesicles docked at perisomatic GABAergic synapses without affecting vesicles farther from the synapse (Ledoux and Woolley 2005); the decrease in docked vesicle number is paralleled by decreased GABA release (Rudick and Woolley 2003). However, the previous study of GABAergic vesicles was not designed to distinguish between vesicles that contain ERα and those that do not. The estrogen effect on vesicles reported in Ledoux and Woolley was observed within 30 nm of synapses, and vesicles further than 100 nm from synapses were not analyzed. Only a small percentage of the ERα-IR vesicles quantified in the current study were located within 100 nm of synapses and very few vesicles were located within 30 nm. Therefore, it is quite likely

that vesicles expressing ERα were not included in the analysis by Ledoux and Woolley. Thus, the finding in this experiment that estrogen mobilizes specifically ERα-IR vesicles toward synapses and not unlabeled ones does not conflict with the previous report of fewer docked vesicles following estrogen treatment and is further evidence that ERα confers specialized estrogen sensitivity to a distinct subset of presynaptic vesicles in inhibitory boutons.

Extranuclear ERα-IR at the bouton plasma membrane

In addition to the possibility that ERα-IR vesicles are neurotransmitter vesicles, ERα-IR vesicles may serve as a mechanism to deliver and retrieve ERα and/or other proteins to and from the bouton plasma membrane. Patches of ERα-IR on the bouton membrane, usually near labeled vesicle clusters, are consistent with this idea. Also, ERα-IR vesicle clusters were occasionally observed in stretches of axons between boutons, further suggesting that the clusters are a functionally discrete subpopulation of vesicles that may play a role in the trafficking of ERα. As discussed in Chapter 1, studies in a variety of cell types have demonstrated ERα expression at the plasma membrane where it functions as a G-protein coupled receptor and can influence secretion of neuropeptides (Navarro et al. 2003). If ERα-IR vesicles are involved in regulating plasma membrane-associated ERα, then estrogen effects on ERα-IR vesicle location could result from exo- and/or endocytosis at different sites relative to synapses. Interestingly however, the number of vesicles per ERα-IR cluster was not affected by

estrogen treatment, arguing against the idea that estrogen specifically stimulates ERα delivery to or retrieval from the plasma membrane.

Extranuclear ERα at the bouton plasma membrane may provide a link between rapid estrogen signaling and our previous observation of an estrogen-induced decrease in the number of docked vesicles at GABAergic synapses. As discussed earlier, estrogen acting through ERα associated with signaling complexes on caveolin rafts has been shown to rapidly increase cAMP and activate the MAPK and PI3K pathways. One substrate for MAPK in presynaptic boutons is synapsin-I, which anchors synaptic vesicles to the actin cytoskeleton in a phosphorylation-dependent manner (Jovanovic et al. 1996). Phosphorylation of synapsin-I at MAPK sites is involved in mobilization of synaptic vesicles for release, resulting in impaired efficiency of vesicle pool turnover (Chi et al. 2003), and at inhibitory synapses, synapsins are critical for maintaining the readily releasable pool of presynaptic vesicles (Gitler et al. 2004). Therefore, it is possible that ERα inserted into inhibitory bouton membranes could mediate the estrogen-induced decrease in docked vesicle number discussed above, through regulation of MAPK activity and synapsin phosphorylation.

What is the nature of ERα-IR presynaptic vesicles?

As discussed above, ERα-IR vesicles may not contain any neurotransmitter, in which case, their most likely function is to deliver and insert ERα and/or other proteins to the bouton membrane. *In vitro* studies demonstrate a requirement for ERα to associate with caveolin in order to localize to cell membranes and function as a membrane receptor

(Razandi et al. 2002; Razandi et al. 2003; Acconcia et al. 2005). Since ERα lacks a sequence of hydrophobic residues, a common feature of proteins that insert into membranes, it is plausible that ERα interacts with caveolin in order to remain associated with vesicular membranes. Presynaptic vesicles could then serve as a mechanism to regulate the estrogen response within a bouton by delivering and retrieving ERα to and from the plasma membrane. Once it is at the bouton membrane, ERα would be positioned to interact with numerous signaling proteins recruited to and associated with caveolin rafts. Disrupting the synthesis of cholesterol, a major component of the caveolin raft complex, could potentially test whether or not interaction with caveolin is necessary for ERα to associate with presynaptic vesicles and, indirectly, test the role of vesicles in delivering ERα to the bouton membrane. Care would have to be taken, however, to not disrupt cholesterol synthesis to the point that plasma membranes are compromised.

Alternatively, ERα-IR vesicles may mediate a neuroprotective effect of estrogen. High levels of glutamate are toxic to neurons. Many interneurons, including basket cells, monitor the activity of and provide feedback inhibition to CA1 pyramidal cells, thus acting to limit excessive excitation and glutamate release. Additionally, heat shock proteins (HSP), especially members of the HSP70 family, have been shown to play a role in protecting neurons from fatal damage after a variety of insults and, interestingly, have been localized near synapses (Gioio et al. 2001; Lancaster and Febbraio 2005; Mariucci et al. 2007). The interaction between ERα in the nucleus and HSP90 is well established (Segnitz and Gehring 1995; Aumais et al. 1997; Fliss et al. 2000; Bagatell et al. 2001; Bouhouche-Chatelier et al. 2001). Perhaps, ERα in perisomatic inhibitory boutons is

complexed with HSP70 in a similar manner. Binding of ERα by estrogen would then cause HSP70 to dissociate, resulting in an increase of HSP70 available for release from vesicles at the synapse or bouton membrane, thus providing a neuroprotective effect. This hypothesis could be tested using a synaptosome preparation extracted from hippocampal tissue. Estradiol- and vehicle-treated preparations could be assayed in order to determine if estradiol treatment caused a release of HSP from synaptosomes. If the level of HSP increased following exposure to estradiol, then further experiments could be done using ER antagonists to determine the role of ERs.

Another interesting possibility is that ERα-IR vesicles in perisomatic inhibitory boutons contain a neurotransmitter other than GABA. It has been reported that some GABAergic boutons in the CA1 pyramidal cell layer express vesicular glutamate transporter type 3 (VGLUT3; (Somogyi et al. 2004), a protein that transports glutamate into presynaptic vesicles (Fremeau et al. 2002; Schafer et al. 2002; Takamori et al. 2002). This is somewhat surprising since in these same boutons GAD converts glutamate into GABA, which is loaded into presynaptic vesicles by vesicular GABA transporter (VGAT; (Fykse and Fonnum 1988; Kish et al. 1989; McIntire et al. 1997; Chaudhry et al. 1998), and GABA is released at the synapse upon stimulation. Similar to ERα, VGLUT3 immunoreactivity is only observed in a subset of CCK basket cell boutons in the pyramidal cell layer (Somogyi et al. 2004). It remains to be determined whether a subset or all of the vesicles in CCK basket cell boutons express VGLUT3. The function of VGLUT3 in inhibitory boutons is not known, but its presence in perisomatic boutons

suggests the possibility that, upon stimulation, both GABA and glutamate are released at a subset of CCK basket cell synapses.

While postsynaptic glutamate receptors have not been reported at perisomatic inhibitory synapses on CA1 pyramidal cells, many studies demonstrate the ability of presynaptic glutamate receptors to regulate pyramidal cell inhibition. Both presynaptic metabotropic glutamate (Poncer et al. 1995; Poncer et al. 2000; Semyanov and Kullmann 2000) and presynaptic kainate receptors (Clarke et al. 1997; Rodriguez-Moreno et al. 1997; Bureau et al. 1999; Min et al. 1999; Rodriguez-Moreno et al. 2000) have been shown to modulate GABAergic inhibition in the hippocampus. Specifically, activation of kainate receptors decreases GABA release at synapses on CA1 pyramidal cell somata (Rodriguez-Moreno et al. 1997; Min et al. 1999; Rodriguez-Moreno et al. 2000). It would be particularly interesting if VGLUT3 were expressed on a subset of presynaptic vesicles in a bouton that also belonged to an ERα vesicle cluster. It would then be plausible to hypothesize that VGLUT3/ERα vesicles are mobilized by estrogen (ERα-IR vesicles are significantly closer to synapses in estradiol-treated animals compared to controls) and release glutamate at perisomatic inhibitory synapses. At these same synapses, autoactivation of presynaptic kainate receptors could then decrease GABA release, thus providing a possible mechanism for estrogen-induced disinhibition of CA1 pyramidal cells (Rudick and Woolley 2001; Rudick and Woolley 2003). Whether or not VGLUT3-IR and ERα-IR localize to the same vesicles in CCK basket cell boutons remains to be determined. Immunofluorescence confocal microscopy of tissue double labeled for VGLUT3 and ERα could determine if both proteins are present in the same boutons. If

VGLUT3 and ERα do colocalize, postembedding electron microscopy experiments employing different sized gold particles for each label may be able to determine if VGLUT3 and ERα are expressed on the same vesicles. Additionally, it is not known if perisomatic inhibitory boutons in the CA1 pyramidal cell layer express kainate receptors; previous reports of the ultrastructural localization of kainate receptors did not discuss this area of the hippocampus (Petralia et al. 1994; Fogarty et al. 2000). Electron microscopic examination of tissue immunolabeled for one or more kainate receptor subunits should be able to determine if presynaptic kainate receptors are present on perisomatic inhibitory boutons.

In this experiment, I have quantified ERα-IR in perisomatic inhibitory boutons in the dorsal CA1 pyramidal cell layer. Two neurochemically distinct populations of basket cells provide somatic inhibition to pyramidal cells in this area. Is ERα expressed in boutons from PV or CCK basket cells, or both? Since ERα-IR is observed in only 1-3 serial thin sections through a bouton, and many boutons do not contain any ERα-IR, it would be more appropriate to utilize a different method of visualizing and quantifying immunolabeling to answer this question.

CHAPTER 4 - NEUROCHEMICAL IDENTITY OF EXTRANUCLEAR ERα-IR STRUCTURES IN THE DORSAL CA1 PYRAMIDAL CELL LAYER

ABSTRACT

Estrogen has been shown to regulate GABAergic inhibition of CA1 pyramidal cells in the dorsal hippocampus of the rat. It has also been determined that, in this same region, nuclear estrogen receptors are expressed exclusively in inhibitory interneurons, but are limited to a small subset of those. In contrast, extranuclear ERα is present in many structures of the dorsal CA1, including inhibitory boutons. Using electron microscopy, I have demonstrated the existence of estrogen-sensitive ERα-IR vesicle clusters in perisomatic inhibitory boutons. The presence of ERα-IR near inhibitory synapses suggests that estrogen may act directly in boutons through non-nuclear ERs to regulate GABAergic inhibition. However, the proportion of inhibitory boutons containing ERα-IR and the neurochemical phenotypes of ERα-IR boutons is not known. In this study, I used double-label immunofluorescence to quantify the colocalization of extranuclear ERα-IR with 4 interneuronal markers, glutamic acid decarboxylase (GAD65), parvalbumin (PV), cholecystokinin (CCK), and neuropeptide Y (NPY), in hippocampi from animals treated with estradiol or oil vehicle. Quantitative analysis revealed that approximately one-third of GABAergic axonal varicosities in the cell body layer contains ERα. Additionally, I have determined that extranuclear ERα colocalizes with CCK and NPY, but not PV. Estradiol treatment did not affect immunofluorescence or colocalization of any of the labels used. These findings reveal that ERα is expressed in

a substantial portion of GABAergic varicosities in the pyramidal cell layer and suggests that the ability of estrogen to regulate GABAergic inhibition directly at perisomatic synapses through extranuclear ERs is limited to CCK basket cell boutons.

INTRODUCTION

The presence of estrogen-sensitive ERα-IR vesicle clusters in perisomatic inhibitory boutons suggests that extranuclear ERα in these boutons may play a role in estrogen-induced disinhibition of CA1 pyramidal cells. In order to understand the possible contribution of extranuclear ERα, a question that must be answered is: what proportion of perisomatic inhibitory boutons contain ERα-IR vesicle clusters? EM analysis of axon segments clearly indicated that only a subset of boutons contains ERα-IR clusters, and further suggested that no more than one-third were ERα-IR. However, the actual percentage must be empirically determined. EM is not suitable for this purpose because the area sampled is very small and less than 10% of the serial sections that span the full volume of a bouton contain ERα-IR; therefore, many ERα-IR clusters would be missed if only single sections were analyzed, and a serial EM study for this purpose is not feasible.

In this experiment, I have used double-label immunofluorescence to answer the question of how many GABAergic varicosities contain ERα-IR. I also assessed the degree of colocalization between ERα-IR and PV- or CCK-IR to determine if the ERα-IR perisomatic boutons that we analyzed with EM belong to a specific type of basket cell. Additionally, I also double-labeled tissue for ERα and NPY. I hypothesized that

extranuclear ERα might colocalize with NPY because NPY is expressed in a subpopulation of inhibitory interneurons in the hippocampus and some NPY neurons have been shown to express nuclear ERα.

METHODS

Animals

Ten adult female Sprague-Dawley rats (~250 g) were ovariectomized under ketamine (85 mg/kg) / xlyazine (13 mg/kg, i.p.) anesthesia and 3 days later, injected (s.c.) `with 10 μg 17β-estradiol benzoate or oil vehicle as previously described (Rudick and Woolley 2001). Twenty-four hours after injection, animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and intracardially perfused with 180 ml of 4% paraformaldehyde in PB. Efficacy of estradiol treatment was verified by visual assessment of the uterus at the time of perfusion. Two additional intact adult female rats were anesthetized and perfused as above and used for the PV/GAD65/67 labeling experiment described below, and 2 more intact adult female rats were anesthetized, gravity perfused with 2% glutaraldehyde and 2% paraformaldehyde, and processed for EM immunohistochemistry exactly as described in Chapter 3 Methods. All animal procedures were approved by the Northwestern University Animal Care and Use Committee.

Immunofluorescence and quantitative analysis

Brains were removed, blocked to contain the hippocampus, postfixed overnight at 4^{o}C, cryoprotected with 30% sucrose, cut on a freezing microtome into coronal sections (40 μm) spanning the rostro-caudal extent of the dorsal hippocampus, and systematically distributed into 4 groups double-labeled for ERα (0.1 μg/ml, MC-20) and one of the following: mouse monoclonal GAD65 (0.3 μg/ml, MAB351, Chemicon), mouse monoclonal PV (1:8000, P3088, Sigma), rabbit polyclonal CCK (1:1000, PC206L, Calbiochem), or rabbit polyclonal NPY (1:4000, N9528, Sigma). Tissue processing was as described in Chapter 2 except for visualization with species-specific IgG directly coupled to a fluorochrome. For ERα colocalization, ERα-IR was visualized with Alexa Fluor 568, while GAD-, PV-, CCK-, and NPY-IR were visualized with Alexa Fluor 488 (each 2.5 μg/ml, Molecular Probes). For NPY colocalization with rabbit polyclonal GAD65/67 (1:7500, G5163, Sigma), NPY-IR was visualized with Alexa Fluor 568, while GAD-IR was visualized with Alexa Fluor 488. A few sections from each brain were singly labeled for ERα (MC-20) and visualized with DAB. Incubations were simultaneous in cases where primaries were raised in different species (ERα and GAD65 or PV); controls included omission of primary antiserum or antibody. Incubations were sequential in cases where primaries were raised in the same species (ERα and CCK or NPY; NPY and GAD65/67). In these cases, a second blocking step was included after the first secondary incubation, and some tissue was processed with the second primary antiserum omitted as an additional control. No labeling of the first primary with the second secondary was detected. All sections were mounted in order on subbed slides

dehydrated, cleared, rehydrated, coverslipped under Vectashield (Vector), and sealed with nail polish.

Slides were coded prior to image collection, so the experimenter was blind to the treatment condition of the brain during all phases of image collection and analysis. Tissue was imaged using a spinning disc laser confocal system (Perkin-Elmer) with a 100x oil objective. For each brain, 3 image stacks in each hemisphere were collected from 3 sections in each brain, for a total of 18 stacks per brain. The volume of each stack was 21,600 μm^3 (ERα and GAD or PV) or 10,800 μm^3 (ERα and CCK or NPY) and consisted of a 70 μm x 70 μm image field taken at 0.2 μm Z-steps with the CA1 pyramidal cell layer in the center of the field. For PV and GAD65/67 colocalization analysis, 3 image stacks in both hemispheres were collected from 4 sections in each brain, for a total of 24 stacks per brain, each with a volume of 21,600 μm^3. Quantification of immunofluorescence and colocalization were performed using Volocity software (Improvision). The number of fluorescent objects labeled by each antibody or antisera and percentage of colocalization were calculated for each brain and treatment groups were compared using Student's t-test (unpaired, two-tailed, n=4) using SPSS software. All figures were prepared with Photoshop (Adobe).

RESULTS

In order to interpret the results of the immunofluorescence colocalization analysis within the context of the previous electron microscopy study, I first had to confirm that the lot of MC-20 antisera used in this experiment labeled for ERα in hippocampal tissue

the same way as the older lot used in both previous experiments. The newer lot used in this study labeled much more robustly than the MC-20 antisera used to label the tissue for the EM experiment and had to be used at lower concentrations to avoid high background labeling. Light microscopic examination of the DAB sections labeled with new MC-20 antiserum showed a pattern of ERα-IR that was similar to previous experiments, with many ERα-IR puncta visible in the cell body layer. EM verified that ultrastructural labeling for ERα by new MC-20 (**Fig. 11A**) was qualitatively the same as older MC-20, with the same types of structures labeled in equivalent proportions. Additionally, Western blots probed with new MC-20 (**Fig. 11B**) show a discrete protein band at the predicated MW of ERα, ~ 67 kDa, the same as older antisera. I am confident that the ERα-IR quantified in this experiment is comparable to the ERα-IR analyzed in the earlier experiments, thus allowing the results from all the experiments to be considered together.

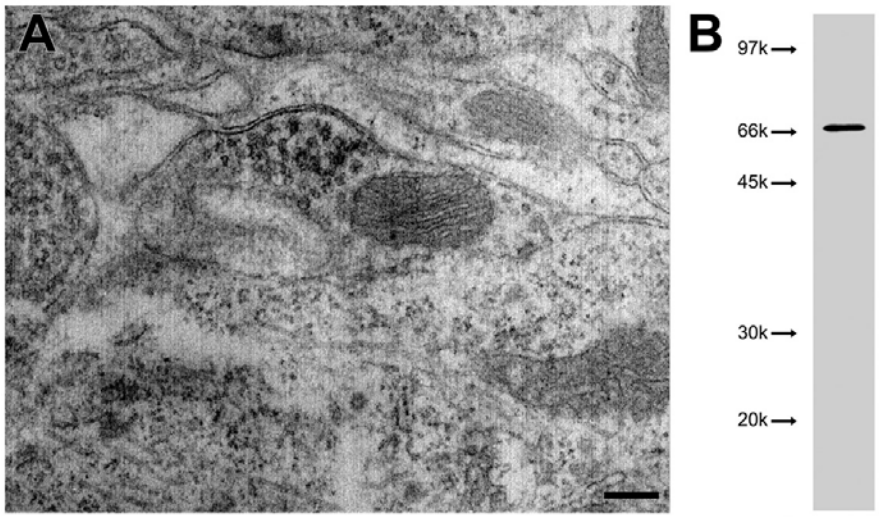

Figure 11. Ultrastructural localization of ERα-IR with the same MC-20 antiserum used for immunofluorescence experiments.

(**A**) Perisomatic inhibitory bouton in the cell body layer containing ERα-IR vesicles in tissue labeled with the new lot of MC-20 antiserum. Note that labeling is similar to that in Fig.6. (**B**) Western blot showing a single band at ~67 kDa labeled with new MC-20 antiserum. Scale bar = 200 nm.

Colocalization of extranuclear ERα-IR with GAD65-IR

To investigate whether ERα is expressed in axonal varicosities of GABAergic neurons, I double-labeled hippocampal tissue for ERα and GAD65 (**Fig. 12**). I was especially interested in the possibility that ERα is expressed in GABAergic boutons because it has been shown previously that estrogen suppresses GABAergic synaptic transmission in CA1 (Rudick and Woolley 2001; Rudick and Woolley 2003). I evaluated GAD65-IR and ERα-IR in 5 ovariectomized oil-treated (OVX+O) and 5 ovariectomized, estrogen-treated (OVX+E) adult female rats. Projected images from stacks of optical sections showed characteristic GAD65-IR in the cell body layer, with varicosity-like structures arranged in rings around unlabeled pyramidal cell somata (**Fig. 12A**). For both GAD65-IR and ERα-IR, measurements of fluorescence intensity, volume, number of labeled objects, and percentage of colocalization were unaffected by estrogen, so data from both treatment groups were combined (n=10). Quantitative analysis of 18 image stacks per brain (21,600 μm^3 per stack) showed that 32.4±0.6% of the 3,815±141 GAD-IR varicosities per stack contained ERα-IR puncta (**Fig. 12B,C**) and 24.0±0.5% of the 5,549±187 ERα-IR puncta per stack were located in GAD65-IR varicosities (**Fig. 12C**). This analysis indicated that extranuclear ERα-IR is expressed in approximately one-third of GABAergic varicosities in the cell body layer, but that a substantial fraction of punctate ERα-IR is expressed in non-GABAergic structures as well. The observation that not all ERα-IR puncta colocalize with GAD-IR varicosities is consistent with previous qualitative EM observations of ERα-IR in multiple extranuclear sites, such as dendrites, glia, excitatory axonal boutons, and somatic organelles (Milner et al. 2001).

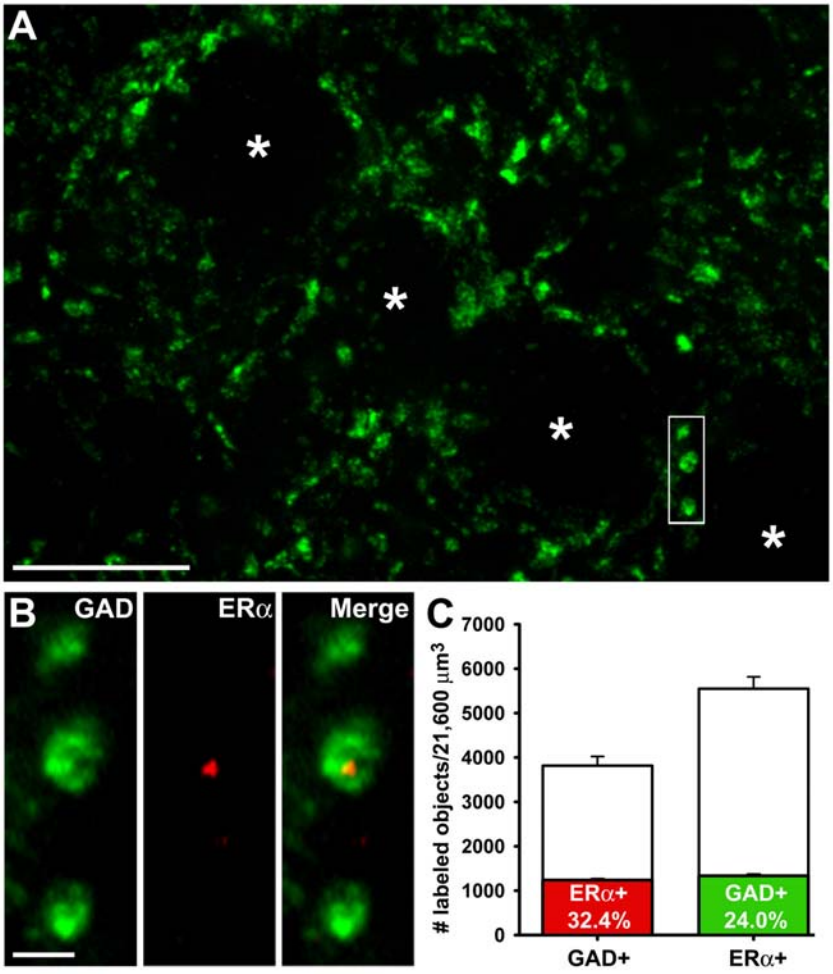

Figure 12. Colocalization and quantification of GAD-IR varicosities and ERα-IR puncta.

(**A**) A 2 μm thick image stack shows that GAD-IR labels varicosities surrounding unlabeled pyramidal cell somata (asterisks). The panels in (**B**) are higher magnification views of the boxed area in **A** through 0.4 μm of tissue. Portions of 3 varicosities labeled with GAD-IR (green) are shown, one of which contains punctate ERα-IR (red). (**C**) Quantification of GAD-IR and ERα-IR co-localization. The entire height of each bar represents the average total number of labeled varicosities (GAD-IR) or puncta (ERα-IR) per 4 μm stack of optical sections (21,600 μm^3 volume); the colored portion of each bar

represents the average number and percent of varicosities or puncta that are double-labeled. Scale bar = 10 μm in **A**, and 1μm in **B** (applies to all panels in **B**). Error bars are s.e.m.

Colocalization of extranuclear ERα-IR with PV- or CCK-IR

The perisomatic inhibitory boutons I reconstructed in the previous EM study arise from basket cells, a population of GABAergic neurons that can be divided into two distinct subtypes based on PV or CCK immunolabeling, each of which have distinct physiological roles in the hippocampus as reviewed in Chapter 1. To determine whether ERα-IR was located specifically in one class of basket cell, I used double-label immunofluorescence to quantify colocalization of ERα-IR with PV- or CCK-IR in the cell body layer (**Figs. 13 and 14**) in 5 OVX+O and 5 OVX+E animals. Estrogen treatment did not affect any parameter of PV-IR, CCK-IR, or colocalization, so data from both groups were combined (n=10). Tissue double-labeled for PV and ERα showed the characteristic 'beads on a string' PV staining of axons and axonal varicosities, but there was no colocalization of ERα-IR and PV-IR (**Fig. 13**). In contrast, ERα-IR did colocalize with CCK-IR, which labels in a more punctate pattern (Morales and Bloom 1997) than GAD or PV (**Fig. 14A**). Quantitative analysis of 18 image stacks per brain (10,800 μm^3 per stack) showed that 25.2±0.6% of 3,817±177 CCK-IR puncta per stack colocalized with ERα-IR, and 36.2±0.8% of 2,762±72 ERα-IR puncta per stack colocalized with CCK-IR (**Fig. 14B**). Thus, the axonal boutons containing ERα-IR vesicle clusters that we analyzed with serial EM likely belong to CCK basket cells and not PV basket cells.

Because the number of CCK-IR puncta was greater than the number of GAD-IR varicosities in the same volume, either there are multiple CCK-IR puncta per varicosity and/or some CCK-IR puncta are located in structures other than GABAergic varicosities,

such as dendrites that do not contain GABA. Consistent with the second possibility, CCK labeling has been shown previously in dendrites in CA1 (Harris et al. 1985; Morales and Bloom 1997). The number of ERα/CCK-IR structures per stack was 902±42, which is similar to the number of ERα/GAD-IR structures calculated for the same volume, 624±31. Thus, most of the puncta that colocalize ERα-IR and CCK-IR are likely to be in GABAergic boutons.

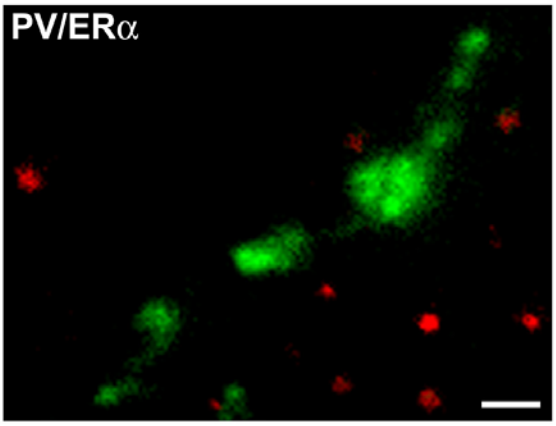

Figure 13. ERα-IR does not colocalize with PV-IR.

ERα-IR puncta (red) did not co-localize with PV-IR structures (green). Scale bar = 1 μm.

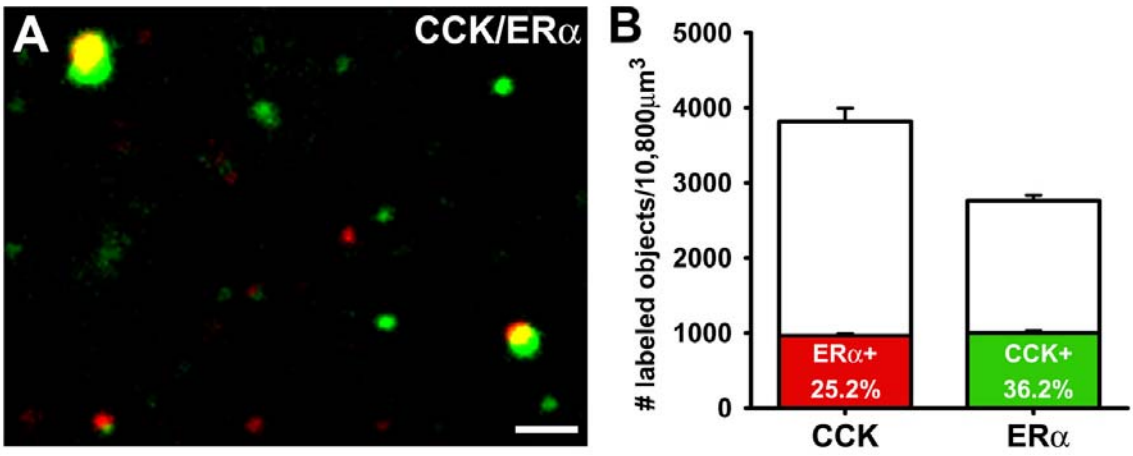

Figure 14. Colocalization and quantification of ERα-IR with CCK-IR.

(**A**) Some ERα-IR puncta (red) did co-localize (yellow) with CCK-IR puncta (green). (**B**) Quantification of CCK- and ERα-IR co-localization. The entire height of each bar represents the average total number of labeled puncta per 2μm stack of optical sections (10,800 μm^3 volume); the colored portion of each bar represents the average number and percent of puncta that are double-labeled. Scale bar = 1 μm in. Error bars are s.e.m.

Colocalization of extranuclear ERα-IR with NPY-IR

A subset of GABAergic neurons in CA1 also expresses NPY (Hendry et al. 1984; Chronwall et al. 1985; de Quidt and Emson 1986) and some NPY neurons express nuclear ERα (Sar et al. 1990; Skinner and Herbison 1997), suggesting that extranuclear ERα-IR also might be found in NPY neurons. To investigate this, I analyzed colocalization of ERα-IR and NPY-IR (**Fig. 15**) in the same tissue used for PV and CCK analyses. No measure of immunofluorescence or colocalization was affected by estrogen treatment, so data from both groups were combined (n=10). Quantitative analysis of ERα-IR and NPY-IR in the cell body layer (**Fig. 15A**) showed that 30.3±0.6% of 4,165±170 NPY-IR puncta colocalized with ERα-IR, and 49.9±2.2% of 2,662±44 ERα-IR puncta colocalized with NPY-IR (**Fig. 15B**).

Because the number of ERα/NPY-IR objects was greater than the number of ERα/GAD-IR objects in the same volume, this suggested that a substantial fraction of ERα/NPY-IR might not be GABAergic. Thus, I performed an additional experiment to quantify NPY/GAD-IR colocalization (**Fig. 16**) and found that ~73% NPY-IR puncta in the cell body layer co-localized with GAD65/67. The remaining ~27% of NPY-IR puncta likely belong to dendrites, excitatory boutons, or cytoplasmic organelles (Milner and Veznedaroglu 1992). Interestingly, the number of ERα-IR puncta per stack that colocalized with NPY-IR (~1,250) is very similar to the number of NPY-IR puncta that were negative for GAD (~1,125). Thus, it is possible that many, if not most, ERα/NPY-IR puncta are not in GABAergic boutons.

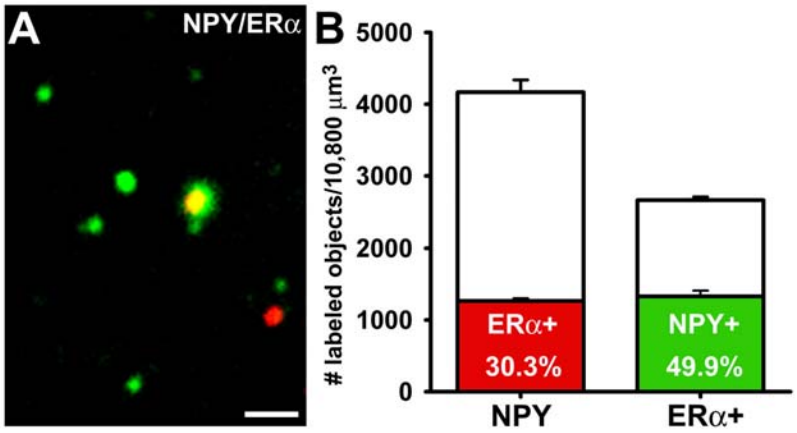

Figure 15. Colocalization and quantification of ERα-IR.

(**A**) A portion of ERα-IR puncta (red) co-localized (yellow) with NPY-IR (green). (**B**) Quantification of NPY- and ERα-IR co-localization. The entire height of each bar represents the average total number of labeled puncta per 2μm stack of optical sections (10,800 μm^3 volume); the colored portion of each bar represents the average number and percent of puncta that are double-labeled. Scale bar = 1 μm. Error bars are s.e.m.

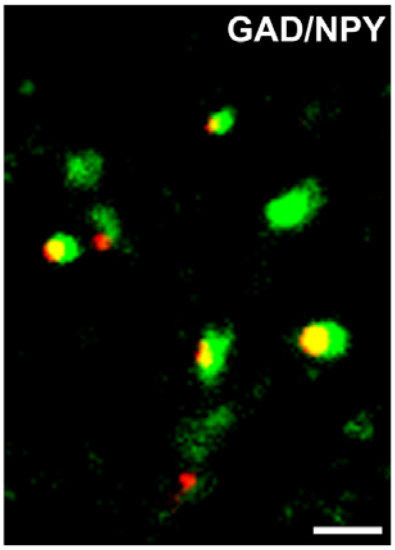

Figure 16. NPY-IR colocalization with GAD-IR.

The majority (~73%) of NPY-IR puncta (red) are co-localized (yellow) with GAD-IR (green). Scale bar = 1 μm

DISCUSSION

In this study, I used double-label immunofluorescence to quantify the colocalization of extranuclear ERα-IR with GAD-, PV-, CCK-, or NPY-IR. Approximately ~32% of the GABAergic boutons in the CA1 cell body layer contained ERα-IR puncta, consistent with the prediction from my EM analysis. Extranuclear ERα-IR also colocalized with CCK- and NPY-IR, but not PV-IR indicating that different subpopulations of GABAergic interneurons are differentially sensitive to estrogen.

GAD65-IR vs. GAD65/67-IR

I used two different GAD antibodies or antisera in this experiment to best achieve the goals of this study. By using a mouse monoclonal GAD65 antibody in conjunction with a rabbit polyclonal ERα antiserum, we eliminated the chance of secondary antibody crossreactivity erroneously increasing colocalization. Furthermore, the GAD65 isoform predominates in GABAergic axon terminals (Esclapez et al. 1994). Since my primary goal for this analysis was to assess the proportion of inhibitory boutons that colocalized ERα, the structures I reconstructed with EM, using a GAD65 antibody was a good choice because it allowed me to more accurately determine the number of GABAergic varicosities that contained ERα-IR. If I had labeled for GAD65/67 instead, I would have increased my fidelity in assessing the number ERα-IR puncta contained within any GABAergic structure, including axons and somata; that information is not as useful for answering my question precisely because ERα-IR is present in so many structures that are not boutons. However, determining the number of NPY-IR puncta contained within

GABAergic structures of any kind was the goal of the NPY/GAD colocalization analysis, and so, I used an antiserum for GAD65/67. When I compared the numbers of GAD-IR varicosities identified by both the GAD65 antibody and GAD65/67 antiserum (GAD65/67-IR > 1 μm^3 was considered to be non-axonal and thus excluded for this comparison), I found that they were not quantitatively different, indicating that the GAD65 antibody had labeled virtually all GABAergic varicosities in the cell body layer.

Colocalization of ERα and CCK

The finding that extranuclear ERα-IR colocalizes with CCK, but not with PV, indicates that direct effects of estrogen on presynaptic boutons likely occur only in the CCK subtype of basket cell. This is significant because PV and CCK cells differ in their electrophysiological and anatomical properties and in their influence on pyramidal cells. Most PV basket cells are capable of firing at high frequencies and without accommodation, whereas CCK cells fire at lower frequencies and do show accommodation (Cauli et al. 1997; Maccaferri et al. 2000; Thomson et al. 2000; Pawelzik et al. 2002). These and other findings have been interpreted to indicate that PV cells play a greater role than CCK cells in entraining rhythmic activity of pyramidal cells, particularly at gamma frequency (Freund 2003). This idea is supported by the observations that PV basket cells fire counter-phase with pyramidal cells (Klausberger et al. 2003) and the power of gamma oscillations is 2-3 fold greater in PV-knockout mice compared to wildtypes (Vreugdenhil et al. 2003).

Additionally, anatomical studies show that PV cells receive, on average, ~15,000 excitatory and ~1,000 inhibitory inputs (Gulyas et al. 1999), whereas CCK cells receive only ~5,000 excitatory but ~3,000 inhibitory inputs (Matyas et al. 2004); CCK cells also receive GABAergic input from a specialized, interneuron-driven population of calretinin-positive neurons (Gulyas et al. 1996), and serotonergic input from neurons in the median raphe, neither of which innervate PV cells (Miettinen et al. 1992; Morales and Bloom 1997). Thus, PV and CCK cells appear to monitor the activities of different populations of neurons, and CCK cells are in a better position to respond to modulatory GABAergic and serotonergic inputs. These differences have led to the hypothesis that relatively non-plastic ensembles of PV basket cells are responsible for synchronizing the activity of pyramidal cells giving rise to gamma and theta oscillations, while more modifiable groups of CCK basket cells integrate local and subcortical inputs and provide a mechanism to fine-tune pyramidal cell activity (Freund 2003). The colocalization of ERα-IR with CCK-IR but not PV-IR suggests that estrogen may act through ERα in CCK basket cell axonal boutons to influence such fine-tuning of hippocampal activity.

What percentage of CCK basket cell boutons contains ERα-IR vesicle clusters?

The percentage of CCK basket cell boutons that contain ERα-IR vesicle clusters speaks to the magnitude and scope of estrogen effects mediated through extranuclear ERα in perisomatic inhibitory boutons. Because CA1 pyramidal cells receive inhibition from both CCK and PV basket cells and ERα-IR puncta colocalize with CCK- but not PV-IR, it follows that more than one-third of the CCK perisomatic inhibitory boutons

must contain ERα-IR vesicle clusters in order to account for the observation that one-third of GABAergic axonal varicosities in the CA1 pyramidal cell layer contain ERα-IR. **Figure 17** summarizes some of the data presented in Chapters 3 and 4 in pictorial form. Forty percent of the perisomatic boutons are depicted as arising from PV basket cells. This is consistent with an electron microscopy study that determined that 36% of the inhibitory boutons forming synapses on CA1 pyramidal cell somata were immunoreactive for PV (Ribak et al. 1990). Presumably, the remaining 64% of inhibitory perisomatic boutons that were unlabeled arise from CCK basket cells, and approximately half of them will contain ERα vesicle clusters to achieve the observed ~32% of GABAergic varicosities that are ERα-IR, as determined by immunofluorescence colocalization analysis. In the serial EM experiment discussed in Chapter 3, I always started with an ERα-IR bouton in the middle and reconstructed the axon and boutons on both sides. No ERα-IR was present in boutons adjacent to a bouton containing an ERα-IR vesicle cluster along the same axon, the pattern that would be observed if every other bouton on an axon (50%) contained ERα-IR, as shown in the **Figure 17**. Longer lengths of the ERα-IR axon segments would have to be reconstructed (2 or more boutons on either side of the ERα-IR bouton) to more accurately estimate the percentage of boutons with ERα-IR vesicle clusters. Technical considerations, such as depth of ERα-IR labeling in tissue and the increased difficulty of reconstructing a small structure with an irregular path, such as an axon, through 200+ serial sections, makes the use of electron microscopy for this task prohibitive.

Immunofluorescence colocalization analysis is a more appropriate technique for estimating the percentage of CCK basket cell boutons containing ERα-IR clusters.

In double labeled tissue, I determined that ~25% of CCK-IR puncta were also immunoreactive for ERα, a percentage that is quite a bit lower than the proportion estimated by serial reconstructions and lower than the ~32% of GABAergic axonal varicosities that contain ERα-IR. The actual numbers of CCK-, ERα-, and GAD-IR objects counted indicate that the majority of puncta immunoreactive for both CCK and ERα are likely to be in GABAergic boutons. However, one would expect a higher percentage of CCK-IR puncta to be ERα-IR, since CCK basket cell boutons containing ERα-IR vesicle clusters should account for the majority of ERα-IR GABAergic axonal varicosities in the pyramidal cell layer. It is likely that 25% is an underestimate of the

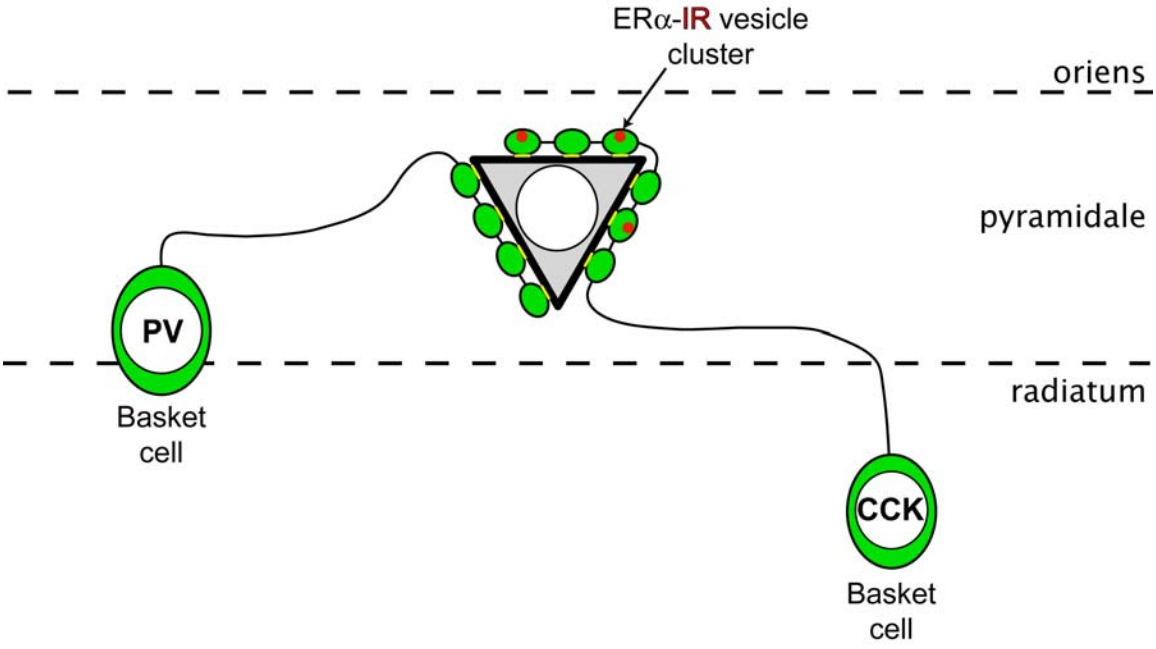

Figure 17. Diagram of extranuclear ERα in perisomatic inhibitory boutons in the CA1 pyramidal cell layer.

actual proportion of ERα-IR CCK basket cell boutons for at least two reasons. First, both ERα- (Milner et al. 2001) and CCK-IR (Harris et al. 1985; Morales and Bloom 1997) label dendrites as well as boutons, and it is not possible to distinguish between immunoreactive puncta in multiple cellular compartments using double label immunofluorescence. Inclusion of CCK- and/or ERα-IR puncta that are located in dendrites in the colocalization analysis will result in an underestimate of the percentage of CCK boutons that contain ERα-IR. Morales and Bloom (1997) show that a single labeled dendrite contains many CCK-IR puncta, while only a few ERα-IR puncta are present in a single dendrite (Milner et al. 2001). Consequently, only a very small percentage of CCK-IR puncta will colocalize with ERα-IR in dendrites. Second, a single bouton arising from a CCK basket cell axon may contain more than one CCK-IR punctum (Harris et al. 1985), only one of which will colocalize with ERα-IR, thus underestimating the proportion of CCK boutons containing ERα-IR clusters. The most accurate determination of how many CCK basket cell boutons contain ERα-IR may be obtained by analysis of tissue triple labeled for CCK, ERα, and GAD immunofluorescence, which should make it possible to identify and limit analysis to CCK- and ERα-IR in axons and boutons.

Is the percentage of ERα-IR CCK basket cell boutons variable?

Another possible explanation for the relatively low percentage of colocalization of CCK- and ERα-IR puncta is that the proportion of CCK boutons containing ERα vesicle

clusters varies. This could result from a change in the level of CCK expression, analogous to what is observed for GAD-IR. Analysis of hippocampal CA1 tissue collected at multiple time points after the initiation of estradiol treatment showed that the number of interneurons in the radiatum and oriens expressing detectable levels of GAD-IR decreased significantly after 24 hours. Just 2 hours later, following a second estradiol injection, the number of interneurons positive for GAD-IR rebounded to pre-treatment levels and was the same as in oil-treated controls (Rudick and Woolley 2001). CCK expression may also fluctuate in a similar manner but with different timing. Experiments have shown that CCK mRNA levels are increased in the medial preoptic nucleus as early as 12 hours following estradiol treatment (Micevych et al. 1996).

Analysis of tissue collected 24 hours following treatment with either estrogen or oil vehicle did not reveal any differences in the percentages of colocalization of CCK- and ERα-IR puncta between estrogen- and oil-treated animals, the measure that would be predicted to change if fewer CCK boutons contained ERα-IR clusters in one of the treatment conditions. Additionally, a recent study reported no difference in CCK mRNA expression in the hippocampus between estradiol- and oil-treated controls 24 hours after treatment (Nakamura and McEwen 2005). Twenty-four hours, however, may not be the optimal time point to observe changes in CCK-IR and/or the proportion of ERα/CCK boutons. If CCK-IR expression is maximal sometime earlier than 24 hours, it may revert back to pre-estradiol levels sooner, as well. Thus, any fluctuation in CCK-IR and percentage of colocalization with ERα-IR would be missed. Quantification of CCK and ERα immunofluorescence at an earlier time point, perhaps 12 hours after injecting

estradiol, may reveal fluctuations in CCK-IR and differences in colocalization that would suggest that the proportion of CCK boutons containing ERα-IR vesicle clusters is dynamic. Experiments examining CCK levels at earlier time points, however, would need to be designed to take into account the diurnal variation of CCK. Fluctuating levels of CCK over a 24 hour time period has been demonstrated in both humans and rodents (Burhol et al. 1980; Nicholson et al. 1983; Pasley et al. 1987; Schade et al. 1993; Lundberg et al. 2007). Diurnal variation of CCK was not a factor in the experiment reported in this chapter. Estradiol- and oil-treated animals were perfused alternately, minimizing differences in timing of treatment and tissue fixation. Additionally, all procedures and treatments were performed during the middle part of the light cycle for the rats, and CCK variation has been shown to occur between light and dark cycles (Pasley et al. 1987).

Colocalization of ERα and NPY

The colocalization of ERα-IR with NPY-IR provides a further clue to the role of extranuclear ERα in the hippocampus. In CA1, NPY-IR is expressed primarily in GABAergic interneurons (de Quidt and Emson 1986; Freund and Buzsaki 1996), but also can be expressed within excitatory neurons (Milner and Veznedaroglu 1992). NPY inhibits presynaptic glutamate release in the hippocampus (Colmers et al. 1988), and in cortical synaptosomes, this occurs through Y1 receptor-dependent reduction in Ca^{++} influx through N- and/or P/Q-type Ca^{++} channels (Wang 2005). Although it is likely that ERα/NPY sites in the CA1 cell body layer are located too far from most glutamatergic

inputs to influence them, it is possible that a similar Y1 receptor-mediated mechanism could regulate GABA release at perisomatic synapses. While it has been demonstrated that NPY has no effect on synaptically mediated inhibition in the str. radiatum (Klapstein and Colmers 1993), similar experiments have not been done in the cell body layer. Calcium-dependent GABA release in CA1 also depends on N- and/or P/Q-type Ca^{++} channels (Doze et al. 1995). Additionally, NPY has been shown to suppress GABAergic synaptic transmission in the spinal cord (Moran et al. 2004) and paraventricular nucleus (Pronchuk et al. 2002), and at least some GABAergic neurons express Y1 receptors (Oberto et al. 2001). Thus, sites at which ERα and NPY are co-localized, whether in presynaptic boutons or other structures in the cell body layer, could provide an additional means by which estrogen suppresses GABAergic synaptic transmission, in this case by modulating the release of NPY.

CHAPTER 5 - CONCLUSION

Collectively, the experiments presented here begin to reveal the complexity of estrogen and estrogen receptor interaction in the hippocampus. Estrogen regulates both excitation (Weiland 1992; Cordoba Montoya and Carrer 1997; Woolley et al. 1997; Foy et al. 1999) and inhibition in the dorsal CA1 (Rudick and Woolley 2001; Rudick and Woolley 2003). When I began these experiments, relatively little was known about ERs in the hippocampus and their role in the mechanisms of estrogen action on the structure and physiology of the CA1 region. Previous studies described a few scattered cells labeled by nuclear ERα-IR (DonCarlos et al. 1991; Weiland et al. 1997), but the extent of ERα expression and the neurochemical nature of cells expressing nuclear ERα were not known. Additionally, even though rapid estrogen effects have been known for more than 20 years (Teyler et al. 1980; Wong and Moss 1992), extranuclear ERα-IR in the hippocampus was only reported for the first time in 2001 (Milner et al. 2001), and quantitative studies were non-existent. This gap in our knowledge had to be filled in order to understand how ERα could mediate the effects of estrogen in the hippocampus.

The results of the experiments discussed in the previous chapters provide answers to many of the fundamental questions about ERα that are important for elucidating the mechanism of estrogen effects on inhibition in the hippocampus. These questions are as follows: 1) What is the distribution of nuclear ERα in the hippocampus? 2) Is nuclear ERα expressed only in GABAergic interneurons? 3) What is the distribution and proportion of GABAergic neurons that express nuclear ERα? 4) Is a specific population

of inhibitory interneurons suggested by the distribution pattern of GABAergic cells expressing nuclear ERα? 5) Does estrogen affect the expression of nuclear ERα or the proportion of GABAergic cells expressing nuclear ERα? 6) Do perisomatic inhibitory boutons contain extranuclear ERα? If so: 7) What structures in the bouton contain ERα? 8) Are boutons that contain extranuclear ERα structurally different from boutons that do not? 9) Is ERα expressed in only a subset of boutons on any given axon? 10) How does estrogen affect ERα in perisomatic inhibitory boutons? 11) What proportion of inhibitory boutons in the pyramidal cell layer contains extranuclear ERα? 12) Is extranuclear ERα present in the most common types of neurochemically distinct inhibitory boutons or is it only expressed in certain cell types? 13) Does estrogen affect the expression of extranuclear ERα in neurochemically distinct inhibitory cell structures in the pyramidal cell layer?

The first 5 questions concerning nuclear ERα expression in inhibitory interneurons and in the hippocampus in general are answered by the analysis of tissue double labeled for ERα and GAD (Chapter 2). Cells expressing nuclear ERα are present in all layers throughout the hippocampus, and distinct patterns of distribution are observed between regions. In the rostral hippocampus, virtually all cells expressing nuclear ERα in the CA1, subiculum, dentate gyrus, and non-principal cell layers of CA2 and CA3 are GABAergic interneurons. Conversely, a relatively small subset of GABAergic interneurons throughout the hippocampus expresses nuclear ERα. The portion of inhibitory interneurons containing nuclear ERα varies by layer, with the smallest percentage in the oriens (~ 5%) and the highest concentration located at the

border of the radiatum and lacunosum-moleculare (~ 32%). For the most part, this pattern of nuclear ERα expression in GABAergic neurons is maintained throughout the rostral-caudal extent of the hippocampus and is unchanged by estrogen treatment. The pattern of GABAergic neurons that express nuclear ERα is not characteristic of one particular subpopulation, suggesting that estrogen may act through nuclear ERα in more than one type of inhibitory interneuron.

In contrast, nuclear ERα expression is not limited to inhibitory interneurons in the pyramidal cell layers of CA2 and CA3. In the rostral hippocampus, ~ 46 and ~ 77% of cells expressing nuclear ERα in CA2 and CA3, respectively, are non-GABAergic cells. Additional analysis of tissue labeled for ERα and counterstained with cresyl violet, revealed that this corresponds to 20-30% of the pyramidal cells in the rostral CA2/CA3 express nuclear ERα. The expression of nuclear ERα in non-GABAergic cells is particularly striking more caudally in the hippocampus. In the dorsal CA2/CA3 of the middle hippocampus, 70-85% of cells expressing nuclear ERα are not inhibitory interneurons. This percentage increases to ~ 94% in the ventral CA2/CA3, while the total number of cells expressing nuclear ERα increases almost 7-fold between the dorsal and ventral hippocampus. Further analysis determined that 50-60% of pyramidal cells in the ventral CA2/CA3 express nuclear ERα. Such a high degree of ERα expression had not been reported previously and suggests that estrogen may act directly in pyramidal cells through nuclear ERα to regulate excitation in the ventral hippocampus. Additionally, the expression of nuclear ERα in 20-30% of pyramidal cells in the dorsal hippocampus reveals the potential for estrogen to act directly in those cells as well, but presumably

with a reduced overall effect compared to the ventral hippocampus. Estrogen treatment did not affect the number or distribution of cells expressing nuclear ERα in any region of the hippocampus.

The next 5 questions concerning the expression of extranuclear ERα in inhibitory structures in the dorsal CA1 pyramidal cell layer are answered by the ultrastructural analysis of tissue labeled for ERα (Chapter 3). The vast majority of ERα-IR within perisomatic inhibitory boutons is located on the vesicular membrane of presynaptic vesicles. Vesicles labeled with ERα-IR are always clustered together within a bouton. Furthermore, three-dimensional serial reconstructions of axon segments revealed that only a subset of boutons on an axon contains ERα vesicle clusters, and boutons immediately adjacent to a bouton with an ERα vesicle cluster do not contain any ERα-IR. Boutons that have an ERα vesicle cluster are not structurally different from boutons that do not.

Estrogen did not affect the degree of expression of extranuclear ERα-IR or the structural characteristics of boutons, but estrogen did have a dramatic effect on the distribution of ERα-IR within a bouton. Approximately 10% of vesicles are labeled in boutons containing ERα-IR, regardless of hormone status. Twenty-four hours after treatment, however, the distance between ERα vesicle clusters and the nearest synapse in estrogen-treated animals is half that of oil-treated animals. This novel finding reveals the presence of a subpopulation of presynaptic vesicles that is sensitive to estrogen and suggests that estrogen may regulate inhibition to CA1 pyramidal cells directly at synapses in perisomatic inhibitory boutons.

This leads us to the remaining questions, which concern the extent of extranuclear ERα expression in inhibitory structures, and are answered by the results of double-label immunofluorescence experiments (Chapter 4). Extranuclear ERα is expressed in ~32% of GABAergic axonal varicosities in the CA1 cell body layer, the majority of which are perisomatic inhibitory boutons forming synapses with pyramidal cells. ERα-IR colocalized with CCK-IR, but not PV-IR, indicating that perisomatic inhibitory boutons containing ERα vesicle clusters arise exclusively from axons of CCK basket cells. Furthermore, approximately 50% of extranuclear ERα-IR puncta colocalized with NPY-IR. Estrogen treatment did not affect the colocalization of ERα and GAD, PV, CCK, or NPY. Taken together, these findings indicate that estrogen affects a subpopulation of presynaptic vesicles, ERα vesicle clusters, in a portion of CCK basket cell boutons, representing approximately one-third of the perisomatic inhibitory boutons surrounding CA1 pyramidal cells.

The answers to the above questions can be summarized pictorially (**Fig. 18**). Perisomatic inhibition of CA1 pyramidal cells is a result of the activity of GABAergic basket cells that express either PV or CCK. Multiple boutons from each basket cell form synapses with the pyramidal cell soma, and one-third of the perisomatic boutons contain clusters of ERα-IR vesicles. ERα-IR vesicle clusters are only present in boutons of CCK basket cells, and these clusters are significantly closer to the synapse following estrogen treatment. **Figure 18** incorporates the extranuclear ERα-IR data from the experiments discussed in Chapters 3 and 4 and will be used to illustrate different ERα-IR scenarios.

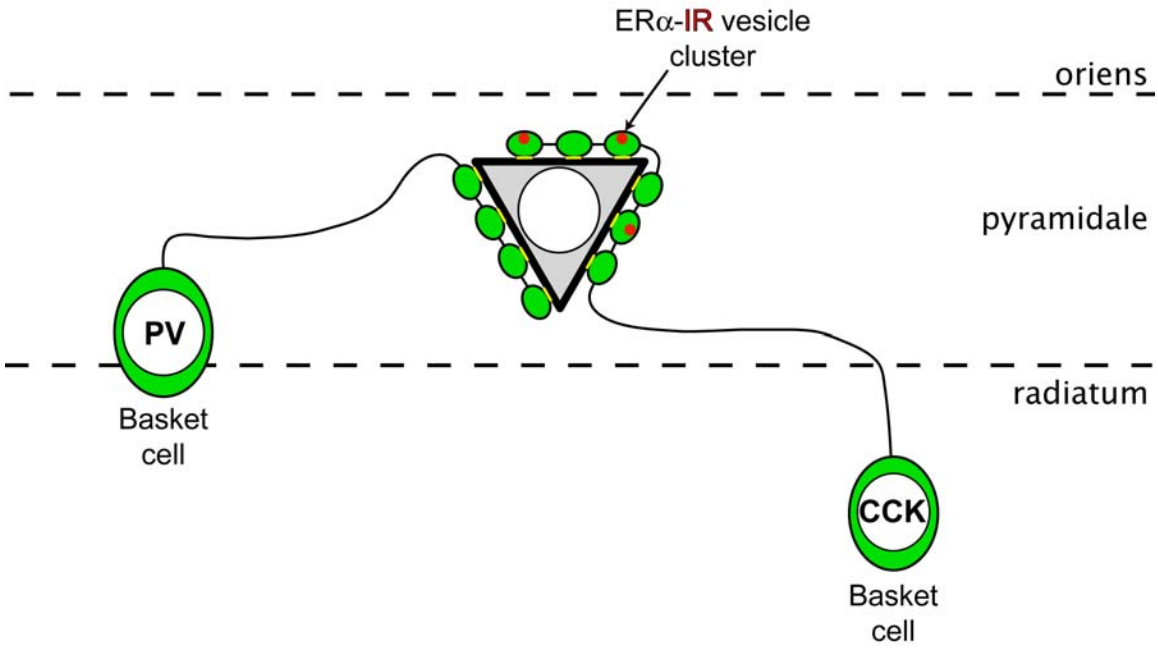

Figure 18. Diagram of extranuclear ERα in perisomatic inhibitory boutons in the CA1 pyramidal cell layer.

Do ERα-IR clusters migrate between boutons?

The location of ERα-IR in CCK perisomatic inhibitory boutons may be dynamic in a manner that would not be detectable by quantitative immunofluorescence: the intriguing possibility that ERα-IR vesicle clusters migrate between boutons. Examination of serial EM sections show that ERα-IR vesicle clusters were often observed in axon segments between boutons, and occasionally, ERα-IR clusters were located half in a bouton and half in the axon. These observations suggest that ERα-IR vesicles are transported along axons and delivered to boutons as clusters. ERα-IR clusters may represent a pool of vesicles that can be deployed in and out of boutons in response to different stimuli or states of the CCK basket cell. A few of the reconstructed boutons contained two ERα-IR vesicle clusters, which may reflect increased mobilization of clusters to meet a higher demand for an estrogen response through extranuclear ERα in the bouton.

Vesicles, including newly recycled vesicles, have been observed to travel between neighboring boutons on axons in primary hippocampal cultures. Using both FRAP and EM, Darcy et al. (2006) observed that within 18 minutes after bleaching, at least 18% of vesicles within a bouton were imported from a neighboring bouton. Newly arrived vesicles were spatially distributed throughout the bouton and underwent exocytosis in a manner similar to native vesicles, although, fewer imported vesicles were available for release. Replenishing vesicle packets contained a mix of vesicles from the recycling and resting pools of the donor bouton. It is possible that ERα-IR vesicle clusters travel between boutons in a similar manner. As stated above, ERα-IR clusters are observed in

axons and entering/exiting boutons, suggesting a dynamic distribution. One potential scenario is that ERα-IR vesicle clusters could mediate an estrogen response that modifies non-ERα-IR vesicles within the same bouton, and then these modified vesicles could migrate to neighboring boutons, thus spreading the estrogen effect between all boutons on an axon (**Fig. 19**).

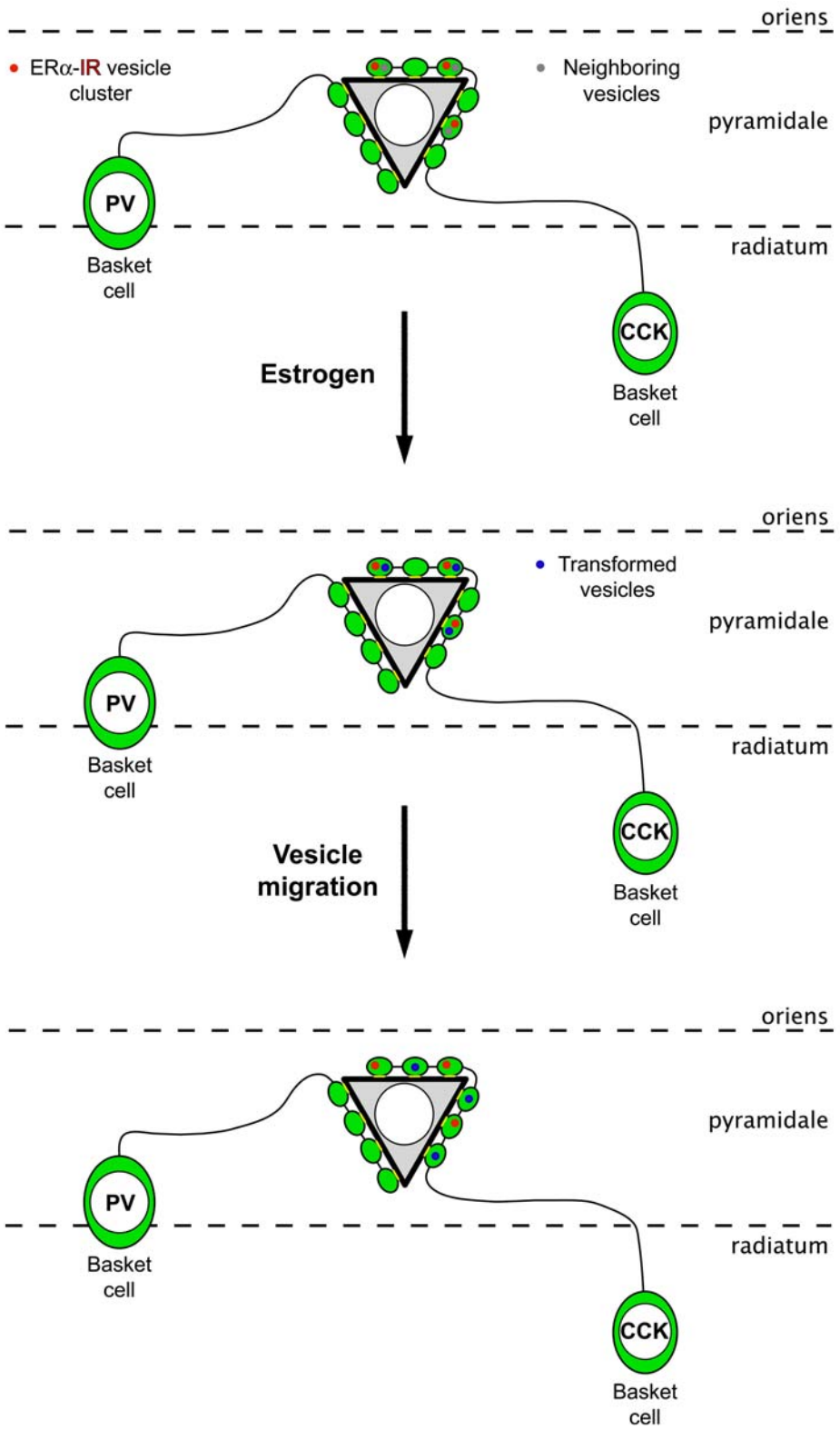

Figure 19. ERα-IR clusters might transform non-ERα vesicles.

How does ERα in perisomatic boutons mediate the observed effects of estrogen on inhibition?

By placing what I have discovered about ERα in perisomatic inhibitory boutons (experimental results discussed in Chapters 3 and 4) within the context of what is already known about the mechanisms of neurotransmitter release, we can begin to develop hypotheses as to how ERα may mediate the effects of estrogen on inhibition. As stated earlier, GABA release from perisomatic inhibitory boutons in the CA1 pyramidal cell layer is decreased 24 hours after estradiol treatment (Rudick and Woolley 2001; Rudick et al. 2003), and ERα-IR vesicle clusters are half again as close to synapses in these same boutons at the same time point in estradiol-treated animals (Chapter 3). The presence of estrogen-sensitive ERα-IR presynaptic vesicles near synapses suggests that extranuclear ERα is poised to mediate the effects of estrogen on GABA release directly in boutons and at potentially multiple points in the synaptic vesicle cycle.

Potential mechanisms affecting exocytosis

One point at which estrogen acting through ERα in the bouton could reduce GABA release from perisomatic inhibitory synapses is during the exocytosis of neurotransmitter vesicles. Multiple proteins are involved in determining the availability of vesicles for exocytosis from the readily releasable pool (RRP). Actin filaments (F-actin) and monomers (G-actin) are ubiquitous cytoskeletal elements in presynaptic boutons and are in equilibrium with each other within the bouton due to the constant assembly and disassembly of actin filaments. F-actin is intimately associated with

synaptic vesicles (Landis et al. 1988; Hirokawa et al. 1989; Gotow et al. 1991) and has been shown to surround clusters of recycling vesicles (Sankaranarayanan et al. 2003), suggesting a corralling function. Actin monomer sequestering agents, such as latrunculin and cytochalasin, disrupt F-actin, thereby shifting the equilibrium in favor of G-actin, and increase exocytosis in primary hippocampal cultures (Sankaranarayanan et al. 2003; Jordan et al. 2005). Estrogen has recently been shown to stimulate the formation of structures composed of actin filaments (lamellipodia, filopodia, and membrane spikes) in endometrial cancer cells (cells rich in extranuclear ERα-IR), suggesting an effect of estrogen to shift the equilibrium in favor of F-actin (Acconcia et al. 2006). It is possible that ERα within perisomatic inhibitory boutons mediates a similar effect on the actin equilibrium in the hippocampus. If so, then it is reasonable to hypothesize that estrogen, in these same boutons, favors the assembly of F-actin. More F-actin would decrease exocytosis and result in less GABA release. Disrupting F-actin with latrunculin or cytochalasin and then observing if estradiol treatment was still able to decrease GABA release could test this idea. If estradiol no longer decreased GABA release, then that would suggest that estradiol uses a mechanism involving F-actin.

F-actin not only potentially acts as a barrier to vesicle movement, but also serves as a scaffold within the bouton. Synaptic vesicles are tethered to F-actin via the protein synapsin (Hirokawa et al. 1989; Greengard et al. 1993). Additionally, there is evidence that synapsin also tethers vesicles to each other (Sankaranarayanan et al. 2003; Jordan et al. 2005) and may be the mechanism by which ERα-IR vesicles cluster together. Synaptic vesicles are liberated from F-actin and become available for release following the

phosphorylation of synapsin (Greengard et al. 1993; Hosaka et al. 1999; Chi et al. 2001; Feng et al. 2002; Chi et al. 2003). When F-actin formation is decreased by cytochalasin, inhibition of Src, a tyrosine kinase that phosphorylates synapsin throughout the brain, no longer results in decreased neurotransmitter release, presumably because there is less F-actin to keep vesicles tethered to (Shyu et al. 2005). Furthermore, inhibition of phosphatases results in increased vesicle mobility, an effect enhanced by the disruption of actin, underscoring the importance of synapsin phosphorylation in vesicle availability for exocytosis (Jordan et al. 2005).

Most experiments examining vesicle mobility have been conducted in excitatory boutons, and it is important to note that synapsins have a significantly different role in inhibitory boutons. In excitatory boutons, synapsins have been shown to regulate the size of the reserve pool (RP) of presynaptic vesicles. However, in inhibitory boutons, synapsins are responsible for maintaining both the RP and RRP (Gitler et al. 2004). In synapsin knockout mice, GABA release at inhibitory synapses is decreased in response to evoked action potentials, while glutamate release at excitatory synapses is unchanged compared to wild-types. Spontaneous release of both GABA and glutamate were unaffected in knockout mice. Gitler et al. (2004) also showed that overall the number of vesicles in boutons is decreased in mice lacking synapsins. Consistent with their electrophysiology data, the number of vesicles within 50nm of the presynaptic density (presumably docked vesicles belonging to the RRP) was decreased in inhibitory boutons but was the same in excitatory boutons compared to wild types, indicating that synapsins do not regulate the size of the RRP in excitatory boutons but do in inhibitory boutons.

Any mechanism that alters vesicle mobility via synapsin in inhibitory boutons will also affect the size of the RRP and the release of GABA at the synapse.

Estradiol treatment has been shown to decrease synapsin phosphorylation in the hippocampus and hippocampal cells, both *in vivo* and *in vitro* (Rebas et al. 1995; Rebas et al. 2005). Estradiol decreases synapsin phosphorylation via PKA, PKC, and CAM Kinase pathways, the same pathways through which synapsin phosphorylation occurs (Hosaka et al. 1999). Extranuclear ERα has been demonstrated to mediate estradiol activation of these same intracellular signaling pathways (Yoona et al. 2001; Shingo and Kito 2005; Li et al. 2006), suggesting that estradiol may decrease synapsin phosphorylation via extranuclear ERα located in the bouton. The effect of estrogen to decrease synapsin phosphorylation is rapid, within 5 minutes of estradiol exposure, and occurs *in vitro* in the absence of nuclei (Rebas et al. 2005), further arguing for a role for extranuclear ERα. Since estradiol does not activate kinase pathways in the absence of extranuclear ERα (Yoona et al. 2001; Li et al. 2006), estradiol-induced decreases in synapsin phosphorylation require ERα and are not simply due to the presence of a steroid compound.

The mechanism regulating exocytosis and, consequently, neurotransmitter release is plastic. Kushner et al. demonstrated that the mobilization of vesicles from the reserve pool (RP) to the RRP is upregulated in transgenic mice expressing a constitutively active form of H-ras (Kushner et al. 2005). H-ras, localized to axon terminals, activated ERK that then increased synapsin phosphorylation. Activation of this signaling chain resulted in an increase in the number of docked vesicles and increased frequency of miniature

excitatory postsynaptic currents (mEPSCs) without a change in mEPSC amplitude, indicating the mobilization of vesicles from the RP to the RRP. When ERK was inhibited, the increase in frequency of mEPSCs in transgenic mice was abolished.

If vesicle mobilization can be upregulated, then it is likely that it can also be downregulated. Kushner et al. also reports a significant decrease in frequency of mEPSCs without a change in amplitude in wild-type mice following ERK inhibition (Kushner et al. 2005). These data suggest a dynamic system regulating the availability of vesicles for release, via synapsin phosphorylation, that can be adjusted up or down. This decreased frequency of mEPSCs is similar to the observed effects of estradiol on mIPSCs in CA1 pyramidal cells, an effect that is ER dependent (Rudick and Woolley 2001; Rudick et al. 2003). It is possible that estrogen acts through extranuclear ERα to decrease synapsin phosphorylation, thus reducing the mobilization of neurotransmitter vesicles from the RP to the RRP and resulting in fewer docked vesicles and decreased GABA release at perisomatic inhibitory synapses.

Potential mechanisms affecting endocytosis

Endocytosis is another point where estrogen could potentially affect GABA release at perisomatic inhibitory boutons. After fusion and release of neurotransmitter into the synaptic cleft, vesicles are recovered from the plasma membrane a short distance from the synapse and recycled within the bouton. Mammalian actin binding protein (mABP), a tyrosine kinase substrate that associates with F-actin, is important for maintaining a functional recycling vesicle pool (Connert et al. 2006). The reformation of

fusion competent vesicles in mice lacking mABP is severely diminished. Interestingly, mABP knockout mice have the same vesicle density and numbers of vesicles near synapses as wild-type mice, but their release probability is lower. Effectively, the loss of mABP results in a smaller RRP without altering the ultrastructural appearance of boutons. In my experiments, I found that vesicle density and overall distribution of vesicles within boutons were not affected by estrogen treatment. If estrogen interfered with mABP in perisomatic inhibitory boutons, then the result would be boutons that are structurally indistinguishable from unaffected boutons, but release less GABA, as is observed following estradiol-treatment. Evidence suggesting how estrogen might interact with mABP is scarce. However, since mABP is a tyrosine kinase substrate and estrogen is known to act through tyrosine kinase pathways in the hippocampus (Bi et al. 2000), it seems plausible that estrogen could affect mABP function. Alternatively, as discussed earlier, estrogen may shift the actin equilibrium in favor of F-actin. mABP colocalizes with F-actin. So, the formation of more F-actin would change the ratio of mABP to F-actin and possibly diminish the ability of mABP to facilitate the formation of competent recycled vesicles.

The possible connection between estrogen, mABP, and GABA release at perisomatic boutons in the CA1 pyramidal cell layer could be tested by electrophysiological experiments utilizing tyrosine kinase inhibitors. The tyrosine kinase inhibitor PP2 has been shown to decrease glutamate release by interfering with vesicle mobilization (Shyu et al. 2005). Experiments could be done comparing the effects of PP2 and estradiol on pyramidal cell inhibition, both individually and combined. If PP2 decreases estradiol-induced disinhibition, then that would suggest that estradiol reduces

GABA release via tyrosine kinase, an effect that could possibly involve mABP since mABP is phosphorylated by tyrosine kinase. If PP2 alone decreases GABA release and does not enhance the decrease in GABA release following estradiol treatment, then that would suggest that estradiol decreases GABA release via a mechanism involving tyrosine kinase inhibition, which would also reduce mABP phosphorylation. It is interesting to note that genistein is both a tyrosine kinase inhibitor (Akiyama et al. 1987) and an ER agonist (Kuiper et al. 1997; Barkhem et al. 1998).

What type of interneuron expresses nuclear ERα-IR?

I have discovered that a portion of perisomatic inhibitory boutons on CA1 pyramidal cells contain ERα-IR vesicle clusters, and I have also determined that a subpopulation of GABAergic interneurons in the hippocampus express nuclear ERα-IR. While the distribution of nuclear ERα-IR cells was not unique to a specific population of interneurons, the pattern is suggestive. In the dorsal CA1, the percentage of GAD-IR cells that also expressed nuclear ERα was lowest in str. oriens, ~5%, and highest at the border between str. radiatum and str. lacunosum-moleculare, ~32%. This suggests that very few if any interneurons that are immunoreactive for somatostatin express nuclear ERα-IR, since virtually all of the somata for these interneurons are located in str. oriens (Kohler and Chan-Palay 1982; Morrison et al. 1982; Johansson et al. 1984; Sloviter and Nilaver 1987). It would also seem that very few PV-IR interneurons express nuclear ERα, since PV-IR somata are limited to str. oriens and the cell body layer (Celio 1986; Kosaka et al. 1987), a layer in which only ~10% of GABAergic cells are ERα-IR. The

low likelihood of PV interneurons expressing nuclear ERα is consistent with the observation that ERα-IR does not colocalize with PV-IR perisomatic boutons.

The neurochemical identity of interneurons with somata distributions somewhat similar to the observed nuclear ERα-IR pattern are those that are immunoreactive for calretinin (CR), NPY, and CCK (Greenwood et al. 1981; Handelmann et al. 1981; Chronwall et al. 1985; Kosaka et al. 1985; Nunzi et al. 1985; de Quidt and Emson 1986; Sloviter and Nilaver 1987; Jacobowitz and Winsky 1991; Gulyas et al. 1992; Miettinen et al. 1992; Rogers 1992). All of these interneuron types, however, appear to be expressed in greater numbers than nuclear ERα, suggesting that if ERα is expressed in any of these interneuron types, it is only expressed in a subset. Nuclear ERα-IR has been reported in CR and NPY, but not CCK interneurons in the hippocampus (Nakamura and McEwen 2005). Further characterization of the exact distribution of nuclear ERα expression between interneuron types could be done with double-labeling immunohistochemistry experiments and may provide valuable clues to as to the mechanisms of estrogen actions in the hippocampus.

Potential mechanism for differential release of GABA and CCK from boutons

As discussed in Chapter 4, CCK basket cell boutons contain ERα-IR and presumably, ERα vesicle clusters. Neuropeptides, such as CCK, are stored in and released from dense core vesicles. Dense core vesicles were commonly present in both ERα-IR and non-ERα-IR inhibitory boutons reconstructed in my experiment. It is reasonable to conclude then, that both CCK and GABA are released from these same

boutons. CCK release from dense core vesicles occurs independently from vesicular GABA release at synapses (Shakiryanova et al. 2005). Dense core vesicles do not move within the bouton unless the cell is firing, and exocytosis of the contents of dense core vesicles requires longer stimulation times relative to neurotransmitter release from presynaptic vesicles. Additionally, neuropeptide release is calcium-dependent, but not actin-dependent (Shakiryanova et al. 2005). Since presynaptic neurotransmitter vesicles require the phosphorylation of synapsin to be released from their actin anchors, and estradiol decreases synapsin phorphorylation, it is possible that when a CCK basket cell is stimulated and estradiol is present, ERα-IR boutons release CCK but little or no GABA. CCK rapidly increases pyramidal cell excitability, so both disinhibition and excitation will be increased simultaneously at pyramidal cell somata (Shinohara and Kawasaki 1997). However, the overall effect of CCK in the hippocampus is to increase GABA release and regulate GABA and NPY expression (Miller et al. 1997; Tirassa et al. 2005). These effects are mediated by CCK receptors on select interneuron somata suggesting that they likely occur via a nuclear mechanism. Also, some of the nuclear ERα+ GABAergic interneurons overlap with the distribution of CCK basket cell somata in the str. radiatum (Chapter 2). Potentially, estrogen could act through nuclear ERα in CCK basket cells to regulate inhibition.

Estrogen acting through both nuclear and extranuclear ERα in CCK basket cells could explain the timing of the observed estradiol-induced fluctuations in inhibition. A possible scenario is that after estradiol exposure, increased CCK release and decreased GABA release from CCK basket cell boutons could result in greater pyramidal cell

excitation. This increase in excitation would then facilitate the formation of dendritic spines on the pyramidal cells. After a delay, estradiol acting through nuclear ERα in a small group of interneurons could increase CCK basket cell firing, resulting in an increase in inhibition on CA1 pyramidal cells and preventing excessive excitatory activity. Further neurochemical identification of interneurons expressing nuclear ERα-IR is necessary to determine if CCK basket cells do contain nuclear ERα. However, since nuclear and extranuclear ERs arise from the same gene transcript, it is quite possible that CCK basket cells contain ERα in multiple locations, including the nucleus.

Estrogen, ERs, the hippocampus, and depression

Estrogen significantly affects mood disorders, particularly major depressive disorder (MDD). Women are twice as likely as men to experience MDD during their lifetime (Hasin et al. 2005). Interestingly, this gender difference in MDD prevalence does not exist prior to the increase in sex steroid production during puberty (Angold et al. 1998; Angold et al. 1999; Born et al. 2002; Hayward and Sanborn 2002). HRT has been shown to be effective in decreasing depressive symptoms in menopausal and perimenopausal women, periods during which endogenous estrogen levels are decreased or highly variable, respectively (Zweifel and O'Brien 1997; Schmidt et al. 2000; Soares et al. 2001). Additionally, HRT has been used effectively to treat postpartum depression (Sichel et al. 1995; Gregoire et al. 1996; Ahokas et al. 2001). Throughout pregnancy, estrogen levels are consistently elevated above normal and then drop suddenly following delivery of the fetus. During the first 4-6 weeks after delivery, when estrogen levels are

low, new mothers are more susceptible to developing postpartum depression. In one study, the majority of subjects suffering from postpartum depression had serum estrogen levels lower than the established threshold for the diagnosis of gonadal failure (Ahokas et al. 2001). Estradiol treatment normalized serum estrogen levels and alleviated the symptoms of postpartum depression in these same women. It is important to note, however, that some studies examining MDD patients have reported normal serum estrogen levels (Rubinow et al. 1988; Rubin et al. 1989; Baischer et al. 1995; Schmidt et al. 2002; Ozcan and Banoglu 2003). One possible explanation for this is that the brains of people with MDD require more estrogen to function normally, and so, normal serum estrogen levels are really decreased levels for them. This scenario could also explain why women are more susceptible to MDD; women regularly experience periods of reduced levels of estrogen because of fluctuations over the menstrual cycle for approximately half of their lifetime. It also allows for the observed improvement in depressive symptoms with HRT.

Inherent in this hypothesis is a predisposition for higher estrogen requirements, either genetic or acquired. Patterns of depression within families are common, indicating a genetic component in the development of MDD. The ER has been implicated as one possible genetic source for a predisposition toward depression. Specifically, ERα genetic variants are associated with an increased susceptibility to MDD (Tsai et al. 2003). Postpartum depression could possibly be an example of an acquired requirement for higher estrogen levels. Estradiol has been shown to decrease ER mRNA and shorten the half-life of ERα (Sohrabji et al. 1994; Wijayaratne and McDonnell 2001). Since serum

estrogen levels are elevated for 40 weeks during gestation, it is likely that ERα expression is reduced by the peripartum period. The sudden postpartum drop of estrogen to pre-pregnancy levels or lower would create an estrogen-deficient state and increase the likelihood that a mother will develop depression. While this mechanism of ERα-mediated postpartum depression seems plausible, experiments supporting or refuting it remain to be done. Similarly, why carrying an ERα genetic variant predisposes one to MDD is not known and also requires further investigation.

Estrogen effects in the hippocampus and the hippocampus itself have also been implicated as major players in MDD. Hippocampal volume is decreased, particularly in the left hemisphere, in both males and females with MDD, an effect that occurs early in the progression of the disease (MacMaster and Kusumakar 2004; Saylam et al. 2006). Additionally, antidepressants increase neurogenesis in the dentate gyrus, while disrupting hippocampal neurogenesis prevents the development of the behavioral effects of antidepressants (Malberg et al. 2000; Manev et al. 2001; Santarelli et al. 2003). Estradiol injected directly into the rat hippocampus decreased depression-like behavior to the same degree as systemic estradiol treatment (Walf and Frye 2006). Conversely, the ER antagonist ICI 182,780 increased depressive-like behavior when injected into the hippocampi of intact rats, indicating that estrogen effects on depression-like behavior in the hippocampus are mediated by ERs.

Estrogen not only has positive effects on depressive symptoms when used as a monotherapy, but also when it is taken in conjunction with antidepressants. Estrogen has been shown to enhance the action of antidepressants, particularly the classes of

antidepressants that increase serotonin (5-HT) signaling. In one study, menopausal women demonstrated a blunted response to a serotonin agonist compared to premenopausal controls (Halbreich et al. 1995). HRT restored the serotonin response to normal levels in the menopausal women. Both testosterone and estradiol facilitated the decrease of serotonin receptor binding, in castrated male rats, following treatment with an antidepressant, an effect likely mediated by ERs (Kendall et al. 1982). Mize and Alper (2002) showed that in hippocampal lysates, estradiol, acting through ERs, uncoupled 5-HT_{1A} receptors from their G proteins, effectively deactivating them. This has implications for depression because, as discussed in Chapter 1, SSRIs ultimately increase serotonin signaling by downregulating serotonin receptors.

Serotonin is also a modulator of plasticity in the hippocampus, and the distribution of serotonin receptors hints at the potential for interaction between estrogen and serotonin effects. Depletion of serotonin in the hippocampus resulted in a significant loss of synapses in the CA1 str. radiatum and str. lacunosum-moleculare (Matsukawa et al. 1997). Serotonin has additionally been shown to play a role in maintaining spine density on CA1 pyramidal cells, but it is not necessary for the development of estradiol-induced spines (Alves et al. 2002). Serotonin receptors are expressed in the hippocampal CA1, particularly in the str. radiatum and the cell body layer. In the str. radiatum, 5-HT_3 receptors are often observed on GABAergic interneurons, while in the cell body layer, both 5-HT_3 and 5-HT_1 receptors are observed in a punctate pattern and are likely located on boutons (Morales et al. 1996; Morales et al. 1996; Iritani et al. 2006). Using a preparation of individual neurons with perisomatic boutons still attached, Katsurabayashi and colleagues determined that GABAergic boutons forming synapses with CA1

pyramidal cells contained both 5-HT_{1A} and 5-HT_3 receptors or 5-HT_{1A} receptors alone (Katsurabayashi et al. 2003). Activation of these two receptors mediate different effects: 5-HT_1 decreases inhibitory transmission and 5-HT_3 increases inhibitory transmission at synapses (Ropert and Guy 1991; Schmitz et al. 1995; McMahon and Kauer 1997).

The location of serotonin receptors on perisomatic inhibitory boutons in CA1 is particularly interesting because one-third of those boutons also contain ERα (Chapter 4). Estradiol acts through ERs to decrease GABA release at perisomatic inhibitory boutons, the same as serotonin acting through 5-HT_1 receptors. Is it possible that both estrogen and serotonin employ the same intracellular mechanism to decrease neurotransmitter release directly at synapses? It seems likely, as it is known that many intracellular pathways are activated by more than one signal molecule. The initial step in answering the question posed above would be to use immunohistochemistry techniques to determine if ERα-IR and 5-HT receptor-IR colocalize to the same boutons. Another important step would be to evaluate the ability of estradiol and serotonin to elicit an effect while blocking the action of the other, perhaps with electrophysiology since both have the effect of reducing inhibition. This experiment would also provide information to answer the following questions: If estrogen and serotonin share a common mechanism to decrease GABA release, what happens when both compounds are present? Do their individual effects summate? Do they cancel each other out? Does one compound facilitate the effects of the other in a non-reciprocating fashion? If estrogen and serotonin do not share a common pathway, then does one alter the effect of the other? I would predict that estrogen and

serotonin do share a common pathway to decrease GABA release. However, I would also predict that estrogen or serotonin alone cannot maximally activate this common pathway and the effects of each are additive when both are present. If estrogen and serotonin do not facilitate the action of the other in a reciprocating fashion, then I would predict that estrogen enhances the effect of serotonin, but that serotonin has no effect on the action of estrogen. Of course, experiments to determine what mechanism ERs activate to mediate estradiol-induced decreases in GABA release must also be done, and would greatly benefit from the development of an *in vitro* system that would allow for maximal manipulation of the extracellular milieu and incorporates a fast and reliable method of assessing estrogen action.

The answers to the questions above have far reaching implications for not only estrogen and ERs in the hippocampus, but also for serotonin and its receptors, antidepressant pharmacological therapy, patients afflicted with depression, and finally, women taking or contemplating starting HRT. As discussed previously, sufficient estrogen levels are required for the development of some behavioral and physiological effects of antidepressants. ERs and serotonin receptors are both located in the CA1 pyramidal cell layer and may even be expressed in the same perisomatic inhibitory boutons. Estrogen and serotonin have similar effects on inhibitory transmission in the dorsal CA1 of the hippocampus through their respective receptors. The hippocampus is sensitive to estrogen and serotonin, both of which modulate plasticity in this region of the brain. MDD has profound effects on the hippocampus, and symptoms of both MDD and postpartum depression are alleviated with estrogen and/or drugs that increase serotonin signaling in the brain. According to the CDC, antidepressants are the most widely

prescribed drugs in the United States, with over 200 million prescriptions written last year (CDC 2006). Despite the fact that the number of women on HRT in the U.S. has been declining since 2002, 57 million prescriptions for HRT were written in 2004 (Hersh et al. 2004). A significant portion of the U.S. population is exposed to exogenous or external regulation of estrogen and/or serotonin levels. The experiments I have presented in the previous chapters contribute to the foundation of knowledge that must be acquired before we can begin to understand intracellular effects of estrogen, how ERs mediate these effects within the brain, and the consequences for people predisposed to or already ill with depression.

Depression, epilepsy, memory, and estrogen intersect in the hippocampus

Depression, epilepsy, and estrogen are intimately connected. Major depressive disorder is the most common psychiatric disorder in people with epilepsy (Jones et al. 2005; Prueter and Norra 2005). Temporal lobe epilepsy (TLE; involving the hippocampus) has the highest prevalence of MDD of all forms of epilepsy (Piazzini et al. 2001); people who have complex-partial seizures (i.e. TLE) are more likely to be depressed compared to those who have generalized tonic-clonic seizures (Grabowska-Grzyb et al. 2006). Furthermore, people with TLE are 3 times more likely to have a mood disorder compared to healthy people without epilepsy (Jones et al. 2007). Data from multiple studies are in conflict as to whether anti-depressants have pro- or anticonvulsant effects, and sadly, as a consequence, most epileptics never receive treatment for depression. One recent report, however, showed that less than 10% of children and adolescents with epilepsy experienced an increase in frequency of seizures when taking

SSRIs, demonstrating the relative safety of treating these patients for depression (Thome-Souza et al. 2007). The comorbidities of depression and epilepsy beg the question: Does epilepsy cause depression or vice-versa in these patients? Interestingly, both children under 10 and adults over the age of 55 (the two age groups with the highest incidence of epilepsy) were found to manifest depressive symptoms prior to experiencing their first seizures (Hesdorffer et al. 2000; Hesdorffer et al. 2006). Therefore, depression is a risk factor for developing new-onset seizures.

Because SSRIs alleviate the symptoms of depression in many people, it is thought that depression results from insufficient levels of neurotransmitter, such as serotonin, in select brain areas, including the hippocampus. The current model postulates that serotonin receptors are upregulated in people with untreated depression because of the scarcity of neurotransmitter. Unfortunately, this increase in receptors only serves to further dilute serotonin signaling because reuptake at the presynaptic terminal is also increased. Some studies have shown that low serotonin levels increase seizure susceptibility (Wenger et al. 1973; Lazarova et al. 1983), thereby providing a mechanism by which depression could facilitate the development of epilepsy. Estrogen also increases the frequency of seizures in some epileptics and may utilize the same mechanism to lower the seizure threshold in the hippocampus. Since estradiol has been shown to facilitate decreased serotonin binding and deactivate $5HT_{1A}$, both mediated via ERs (Kendall et al. 1982; Mize and Alper 2002), the overall effect of estradiol would be to decrease serotonin signaling, thus mimicking a low serotonin state. If lack of serotonin does increase seizure susceptibility, then it is possible that this is the mechanism through which estradiol lowers the seizure threshold in the hippocampus.

Estradiol has also been shown to have antidepressant-like effects and to enhance the action of SSRIs. The same deactivation of $5HT_{1A}$ could be the mechanism through which this occurs. Fewer active $5HT_{1A}$ will result in more serotonin available at synapses, thus enhancing signaling and mimicking the effect of SSRIs. While it seems that the estradiol-depression mechanism is at odds with the estradiol-seizure mechanism, they can coexist if the mechanisms require different levels of estradiol to achieve the end effect. It may be that when estradiol levels are low, the uncoupling of $5HT_{1A}$ is sufficient to lower the seizure threshold but not enough to significantly increase serotonin levels at synapses. Higher, but still physiological, levels of estradiol may be necessary to fully deactivate $5HT_{1A}$, which would then result in higher serotonin levels. This scenario accommodates the following observed effects: increased seizure susceptibility in depression, increased depression during periods of low estrogen, and antidepressant effects of estrogen. What it does not account for is the increase in frequency of seizures during periods of high estrogen. However, this hypothesis could still be useful for generating experiments designed to test the complex interactions between estrogen, depression, and epilepsy in the hippocampus. Experiments in which the proposed connections were blocked with inhibitors or antagonists would be illuminating and would provide information useful in revising the hypothesis.

Learning and memory is also a part of the complex pattern of connections between depression, epilepsy, and estrogen in the hippocampus. Many studies have demonstrated that spine density is decreased in humans epileptics and in animal models of epilepsy (Jiang et al. 1998; Swann et al. 2000; Gonzalez-Burgos et al. 2004; Wong 2005). The behavioral effect of this spine loss is decreased performance in hippocampal-

dependent learning tasks and reduced cognition. Similarly, mentally retarded individuals, who have diminished abilities for learning and memory to begin with, are at increased risk for developing a seizure disorder during their lifetime. As discussed in Chapter 1, estrogen increases dendritic spines at a timepoint that is coincident with estradiol enhancement of learning tasks, while depression decreases concentration and cognitive abilities and reduces performance on learning and memory tasks.

A common point of connection between estrogen, memory, depression, and epilepsy in the hippocampus is the perisomatic inhibitory bouton, where ERα and serotonin receptors happen to be located. Estrogen is obviously connected via ERα. Memory is enhanced when dendritic spine density is increased by estrogen, an effect that has been shown to result from disinhibition of pyramidal cells, which occurs at perisomatic inhibitory boutons. Depression is a derangement of neurotransmitter signaling, most often treated with an SSRI (which downregulates serotonin receptors), and that can be triggered by an abrupt loss of estrogen. Epileptic seizures occur more frequently when estrogen levels are high, which is the same time at which spine density and disinhibition are increased, and eventually result in the loss of serotonin receptors (Giovacchini et al. 2005; Kim et al. 2007). The convergence of three behaviors at a single point presents the unique opportunity to have experimental results for one to be relevant for all.

It is important, however, to keep in mind that memory, depression, and epilepsy are complex behaviors and not likely to be explained by intense investigation of only a single structure or mechanism. For example, not all people suffering with depression

respond to SSRI therapy. Other classes of antidepressant drugs shown to be effective in some patients include monoamine oxidase inhibitors, mixed serotonin/norepinephrine reuptake inhibitors, norepinephrine/dopamine reuptake inhibitors, and tricyclic antidepressants. The difficulty in unraveling the complex mechanisms underlying the effects of estrogen on depression is illustrated by bupropion (Wellbutrin), a popular antidepressant and smoking cessation drug. Bupropion works by making more norepinephrine and dopamine available at synapses. Because higher concentrations of neurotransmitter are present for a longer time at the synaptic cleft, it is more likely to diffuse out into the surrounding area. Focusing specifically on dopamine, extensive and intense immunoreactivity for dopamine receptors is observed in the CA1 of the hippocampus, and in particular, the pyramidal cell layer (Khan et al. 1998; Ciliax et al. 2000). It seems likely that there is quite a bit of dopamine around in an area with a high concentration of receptors, and even more dopamine when the uptake is inhibited by bupropion. The result is that more dopamine is in the same general vicinity as the perisomatic inhibitory boutons that contain ERα-IR vesicles. This is potentially important because dopamine has been shown to potentiate effects mediated through ERs via a mechanism known as ligand-independent activation (Power et al. 1991; Ciana et al. 2001; Olesen et al. 2005). Given the relationship between estrogen, ERs, serotonin, and serotonin receptors discussed above, it is reasonable to hypothesize that bupropion, a norepinephrine/dopamine reuptake inhibitor is activating the same ER-mediated pathway as SSRIs. Unfortunately, there is a dearth of studies examining the effects of estrogen on the efficacy of the most common non-SSRI antidepressants. As discussed above, the

number of people treated pharmacologically for depression is enormous, and new antidepressants continue to be introduced. Knowledge of how estrogen interacts with non-SSRI agents would seem to be particularly important, given the well-established connection between estrogen and depression, and is an area that is in sore need of further investigation.

Why are ERs so prevalent in the hippocampus?

It is clear from the results of the experiments discussed in the previous chapters that ERs are prevalent in the hippocampus. In fact, ERs have been detected throughout the brain, as discussed in Chapter 1. But, why is a receptor for a reproductive hormone expressed so widely throughout the body in tissues as diverse as brain, bone, lung, and vasculature? Estradiol is the final product formed in a biosynthetic pathway that begins with cholesterol and produces the steroid hormones (**Fig. 20**). Steroid hormones have a variety of effects throughout the body: metabolic homeostasis, calcium homeostasis, sodium homeostasis, maintenance of cardiovascular function, immune system regulation, reproduction, and modulation of behavior and cognitive function (Friedman 2004). Basically, the steroid hormone family is necessary for life. While estrogen effects do not include all of those listed above, a growing body of literature describes a role for estradiol, and ERs, in a variety of basic cellular functions common to many cell types. How, then, did estradiol, the last product in an impressive line of essential steroid hormones come to influence so many cellular functions?

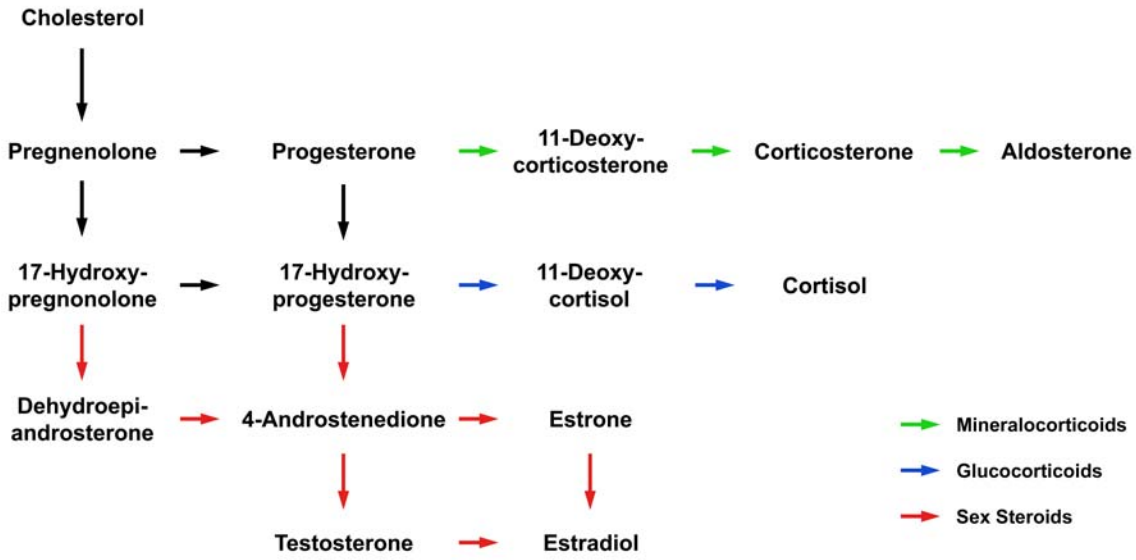

Figure 20. The steroid hormone biosynthetic pathway.

Cholesterol is transformed into steroid hormones via both serial and parallel routes. Green, blue, and red arrows denote routes that lead exclusively to the production of mineralocorticoids, glucocorticoids, and sex steroids, respectively. Black arrows denote routes common to more than one steroid hormone category. Note that progesterone is an intermediate in all three routes.

The answer may be found in the evolutionary history of the steroid receptor family. Using genome mapping and phylogenetic analyses, a model of steroid hormone evolution has been proposed that begins with an ancestral steroid receptor (AncSR) that was an estrogen receptor (Thornton 2001). The AncSR shares a high degree of homology in the DNA- and ligand-binding domains with human ERα and ERβ, 71% and 68% respectively, compared to 33-36% for mineralocorticoid (MR), glucocorticoid (GR), progesterone (PR), and androgen receptors (AR). AncSR and ERs are identical in many respects, including the amino acid sequence that determines the specificity of the receptor for its DNA target sequence, indicating that AncSR activated genes with estrogen-response-elements. According to Thornton's model, AncSR, the first steroid receptor, was an ER, and the last steroid hormone in the biosynthetic pathway, estradiol, was the first steroid hormone to act through an intracellular receptor. Intermediates in the synthetic pathway, such as progesterone and testosterone, were present but did not have a receptor-related function. As the genome expanded, duplications of this first ER gene would occur and be redundant, leaving them free to evolve into receptors with affinities for the intermediates. The development of new steroid receptor-ligand combinations would result in novel signaling functions for the intermediates, some of which were previously the responsibility of estrogen and ERs. The result would be more precise control over specific functions, analogous to the difference in wounds inflicted by a pistol (intermediate steroid hormone/receptor) and a shotgun (estrogen/ER).

If the steroid receptor family did evolve in this manner, then it is reasonable to hypothesize that ERs originally mediated many of the effects of steroid hormones on

physiological and developmental functions. As more complex creatures developed and the need for more specific control of an expanding number of cellular and organ-level functions increased, steroid hormone receptors evolved from the original ER, gained the ability to specifically bind available intermediates in the biosynthetic pathway, and took over some of the signaling functions from the estrogen/ER complex. One could conclude, then, that with the exception of select functions that require more specific control, the plethora of functions originally modulated by estrogen are still modulated by estrogen. If true, then the loss of estrogen should be devastating for maintaining the fitness of an organism.

Indeed, a lack of estrogen induces disease states in multiple organ systems throughout the body. Estrogen depletion, either due to surgery or menopause, results in osteoporosis, which can be reversed with hormone replacement therapy (HRT) (Yasuda et al. 1994; Castelo-Branco et al. 1999; Kanaoka et al. 2003). Estradiol abolishes ovariectomy-induced hypertension (Varbiro et al. 2000). Menopausal women have a higher risk of developing and dying from cardiovascular disease, but are protected from this increased risk by HRT (Mericli et al. 2004; Manson et al. 2007). Lack of estrogen increases the risk of developing metabolic syndrome, an effect that appears to be mediated, at least partially, through ERα, and attenuated by HRT (Salpeter et al. 2006; Gallagher et al. 2007; Musatov et al. 2007). Wound healing is significantly delayed following estrogen depletion in both humans and rodents, an effect that is reversed with HRT (Ashcroft et al. 1997). Cerebral aneurysms are more likely to form when estrogen is absent, and aneurysm formation is arrested by HRT (Jamous et al. 2005; Jamous et al.

2005). Pre-menopausal women who lack estrogen as a result of bilateral salpingoophorectomy (BSO) are more likely to suffer from cognitive impairment and Parkinson Disease compared to women who have had no surgery and post-BSO women who are on HRT (Rocca et al. 2007; Rocca et al. 2007). Overall, increased mortality is observed in women who have undergone BSO prior to menopause and have not taken HRT (Rocca et al. 2006). Clearly, estrogen is necessary for maintaining the proper function of multiple body systems, and it is easy to imagine how strong bones that are more resistant to fracture, a healthy cardiovascular system, quick wound healing, and protection from CNS pathology are beneficial to survival. Could it be that adequate estrogen levels, either from functioning ovaries or from the conversion of testosterone, produced by functioning testicles, is the universal signal that informs the body that it is physically capable of reproduction and, therefore, must expend resources to maintain an appropriate level of fitness? If so, estrogen receptors are surely indispensable in this process.

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