Hypothalamic Gene Expression and Signal Transduction Effects of Estrogen

by

Anna Malyala

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Anna Malyala

has been approved

[redacted]
Mentor/Advisor (Dr. Oline K. Rønnekleiv)

[redacted]
Co-Mentor (Dr. Martin J. Kelly)

[redacted]
Member (Dr. Cynthia L. Bethea)

[redacted]
Member (Dr. Daniel M. Dorsa)

[redacted]
Member (Dr. M. Susan Smith)

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List of Abbreviations

ARC	Arcuate nucleus
BH	Basal hypothalamus
BSA	Bovine serum albumin
DAG	Diacylglycerol
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GABA	Gamma aminobutyric acid
GABARAP	GABA-A receptor associated protein
Gec1	Glandular epithelial cell 1(also known as GABARAPL-1)
GIRK	G-protein coupled inwardly rectifying potassium channel
GST	Glutathione S-transferase
GnRH	Gonadotropin releasing hormone
LH	Luteininzing hormone
IRS	Insulin receptor-substrate
NPY	Neuropeptide Y
PI	Phosphoinositide
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
POA	Preoptic area
POMC	Proopiomelanocortin
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
RTK	Receptor tyrosince kinase
SH	Src homology
VMH	Ventromedial nucleus

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Abstract

The goal of this dissertation is to define the underlying molecular mechanisms of estrogen-mediated effects that influence homeostasis. The rapid and enduring effects of estrogen in the central nervous system are complex and overlap with multiple signaling pathways such that defining its mechanism of action has been difficult. By elucidating hypothalamic genes and signaling pathways regulated by estrogen, we will develop a better understanding of how multiple physiological processes are affected and interconnected. Furthermore, revealing estrogen's molecular effects will enable development of specific and targeted therapies for disorders associated with fertility and homeostasis.

The background for this dissertation is presented in chapter 1. It includes the fundamental role of estrogen in reproduction and its link to energy balance; illustration of the hypothalamus as a key mediator of estrogen action; approaches taken to understand the multifaceted signaling of estrogen; review of phosphatidylinositol 3-kinase signaling; and finally, the putative roles of other regulated estrogen-regulated genes such as gec1 in hypothalamic signaling.

Chapter 2 explains the high throughput approach to identify estrogen-regulated genes in the hypothalamus. We describe the method used to create a guinea pig brain-specific cDNA library that was subsequently used for printing cDNA microarray chips. Microarray analysis of estrogen- and vehicle-treated hypothalamic tissue reveals that many regulated genes are involved in signal transduction and neurotransmission. Futhermore, upon examination of the promoter and enhancer sequences of several

regulated genes, we find that estrogen affects transcription directly via estrogen response elements (ERE) but also via non-classical mechanisms independent of EREs.

Further analysis of the microarray data show that PI3K p55γ and many transcripts encoding pleckstrin homology domain-containing proteins are differentially regulated, suggesting that PI3K signaling is critical for some estrogen-mediated effects. In chapter 3, we present findings on the expression of PI3K regulatory subunits in the central nervous system. We propose a model in which estrogen alters the sensitivity of molecules that signal through the PI3K pathway by changing the regulatory subunit levels. Furthermore, electrophysiology data reveal that PI3K signaling may mediate some of the rapid estrogen effects at the membrane. These data suggest estrogen acts at multiple levels of the PI3K pathway to mediate rapid and sustained effects.

Chapter 4 highlights other genes regulated by estrogen in the hypothalamus. Gec1, a known estrogen-regulated gene in the periphery, is highly homologous to the $GABA_A$ receptor associated protein (GABARAP) and we find that gec1 expression increases with estrogen in the arcuate nucleus. GABARAP facilitates $GABA_A$ receptor trafficking and insertion into the plasma membrane, and evidence suggests that gec1 may also function similarly. Interestingly, PI3K signaling facilitates the insertion of $GABA_A$ receptors to the neuronal membrane and implies that estrogen could act in multiple ways to affect rapid signaling and gene transcriptional events.

Finally in Chapter 5, a general discussion on these research findings is presented. Future experiments building upon the research presented here are listed and thus will provide more insight on the neuronal effects of estrogen to modulate homeostasis.

Chapter 1

Historical Characterization of Estrogen

By the time Starling coined the term "hormone" in 1905 to describe internal secretions, endocrinology as a scientific discipline had taken off. An informative and historical perspective of the isolation and characterization of estrogen in the early 1900s was recently published by Davis et al. (Davis, Dinatale, Rivera-Woll, & Davison, 2005). It was a time of rapid progress, going from isolation of crude organ extracts to pure crystalline hormone preparations to be used for treating various human ailments.

In 1912, Henri Iscovesco, a Parisian gynecologist, reported the extraction of a potent substance from sows' ovaries using lipid solvents (Davis et al., 2005). When injected into young rabbits, the extract induced premature sexual maturation, and when administered to humans, it was said to be a successful therapy for dysmenorrhoea and amenorrhoea. Otfried Fellner independently reported similar findings in 1913, also using lipid extracts from sows' ovaries in young female rabbits and guinea pigs (Davis et al., 2005). Interestingly, in 1917, Charles Stockard and George Papanicolaou observed histological changes in the vaginal mucosa cells that closely paralleled the phases of the menstrual cycle (Davis et al., 2005). They further reported that these changes were abolished after oophorectomy, and their findings subsequently led to the first biological assay to determine the efficacy of ovarian extracts.

More progress in the endocrinology field came from the seminal findings by Edgar Allen and Edward Doisy that were published in 1923. By injecting follicular fluid from hog ovaries to oophorectomized rats and mice, they found that it restored their estrous cycles (Davis et al., 2005). This was determined by the cytological changes in the vaginal epithelial cells as described by Stockard and Papanicolaou's method. Further research at this time was hampered by the limited quantities of follicular raw

material. However, within a decade, chemists detected estrogenic hormones in human urine and even more in the urine of pregnant women, which let to the isolation of estrone and estriol in crystalline form; isolation of the more potent estradiol from sows' ovaries by Doisy came in 1936 (Davis et al., 2005). These early studies provided the scientific platform for subsequent understanding of estrogen and its related forms.

Estrogen Biosynthesis and Physiology

Estrogens are derived from cholesterol via the precursor androgenic steroids androstenedione and testosterone, as shown in Figure 1. Estrogens are a group of steroid compounds, and of these, estradiol has the highest biological activity (Meldrum et al., 1980). Oxidation of the hydroxyl group at C-17 of estradiol gives rise to estrone, which is 50-70% less active than estradiol. Addition of a hydroxyl group at C-16 of estradiol gives estriol, which is 90% less active than estradiol.

During the menstrual cycle, ninety-five percent of circulating estradiol is produced in the ovaries by granulosa cells of the developing follicle and corpus luteum as shown in Figure 2. Estradiol concentration increases fivefold or more from baseline in a natural ovarian cycle, with serum levels ranging from about 30 pg/mL during menses to about 200 pg/mL at ovulation (Baird & Fraser, 1974). In contrast to estradiol, less than half of estrone is produced by direct ovarian secretion. It is converted from estradiol by 17β-estradiol dehydrogenase in the liver and other tissues (Fishman, Bradlow, & Gallagher, 1960). Estrone is also produced by the conversion of estrone sulfate by the adrenal, and by the aromatization of androstenedione whose concentration also peaks at mid-cycle.

Estrogens, like other steroid hormones, are tightly bound to carrier proteins in blood circulation. About 38% of circulating estradiol is bound to sex hormone binding globulin (SHBG), 60% to albumin, and about 2% circulating in the free state (Dunn, Nisula, & Rodbard, 1981). SHBG concentrations increase with estrogen therapy,

pregnancy, hyperthyroidism, and smoking, but decrease with androgen excess, hypothyroidism, obesity, and insulin resistance (Dunn et al., 1981). For example, obese women have up to 30% less SHBG than non-obese women, resulting in a relative increase of free estradiol in obese women (Dunn et al., 1981).

Serum estrogens dramatically decrease in the perimenopause and postmenopausal period (Longcope, 1986). For example, postmenopausal women have serum estradiol levels below 15 ng/mL and mean estrone levels of about 30 pg/mL (Cauley, Gutai, Kuller, LeDonne, & Powell, 1989). Women who have had bilateral oophorectomy show little difference in serum levels of estrone, estradiol, or testosterone when compared with women undergoing a natural menopause (Cauley et al., 1989). Older women convert androstenedione to estrone more efficiently than young women, which may in part be due to the two- to fourfold increase in adipose tissue aromatase mRNA expression between age 20 and 60 (Bulun, Zeitoun, Sasano, & Simpson, 1999).

In females and males, small amounts of estradiol are produced from non-ovarian sources through local aromatase activity on testosterone or androstenedione (MacLusky, Philip, Hurlburt, & Naftolin, 1985; Simpson & Davis, 2001). Aromatase expression in the central nervous system is mainly in hypothalamic (POA and VMH) and limbic areas (BNST, amygdala, hippocampus) and less so in the cerebral cortex (Roselli & Resko, 1987; Roselli, Klosterman, & Resko, 2001; Wagner & Morrell, 1997; Stoffel-Wagner, 2003). Interestingly, aromatase expression is detected in glial cells of particular brain regions (where it is normally not expressed) that have been affected by insult or injury (Azcoitia, Sierrra, Veiga, & Garcia-Segura, 2003). Unfortunately, the localized concentration of estrogen or testosterone in the brain has not been well studied (Bixo, backstrom, Winblad, & Andersson, 1995; Hojo et al., 2006) with most studies correlating levels to those of plasma. The implications of local estrogen concentrations in the central

nervous system may be important especially in terms of neuroprotection, however, sufficient data are not available to make any solid conclusions.

Estrogen Receptors and Mechanism of Estrogen Action

Prior to the tools of molecular biology, [^{3}H]-17β-estradiol was used to demonstrate that estradiol was specifically retained in estrogen target tissues (e.g., uterus), which led to the hypothesis that a receptor must exist for this molecule. The same autoradiography techniques were also used to identify hypothalamic brain regions that concentrate estrogen and included the preoptic area and the arcuate nucleus (Pfaff & Keiner, 1973; Sar & Stumpf, 1975; Warembourg, 1977; Tardy & Pasqualini, 1983). An estrogen receptor (ER) was isolated from rat uterus by Toft and Gorski (Toft & Gorski, 1966) and later from other mammalian species, including human (Toft, Shyamala, & Gorski, 1967; Gorski, Toft, Shyamala, Smith, & Notides, 1968; Jensen, 1968). However, it would be more than a decade before the first ER, now called ERα, was cloned from MCF-7 human breast cancer cells by two independent groups of investigators (Walter et al., 1985; Green et al., 1986; Acuna-Goycolea, Tamamaki, Yanagawa, Obata, & Van den Pol, 2005; Greene et al., 1986). A second ER (ERβ) was later identified in 1996 during investigations of the role of estrogens in the rat prostate and ovary; it was subsequently cloned from several species (Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996; Mosselman, Polman, & Dijkema, 1996; Tremblay et al., 1997).

ERα and ERβ are encoded by separate genes and located on different chromosomes (Freedman & Roehrs, 2006). They are members of the hormone binding nuclear receptor family that were likely created by a series of duplications from a common ancestral receptor gene (Kurrasch-Orbaugh, Watts, Barker, & Nichols, 2003). The 66 kDa ERα and 53 kDa ERβ are hormone-inducible transcription factors that can

act positively or negatively to regulate gene expression to regulate tissue growth and differentiation (Kuiper et al., 1996; Mosselman et al., 1996; Nilsson et al., 2001), in addition to other functions. The structures of both receptors are illustrated in Figure 3.

There are six structural domains (termed domains A–F) of ERα and ERβ. There is a predicted 97% homology in the DNA binding domain (region C), and a 60% homology between the E/F domains, but the A, B, and hinge (region D) domains are even less well conserved between ERα and ERβ. In addition to their structural domains, the ERs contain defined functional domains. For example, the transactivation domain termed activation function (AF-1) resides within the amino-terminal A/B domains and mediates ligand-independent activation function (Metzger, 1995). In addition, the A/B region contains a coregulatory domain, which binds various ER coactivators and corepressors that modulate ER-mediated transcriptional activity. The C domain is composed of two zinc finger motifs and encodes the DNA binding domain that is responsible for binding to specific estrogen response elements (EREs) within the promoters of estrogen-responsive genes (Rebois & Hebert, 2003; Goda & Südhof, 1997). The ER dimerization domain is split between the C and E domains, and it is required for the ERs to dimerize, enabling binding of the entire ERE site (Rebois et al., 2003). The structural D domain contains the hinge region, part of the ligand-dependent, transactivation domain AF-2 and a portion of the ER nuclear localization signal (Hilgemann, Feng, & Nasuhoglu, 2001). The carboxy-terminal E and F regions contain the ligand binding domain and the ligand-dependent AF-2 transactivation domain (Hilgemann et al., 2001). This carboxy-terminal region is also involved in receptor dimerization, and the binding of coregulatory and chaperone proteins, such as heat shock proteins (Klinge, 2000). The high homology of the DNA binding domain between the receptors suggests that they can bind to similar DNA sequences however, the low

homology in the ligand binding and other domains suggests that these are the regions for conferring differential activation by the receptor subtypes.

There are a number of functional similarities between human ERα and ERβ, such as binding to E_2 with similarly high affinities (Kuiper & Gustafsson, 1997; Vertes, Fortin, & Crane, 1999). Ligand-activated ERα and ERβ can form homodimers (ERα-ERα or ERβ-ERβ) or heterodimers (ERα-ERβ), which then bind to various EREs (Garattini, Mennini, Bendotti, Invernizzi, & Samanin, 1986; Vertes et al., 1999; Katzenellenbogen, O'Malley, & Katzenellenbogen, 1996; Nikolov & Ivanova-Nikolova, 2004). However, there also exist significant differences between the receptor subtypes as shown by tissue distribution pattern and functional consequences of gene deletion of ERα or ERβ (Katz, Newman, & Izenwasser, 1997) (Couse, Lindzey, Grandien, Gustafsson, & Korach, 1997; Valdez, Burke, & Hensler, 2002; Saunders et al., 2000; Freedman et al., 2006; Shughrue, Lane, & Merchenthaler, 1997) (Oka, Oka, & Saper, 2003).

A key example is that ERα knockout mice are infertile and do not display typical lordosis behavior, whereas ERβ knockout mice are fertile and do exhibit lordorsis behavior (Ogawa et al., 1998). Receptor expression data in the CNS also show that the two subtypes do not completely overlap, suggesting separate functional roles for ERα and ERβ (Simerly, Chang, Muramatsu, & Swanson, 1990; DonCarlos, Monroy, & Morrell, 1991; Shughrue et al., 1997; Shughrue & Merchenthaler, 2000; Laflamme, Nappi, Drolet, Labrie, & Rivest, 1998; Gundlah et al., 2000; Warembourg & Leroy, 2004). The differences in the ligand-binding domain of both ERs are such that a variety of compounds can bind to these receptors to produce agonist, antagonist or mixed responses. This variety in responses is attributed to the unique three-dimensional conformation induced by the specific ligand binding to the ER, which determines how the ligand-receptor complex will behave (Mao et al., 2004). This multifarious behavior

explains why phytoestrogens, xenoestrogens and selective ER modulators (SERMs) can bind ERs and elicit different responses (Navarro et al., 2004). Moreover, growth factors and other compounds can activate the AF-1 region of the receptor and show estrogen-like actions without acting like traditional ligands. This cross talk between estrogen and growth factors may be important for signal amplification and will be discussed in Chapter 3. Interestingly, binding data highlight the fact that the traditional definitions of agonist and antagonist are not really applicable in the case of estrogen and its receptors, since each receptor, receptor combination, and array of coregulators will produce different effects upon stimulation. The existence of two distinct ERs indicates that the signaling pathways for endogenous estrogens are significantly more complex than originally expected. To add to the complexity is the probability of yet another ER, the "membrane" estrogen receptor (Kelly, Qiu, Wagner, & Rønnekleiv, 2003; Kelly, Qiu, & Rønnekleiv, 2003; Qiu et al., 2003; Qiu et al., 2006b).

Around the same time of the early autoradiography studies trying to localize receptors for estrogen in the periphery, a number of studies showed that estrogen can directly alter the electrical activity of hypothalamic neurons (Barraclough & Cross, 1963; Dyer, Pritchett, & Cross, 1972; Bueno & Pfaff, 1976; Dufy, Partouche, Poulain, Dufy-Barbe, & Vincent, 1976; Yagi, 1970; Yagi, 1973; Yagi & Sawaki, 1971; Whitehead & Ruf, 1974; Moss & Law, 1971; Kubo, Gorski, & Kawakami, 1975; Lincoln, 1967; Lincoln & Cross, 1967). For example, Yagi revealed changes in the firing rate of preoptic and basomedial hypothalamic neurons soon after giving an intravenous injection of estrogen (within 16 minutes) (Yagi, 1970). Furthermore, neuronal firing rates in preoptic area neurons dramatically decreased after microelectrophoresis application of nanomolar concentrations of 17β-estradiol but not 17α-estradiol (Kelly, Moss, & Dudley, 1977; Kelly, Moss, Dudley, & Fawcett, 1977). What is striking about these studies is the rapidity with which the effects took place, similar to the actions of neurotransmitters.

These electrophysiology studies corresponded with the later biochemical studies in the 1980s in which [^{3}H]-17β-estradiol bound with high affinity and specificity to rat brain synaptosomal membrane preparations (Towle & Sze, 1983). Further corroboration that the effects of estrogen were at the membrane came from studies using [^{125}I]-17β-estradiol congjugated with bovine serum albumin (BSA) (Zheng & Ramirez, 1997). Since BSA is a large bulky protein unable to permeate the lipid membrane, the observed binding is thought to be due to specific binding of radiolabled 17β-estradiol to the membrane fraction. These and other studies (Pietras & Szego, 1977; Pietras & Szego, 1979) put forth the concept of membrane steroid receptors mediating the rapid effects of estrogen well in advance of the current scientific debate on this subject.

The Role of Estrogen in the Hypothalamic Pituitary Gonadal Axis

The primary role of estrogen in mammalian brain is its negative and positive feedback actions at the hypothalamic-pituitary axis to orchestrate the menstrual/estrous cycle of females. For most of the cycle, estrogen plays an inhibitory role on the neuronal network regulating GnRH secretion (Sarkar & Fink, 1980; Caraty, Locatelli, & Martin, 1989; Chongthammakun & Terasawa, 1993; Evans, Dahl, Glover, & Karsch, 1994), which is considered the final output of a system that integrates numerous external and internal cues to regulate the release of LH and FSH from the anterior pituitary gland (refer to Figures 4 and 5). Inhibition of GnRH secretion by estrogen is mediated by direct actions on GnRH neurons (Kelly, Rønnekleiv, & Eskay, 1984; Lagrange, Rønnekleiv, & Kelly, 1995; Abraham, Han, Todman, Korach, & Herbison, 2003; Abraham, Todman, Korach, & Herbison, 2004) as well as indirect actions onto GABA and opioid neurons, which synapse onto to GnRH neurons (Herbison & Dyer, 1990; Herbison, Heavens, Dye, & Dyer, 1991; Herbison, 1997; Wagner, Rønnekleiv, Bosch, &

Kelly, 2001). Disruption of the feedback loop by ovariectomy results in the rise of LH and FSH within 1-2 days. Restoration of the feedback loop occurs rapidly by giving exogenous doses of estrogen that mimic physiological levels. After an initial drop in plasma gonadotropin levels (negative feedback effects), the positive feedback actions of estrogen increase GnRH secretion, which then induces an LH surge approximately 36-48 hours later (Sarkar et al., 1980; Moenter, Caraty, & Karsch, 1990; Pau, Berria, Hess, & Spies, 1993), depending on the species (Tsai & Yen, 1971; Yamaji, Dierschke, Bhattacharya, & Knobil, 1972; Terasawa, Rodriguez, Bridson, & Wiegand, 1979; Levine et al., 1985). These effects are time- and dose-dependent, and they are orchestrated with other reproductive tissues to precisely time ovulation and subsequent fertilization.

The Guinea Pig Model, Energy Homeostasis & the Hypothalamus

In addition to its principal role in reproductive physiology, estrogen influences a number of homeostatic functions, including energy balance and thermoregulation. The female guinea pig is an excellent model to study how gonadal steroids regulate such homeostatic functions because of the longer ovulatory cycle (16-18 days) as compared to the rat or mouse (4 days), the estrogen-regulated reproductive functions are more similar to that of primates, and the extensive physiological observations made in this species documenting estrogen's effects on food intake, water intake and thermoregulation (Terasawa et al., 1979; Butera & Czaja, 1984; Czaja & Butera, 1986; Czaja, Butera, & McCaffrey, 1983; McCaffrey & Czaja, 1989). Using a species with similarities to the primate is important, if we are to understand how estrogen regulates the myriad number of homeostatic functions that it does, many of which are dysregulated in aging and human disease (McEwen, 2002; Genazzani, Monteleone, & Gambacciani, 2002; Gibbs & Aggarwal, 1998). Although the use of non-human primates would be ideal because of their similarity to the human reproductive cycle, the costs and level of

involvement to work with them is prohibitive. Thus, much of our work uses the guinea pig as a model.

Not surprisingly, there has been a renewed interest in understanding how food intake and body weight are regulated. Although a number of brain regions play a role in energy homeostasis (for a comprehensive and well-written review, see (Berthoud, 2002) and are targets for estrogen, the hypothalamus is still regarded as a key regulator of energy balance and a primary site of estrogen action. Indeed, the pioneering hypothalamic lesioning studies that were performed to explore central mechanisms of obesity laid the foundation for our current understanding of the neural circuitry underlying energy homeostasis. Hetherington was the first to use a stereotaxic instrument to place discreet hypothalamic lesions in the ventromedial nucleus (VMH) in rats and study the lesion effects on weight (Lober, Pereira, & Lambert, 2006). These and other studies at the time demonstrated that lesions to the VMH, particularly the ventrolateral part, caused obesity due to hyperphagia (Lober et al., 2006). At this time, Kennedy proposed that the VMH was the satiety center in the brain, such that feeding behavior is inhibited when the VMH is activated (Lober et al., 2006). A year later, the lateral hypothalamus (LH) was proposed to be the feeding center of the brain, when Anand and Brobeck reported weight loss due to aphagia by placing lesions in the LH of the cat (Cox & Cohen, 1996). Additional electrical stimulation experiments at the time supporting roles for the VMH and LH in satiety and feeding led Stellar to come up with the dual center hypothesis for motivated behaviors (Vickers, Clifton, Dourish, & Tecott, 1999). While this notion of two specific centers controlling feeding behavior and body weight is too simplistic, and subsequent work has shown that other hypothalamic nuclei and brain regions play a role in energy homeostasis, it did help the move the field forward.

Interestingly, considerable evidence suggests the control of feeding and peripheral fat deposition is sexually dimorphic. Furthermore, food intake varies during

the menstrual cycle in humans and animals, and these physiological observations suggest a role for gonadal hormones in body weight homeostasis (Buffenstein, Poppitt, McDevitt, & Prentice, 1995; Dye & Blundell, 1997; Fong & Kretsch, 1993; Li, Tsang, & Lui, 1999; Paolisso et al., 1999). For example, a luteal phase peak and a periovulatory nadir in food consumption are seen across the menstrual cycle in humans and animals. In addition, it is known that ovariectomized animals gain weight and have a tendency to become obese, and this is reversed by estrogen replacement treatment (Asarian & Geary, 2002). The key candidate in mediating these effects is the gonadal hormone estrogen. Estrogens are considered to be anorectic, thus leading to decreased food intake and reduced body weight. Estrogen affects many peripheral sites involved in energy balance but it also has central effects that reduce food intake and body weight. Although it is known that estrogen acts directly within the hypothalamus to affect energy balance, the specific mechanisms underlying estrogen's actions are not completely understood.

Of the individual hypothalamic nuclei involved in regulating energy homeostasis, neurons of the arcuate nucleus are in a unique position to directly sense peripheral metabolic factors because of the incomplete blood-brain barrier in this area. Figure 6 illustrates the major hypothalamic nuclei involved in sensing and transducing peripheral signals. These neurons receive information from numerous peripheral signals reflecting the energy status of the body (i.e., insulin, leptin, glucose, ghrelin, and many others) and integrate those signals from inputs from other brain regions regarding sensory attributes, reward expectancies and emotional aspects of food(Kelley, Baldo, & Pratt, 2005; Saper, Chou, & Elmquist, 2002). At least two distinct neuronal populations within the arcuate contain receptors for both insulin and leptin and these populations mediate opposing effects on feeding. One group of neurons, containing neuropeptide Y (NPY) and agouti-related protein (AgRP), is considered orexigenic (i.e., stimulates feeding and decreases

energy expenditure), while the other group, containing POMC and cocaine-and amphetamine-regulated transcript (CART), is considered anorectic (i.e., inhibits feeding and increases energy expenditure) (Schwartz, Woods, Porte, Jr., Seeley, & Baskin, 2000). More specifically, it is the posttranslational product of POMC, α-melanocyte stimulating hormone (α-MSH), that is considered to be anorectic via signaling through the melanocortin (MC-3 and MC–4) receptors (Saper et al., 2002). However, recent data suggest the β-endorphin, another posttranslational product of POMC, may also be involved in energy homeostasis(Appleyard et al., 2003). Reduced signaling by either leptin or insulin results in hyperphagia and obesity (Obici, Feng, Karkanias, Baskin, & Rossetti, 2002; Eckel, Rivera, & Atchley, 2005). Evidence points to a partial overlap between insulin and leptin signaling in arcuate neurons, since these hormones exert similar effects on neuronal firing rates and neuropeptide gene expression (Schwartz et al., 1991) (Schwartz et al., 1992; Stephens et al., 1995). For example, both insulin and leptin inhibit NPY gene expression while increasing POMC gene expression (Spiegelman & Flier, 2001). There is considerable evidence that insulin and leptin signaling converge on the PI3K intracellular signal transduction pathway, but the extent of signaling overlap and the phenotype of all neurons that signal via this pathway has not yet been determined (Niswender & Schwartz, 2003). This involvement of the PI3K pathway for metabolic signaling was of particular interest to us and PI3K is described in the following section.

Characterization of PI3K, a Key Signal Transduction Enzyme

Phosphatidylinositol 3-kinases (PI3Ks) are a conserved family of agonist-stimulated enzymes that catalyze the addition of phosphate at the 3'-OH position of inositol head groups of membrane phosphatidylinositol (PtdIns) and phosphoinositides

(PIs). Phosphatidylinositol consists of an inositol ring attached to the 1'-OH group of phosphatidic acid. The free –OH groups of the inositol ring can be phosphorylated in different combinations and these phosphorylated derivatives are referred to as phosphoinositides. See figure 7 for structure of the various phosphorylated species.

Between the late 1970s and 1980s, phosphoinositides emerged from being perceived as a minor group of metabolically active, membrane phospholipids to being superstar members of the phosphatidylinositol cycle involed in signal transduction. It started with the discovery of phospholipase C (PLC)-mediated hydrolysis of PtdIns(4,5)P_2 lipids to generate the second-messenger molecules diacylglycerol (DAG), which activates protein kinase C (PKC) (Goudie, Thornton, & Wheeler, 1976), and inositol 1,4,5-trisphosphate (IP_3), which liberates calcium from intracellular stores (Berridge, 1984). This led to the ensuing interest in identifying the enzymes involved in maintaining the phospholipid pool of the PtdIns cycle. The two distinct kinase activities known at the time included the PI4-kinase activity, which converted phosphatidylinositol to PI(4)P, and the PI5-kinase activity, which catalyzed the formation of PI(4,5)P_2 from PI(4)P. Although it was long suspected that another PI kinase existed based on kinase activities found in association with oncogene products (Wright, Seroogy, Lungren, Davis, & Jennes, 1995) (Kuohung & Kaiser, 2007), the existence of PI3K was not confirmed until 1988 when the different phosphorylated products of two different kinases identified in mouse fibroblasts were structurally verified (Tietjen et al., 2003).

Whitman et al. distinguished PI3-kinase from PI4-kinase because one kinase was inhibited by nonionic detergents but resistant to inhibition by adenosine, while the other was adenosine-sensitive; they also noticed that the products of each kinase exhibited slightly different migration rates using thin layer chromatography (TLC) (Franceschini et al., 2006). To determine the different sites of phophorylation on the inositol ring, PtdIns was labeled in the inositol ring with [^{3}H] and phosphorylated using

unlabeled ATP. The resulting [^{3}H]-PtdIns was purified and separated by high pressure liquid chromatograpy (HPLC), and it was then discovered that one kinase phosphorylated the 3' position, the other the 4' position of the inositol ring (Tietjen et al., 2003). A number of observations suggested the 3'phosphorylated lipids were not precursors for signaling but were signals themselves (Sutton, Patterson, & Berthoud, 2004). First was the failure to find a phospholipase that hydrolyzed the 3' phosphorylated lipids (Bethea, 1993; Van de Kar & Lorens, 1979). Also, PI(4)P and PI(4,5)P_2 are normally present in significant quantities and decline after stimulation by hormones or growth factors, and it was observed that PI(3,4)P_2 and PI(3,4,5)P_3 are barely detectable in quiescent cells and rapidly increase with stimulation (Franklin & Paxinos, 1997; Watson, Elliott, & Brown, 1995). More evidence to support the view that these lipids were indeed signaling molecules came from the identification of a particular phosphatase enzyme that preferentially removed the phosphate group from the 3' position of the inositol ring (Bonhaus et al., 2006; Hewitt, Lee, Dourish, & Clifton, 2002). Thus, a new branch of lipid signaling was initiated, and it has provided a number of novel insights to cellular physiology.

PI3K enzymes were originally isolated and purified from rat liver (Gamper, Li, & Shapiro, 2005), bovine brain (Steiner et al., 2006; Conn, Sanders-Bush, Hoffman, & Hartig, 1986; Samanin, Mennini, & Garattini, 1980) and bovine thymus (van Wijngaarden, Tulp, & Soudijn, 1990). Preliminary characterization of the PI3K enzymes purfied from these various tissues showed some subunit variations; however, all were found to associate with proteins of 85 kDa, 110 kDa or both.

Mammalian cells contain three distinct classes of PI3Ks (I-III) that differ in mechanism of activation, substrate specificity, structure, and subcellular and tissue distribution (Vanhaesebroeck & Waterfield, 1999). These three classes of PI3K produce stereochemically distinct phosphoinositides—PI(3)P, PI(3,4)P_2, PI(3,5)P_2 and

$PI(3,4,5)P_3$. The unique stereochemistry and precise location of phosphoinositides provide the specificity required for the exquisitely controlled functioning of a cell. Indeed, aberrant phosphoinositide signaling has been implicated in cancer and a host of other diseases (Wymann, Zvelebil, & Laffargue, 2003). Of the three classes, class I PI3K has been the most thoroughly investigated and will be described below in detail. While important for cell signaling and membrane trafficking, the specific functions of class II and class III PI3Ks have not been thoroughly characterized and are not well understood at this time, and thus will not be discussed here (Vanhaesebroeck et al., 1999).

Although PI3Ks utilize phosphatidylinositol, PI(4)P, and $PI(4,5)P_2$ *in vitro*, their preferred substrate *in vivo* is $PI(4,5)P_2$ (Guo & Schofield, 2002). Each of these lipid species (in addition to other species that are not 3' phosphorylated) is predominantly enriched in discrete subcellular membranes and function both as signaling molecules and as localization cues (Cooper & Jan, 2003; Bajic, Hoang, Nakajima, & Nakajima, 2004). An excellent comprehensive review on the role of phosphoinositides in signal transduction, defining organelle identities, regulating membrane traffic (i.e., endocytosis, vesicle formation, etc.), and role in cytoskeletal changes and nuclear events, has been recently published by DiPaolo and DeCamilli (Södersten, Bergh, & Zandian, 2006).

In eukaryotic cells, the predominant inositol lipid is phosphatidylinositol, with typical concentrations of 0.5-2.5 μmol/g tissue which is roughly 2-12% of total cellular phospholipids (Breisch, Zemlan, & Hoebel, 1976). The polyphosphoinositides constitute less than 0.1% of total cellular lipids (Guo et al., 2002). And as mentioned before, unlike other PIs whose levels are relatively constant or decrease, the basal levels of $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ are quite low but dramatically rise in response to specific stimuli.

Class I PI3K Catalytic Subunits and Enzymatic Activity

PI3K enzymes are heterodimers composed of one catalytic and one regulatory or adaptor subunit (Li & Van den Pol, 2006; Makarenko, Meguid, & Ugrumov, 2002). The subunit structures and the general mechanism of activation are illustrated in Figure 8. The catalytic subunit displays dual specificity by phosphorylating the 3' position of PtdIns and its phosphorylated derivatives, and also phosphorylating serine residues of PI3K regulatory subunits and insulin receptor substrate (IRS-1) (Li et al., 2006; Oka et al., 2003; Oka et al., 2000; Berg, Maayani, Goldfarb, & Clarke, 1998).

There is a complex interplay between the regulatory and catalytic subunits. For example, when expressed without the regulatory subunit, the catalytic subunit is active but highly unstable at 37°C; when expressed with the regulatory subunit, it is thermally stable but not catalytically active (Watanabe et al., 2002). The lipid kinase activity of the catalytic subunit is regained when the SH2 (Src homology) domain of the regulatory subunit binds to a tyrosine-phosphorylated protein (details of domains and binding is discussed below) (Watanabe et al., 2002). The catalytic subunit is also the binding site for wortmannin (Suh & Hille, 2005), a highly potent inhibitor of PI3K (Hessemer & Brück, 1985). LY294002, an analog of quercetin, is another more selective PI3K inhibitor (Roa et al., 2006). These low molecular weight compounds are structurally unrelated, cell-permeable and specific inhibitors at low concentrations. LY294002 is a competitive inhibitor of the ATP site (Roa et al., 2006). In contrast to LY294002, wortmannin binds covalently to the PI3K catalytic subunits, a reaction that is competed by ATP and $PI(4,5)P_2$ but not PtdIns (Suh et al., 2005).

Class I PI3Ks are subdivided into class I_A and class I_B, and they signal downstream of tyrosine kinases and heterotrimeric G-protein-coupled receptors, respectively. Class I_A PI3K is essential for insulin signaling *in vivo*, and loss of activity of class I_A PI3Ks causes complex metabolic defects that are linked to type II diabetes (Mouihate & Pittman, 2003; Azmitia & Gannon, 1986). In addition, genetic

polymorphisms within the PI3K pathway are also associated with an increased risk of type II diabetes (Mouihate et al., 2003; Azmitia et al., 1986). Moreover, amplification and point mutations of the genes encoding PI3K subunits have been frequently found in human cancers (Saudou & Hen, 1994; Shen, Hamilton, Nathanson, & Surmeier, 2005).

The class I_A catalytic subunits, including p110α, p110β, and p110δ, are encoded by the genes *Pik3ca, Pik3cb,* and *Pik3cd*, respectively (Otsu et al., 1991) (Yu, Rusak, & Piggins, 1993; Li et al., 2006; Stoyanov et al., 1995) and regulate many diverse processes—such as metabolism, proliferation, growth, survival and motility—by acting downstream of hormones, growth factors, cytokines, integrins and other extracellular stimuli (Caterina & Cater, 2006). In contrast to the restricted distribution of p110δ to mainly leukocytes (Elmquist, Coppari, Balthasar, Ichinose, & Lowell, 2005) (Castellano et al., 2006), northern blot analysis from various mouse tissues shows p110α and p110β expression widely distributed, with the highest expression in kidney for the latter (Makarenko et al., 2002). Ablation of p110α or p110β results in embryonic lethality, which indicates non-redundant and essential roles for these subunits, at least during mouse development (Korsgaard et al., 2005; Hoshi et al., 2003). However, a fraction of PI3K activity is apparently sufficient for normal development since heterozygous mice are viable (Korsgaard et al., 2005; Hoshi et al., 2003).

PI3K p110γ (which is not related to the PI3K p55γ regulatory subunit described below) is the sole class I_B enzyme whose activation by βγ-subunits of heterotrimeric G-proteins is potentiated by its association with the p101 or p84 non-catalytic proteins (Stoyanov et al., 1995). In addition, PI3Kγ catalytic activity is directly activated by Ras (Leaney, Dekker, & Tinker, 2001) (Dechering, Boersma, & Mosselman, 2000) and regulates mitogen-activated protein kinase (MAPK) cascades, such as those controlled by Jun-N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK)

(Horowitz et al., 2005; Bonasera & Tecott, 2000). There is no similarity between p110γ and either p110α or p110β in the amino terminal region in which they bind their regulatory subunits (Oka et al., 2003). Unlike the ubiquitous expression of p110α and p110β, p110γ is enriched in cardiomyocytes and white blood cells (Levitan, 2006). It also appears that p110γ knockout mice or mice with targeted mutations of its enzymatic activity are viable, fertile and do not exhibit any overt adverse phenotype (Dhaka, Viswanath, & Patapoutian, 2006; Sodersten, Bergh, & Zandian, 2006; Luquet, Perez, Hnasko, & Palmiter, 2005). These data suggest that the p110γ subunit may not play a large role in PI3K signaling in the central nervous system but this has not been thoroughly investigated to date.

Class I PI3K Regulatory Subunits Provide Signal Specificity

The class I_A catalytic subunits are tightly associated with any of the five PI3K regulatory subunits, which are encoded by 3 distinct genes (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991; Inukai et al., 1996; Inukai et al., 1997; Pons et al., 1995). Please refer to Figure 8 for regulatory isoform description. The p85α regulatory subunit encoded by *Pik3r1* gene also gives rise to the p55α and p50α isoforms by alternative splicing. The *Pik3r2* gene encodes the p85β subunit, while the *Pik3r3* gene encodes the p55γ (also known as PIK) subunit. The carboxy-terminal domains of all 5 regulatory subunits are highly homologous and are composed of two SH2 domains, which have a high affinity for phosphorylated tyrosines in pYXXM motifs of growth factor receptors, their substrates or adaptor proteins (Navarro et al., 2006). For example, the YMDM motif in the platelet-derived growth factor (PDGF) receptor associates directly with the p85 regulatory subunit SH2 domain (Misra et al., 2006), enabling translocation of cytosolic PI3Ks to their lipid substrates in the membrane. The inter-SH2 domain of

the regulatory subunits is the binding site for catalytic subunits. In contrast to the conserved carboxy-terminal sequence among the regulatory subunits, the amino-terminal sequence is unique to each subunit. For example, the p85α and p85β subunits each have a large amino-terminus containing an SH3 and a bcr homology (BH) domain whereas the smaller subunits have much smaller amino-terminal domains consisting of 34 (p55α and p55γ) and 6 (p50α) amino acids (Pons et al., 1995; Inukai et al., 1997).

In contrast to the rapid progress made in delineating the molecular actions of PI3K signaling, there is a relative dearth of knowledge about PI3K expression in the brain. The field has mainly focused on the role of PI3K in insulin signaling and oncogenesis (studies mostly in cell lines and peripheral tissues), and while these are enormously important, PI3K plays key roles in many other physiological functions. Furthermore, considering the high lipid content of the brain, the importance of lipid signaling in the brain, that PI3K activity was originally found in the bovine brain, and the importance of the surrounding lipid environment for modulating some ion channels are, it is surprising that relatively little has been published on PI3K expression and function within the central nervous system. I will highlight what is known about PI3K regulatory isoform expression in the CNS, emphasizing data related to the hypothalamus, and discuss the functional aspects of such expression.

The earliest studies used biochemical and northern blot analyses to identify PI3K regulatory subunits and found them present at high concentrations in the rat CNS and spinal cord (Skolnik et al., 1991; Boulant, 2006). However, these early studies looked at p85 expression because it was the only regulatory subunit known at the time. For example, the study by Folli et al. evaluated the topographic and cellular distribution of p85α and its possible colocalization with insulin receptor substrate 1 (IRS1), insulin receptor (IR), and insulin-like growth factor-I (IGF-I) (Boulant, 2006). They first show

that IR, IRS-1 and p85α are present in the adult rat brain at similar concentrations as those in muscle and liver by western blots. Then using immunoctochemistry, they report a good correlation between the distribution of p85α, IR, and IRS-1 in many brain regions. Their findings were novel in that they found p85α to be a component of growth factor signaling that is expressed in neurons of the adult rat brain and showed this at the cellular level by immunocytochemistry.

Soon after the Folli study was published, Pons et al. screened an adipose-cell cDNA library with [^{32}P]-labeled IRS-1 and isolated a number of IRS-1 binding proteins, one of which was ~80% homologous to p85α in the SH2 domains and named it p55^{PIK} (currently known as p55γ) (Pons et al., 1995). Next, they measured levels of p85α, and p55γ mRNA in mouse tissues by northern blot and immunoblot analysis. Consistent with the mRNA data, western blot data from brain, liver, testes and adipose tissues revealed p55γ expression most abundant in brain and testes; whereas p85α was expressed in all tissues. In summary, this group found a new regulatory element of PI3K, described its expression in comparison with the p85 subunits in the developing and postnatal mouse brain, and partially characterized its function. Their data showing high p55γ expression during development suggest a role in growth and development, and perhaps in plasticity and survival in the adult brain. The possibility of p55γ's role in neuronal plasticity and its connection to estrogen is addressed in Chapters 3 and 5 of this dissertation.

Functional Significance of PI3K Regulatory Subunits

The importance of documenting the distribution and expression levels of the regulatory subunits stems from functional studies that show differential responses with various stimuli depending on the subunit expressed. This suggests that the regulatory subunits are able to fine-tune the signaling from growth factors and other stimuli.

Although the studies described below were performed in cell lines or from peripheral tissue, it is not unreasonable that some of these phenomena might extend to signaling that occurs in the CNS. Moreover, recent data suggests that the regulatory subunits' role in negative feedback signaling may in part underlie the pathophysiology of metabolic syndrome, type II diabetes, pregnancy-induced diabetes, cancer and others (Schiltz & Sawchenko, 2002).

To address how PI3K signaling is altered by the regulatory subunits, Inukai et al. (Tschöpl, Hui, & Horvath, 2007) overexpressed the regulatory isoforms along with various receptor tyrosine kinases (RTKs) in Chinese hamster ovary (CHO) cells. Their data suggest that the combination of a RTK and expression level of each regulatory subunit determines both the degree of PI3K activation and the downstream effectors used for signaling in response to a particular growth factor. This is important because of the N-terminal sequence and structure differences of the regulatory subunits that can recruit different adaptor proteins and have different affinities to RTKs. These effects can be related to physiological consequences such as insulin signaling, based on knockout studies described below. For example, not only does the regulatory subunit protect the catalytic subunit from degradation and inhibit its lipid kinase activity (Watanabe et al., 2002), there is evidence that p85 isoforms are in excess of over p110 isoforms and can compete with the p85/p110 holoenzyme for binding to IRS proteins and thus impair insulin signaling (Ueki et al., 2003). Hence, not only do the regulatory subunits regulate the location and specific activity of the catalytic subunit, but they also act as positive and negative modulators of PI3K activity. The study described above by Inukai et al. and another study showing the p55 subunits bind to tubulin (which is described later) are the only functional studies published on the p55γ regulatory subunit. Furthermore, the lack of knockout studies for this subunit has also made it difficult to assess the importance of this subunit's physiological role.

A major downstream effector of PI3K signaling is the protein serine/threonine kinase Akt (also known as protein kinase B or PKB). Upon membrane recruitment by $PI(3,4,5)P_3$, Akt becomes activated through phosphorylation at Thr-308 in its catalytic loop by the kinase PDK1 (Chang, Zhang, Tam, & Sanders-Bush, 2000). Additional phosphorylation at Ser-473 of Akt leads to its full activation (Bayascas and Alessi, 2005). Akt regulates cell growth and metabolism through the phosphorylation of a number of target molecules. Furthermore, Akt phosphorylates and inactivates the forkhead (FOXO) family of transcription factors that regulate the expression of a wide range of metabolic, survival and cell cycle genes (Pfaffl, 2006; Higashida et al., 2005).

In the central nervous system, the PI3K pathway is involved in the promotion of neuritic growth and synaptic plasticity (Niswender et al., 2003), and interestingly, there is evidence to suggest a role for estrogen interaction with PI3K signaling. For example, remodeling of axosomatic inhibitory synapses prior to the gonadotropin surge has been described (Calizo & Flanagan-Cato, 2000; Calizo & Flanagan-Cato, 2002) that is dependent on Akt activation. In addition, an interaction between the PI3K p85α subunit and ERα takes places within 1-3 h of systemic estrogen administration, and subsequently leads to the the activation of Akt (Cardona-Gómez, Mendez, & Garcia-Segura, 2002; Mendez, Azcoitia, & Garcia-Segura, 2003). Furthermore, estrogen activation of IGF-I receptors in the hypothalamus leads to a functional interaction between IGF-I and ERα (Quesada & Etgen, 2001; Cardona-Gómez et al., 2002; Mendez et al., 2003). To illustrate this interaction, intracerebroventricular (i.c.v.) infusion of a selective competitive antagonist of IGF-1 autophosphorylation, inhibits the estrogen-induced LH surge and sexual behavior in ovariectomized rats (Quesada & Etgen, 2002). Additionally, co-administration (i.c.v.) with blockers of PI3K (wortmannin) and MAPK (PD98059) inhibits the long-term (48 h) effects of estrogen to induce the LH surge and

facilitate lordosis behavior (Etgen & Acosta-Martinez, 2003). These data illustrate the complex interaction between the PI3K, growth factor and estrogen signaling pathways to affect synaptic remodeling, neuronal plasticity, and behavior involved with reproductive function.

PI3K Signaling Affects GABA Neurotransmission

Another observation that peaked our interest in PI3K signaling was that of its role in GABA neurotransmission. GABA is the primary inhibitory neurotransmitter in the brain and acts on three different receptor types including $GABA_A$, $GABA_B$ and $GABA_C$. $GABA_A$ and $GABA_C$ receptors are ionotropic receptors that gate Cl^- channels, whereas the $GABA_B$ receptor is a metabotropic receptor that couples to Ca^{2+} and K^+ channels via G proteins and second messenger systems (Bormann 1988, Bowery and Enna 2000 and Hammond 2001). GABA neurons expressing ERα are found in the preoptic area (POA) (Herbison et al., 1993), the site of the majority of GnRH cell bodies. In addition, close contacts between GABAergic terminals and preoptic GnRH cell bodies, and even GABAergic synapses onto GnRH neurons have been identified (Herbison et al., 1993; Leranth et al., 1985). Administration of GABA, or GABA agonists and antagonists, alters LH secretion (Jackson and Kuehl 2002) suggesting an inhibitory role for GABA in the control of LH secretion. More specifically, there is increased GABAergic transmission in the hypothalamus during the negative feedback phase of the reproductive cycle (Herbison, 1998; Wagner et al., 2001; Jackson & Kuehl, 2002).

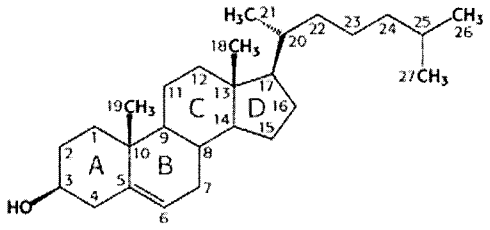
Recently, Tan and colleagues reported that PI3K signaling augments μ-opioid receptor desensitization in sensory neurons (Tan el al., 2003) and data from our laboratory show that μ-opioid and $GABA_B$ receptors are coupled to the same population of GIRKs in hypothalamic neurons. Therefore, we hypothesized that the PI3K may play

a role in the estrogen-induced uncoupling of $GABA_B$ receptors in these neurons. Furthermore, microarray data presented in Chapter 1 of this dissertation showed that estrogen increased gec1 mRNA expression in the hypothalamus. Gec1 encodes a $GABA_A$ receptor associated protein (GABARAP). As mentioned above, $GABA_A$ receptors are ligand-gated ion channels and efficient synaptic transmission depends on the precise spatial and temporal localization of these receptors such that they are in close apposition to presynaptic GABA releasing terminals (Kneussel, 2002). The increased expression of gec1 may result in the targeting of $GABA_A$ receptors to the membrane, contributing to the observed increase in GABAergic transmission during negative feedback (Connolly, Roselli, & Resko, 1991; Wagner et al., 2001; Herbison, 1998; Jackson et al., 2002).

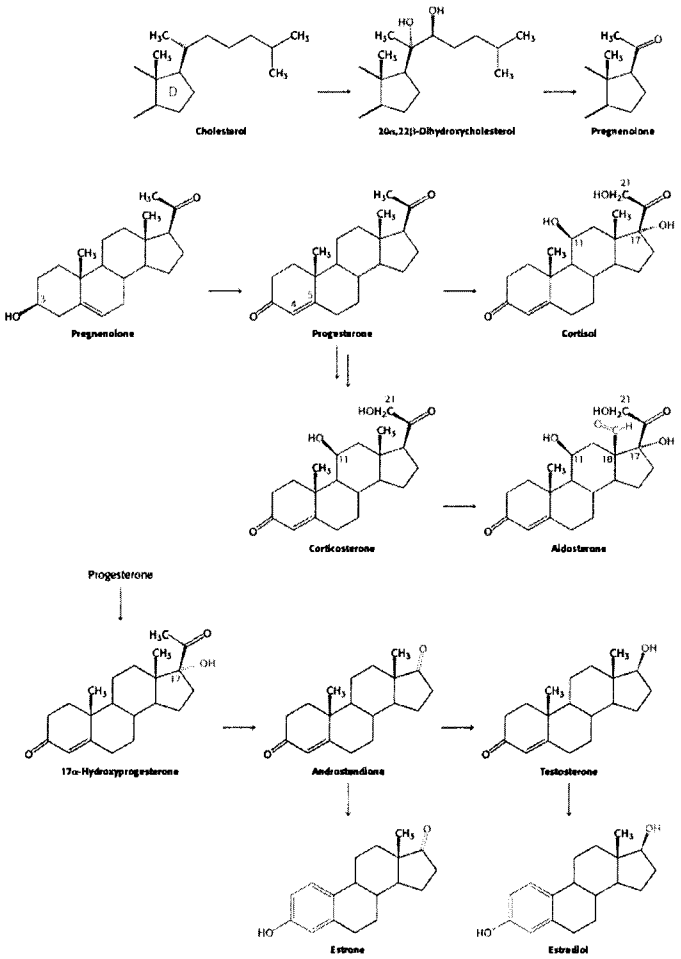
The aim of this thesis is to unravel, at least in part, the signaling events that are affected by estrogen in the hypothalamus to regulate homeostatic functions. To investigate this question, I used microarray analysis to find estrogen-regulated genes in the guinea pig hypothalamus, then followed up on certain genes by characterizing their distribution using *in situ* hybridization, verified that these genes were indeed regulated by estrogen and finally, investigated their possible functional roles of estrogen-mediated effects in hypothalamic neurons. I found a number of genes regulated by estrogen that play a role in signal transduction and synaptic plasticity, and determined the microarray approach used was a useful strategy. I also found that estrogen regulated the expression of p55γ in some hypothalamic nuclei and importantly, the data showed that the PI3K pathway mediates some of the rapid estrogen responses that are initiated at the membrane. Finally, I observed increased gec1 mRNA expression in the hypothalamus after estrogen treatment and this may play a role in GABA and PI3K signaling, both of which are important components of estrogen-mediated events.

Figure 1-1

A. Carbon numbering for cholesterol and other steroids



B. Biosynthesis of steroids



Biochemistry, 5th Edition; Editors: Berg, Tymoczko, Stryer. 2002

Figure 1-2

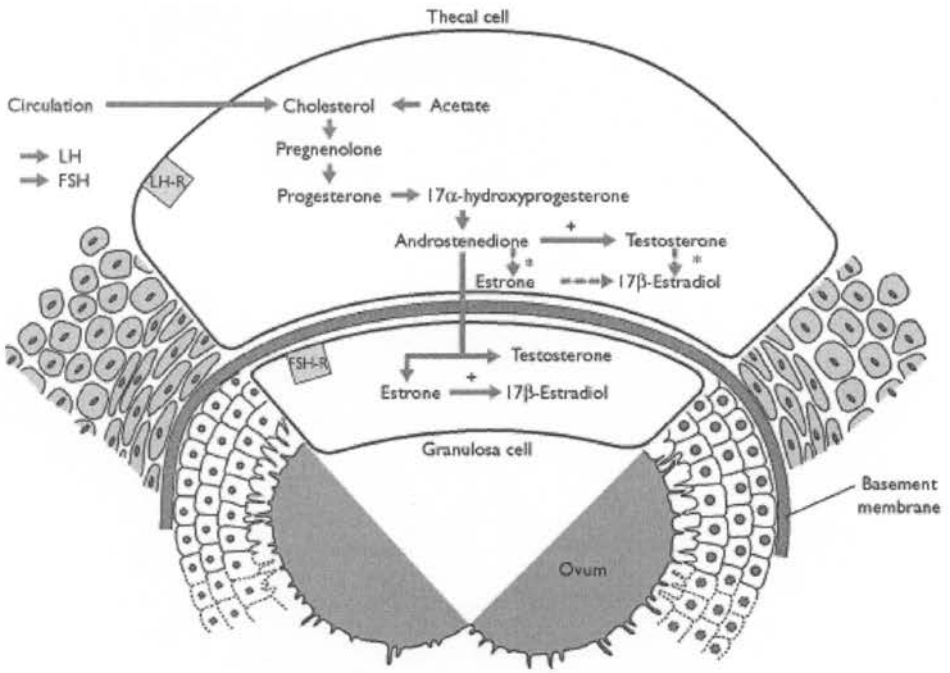


Figure 2

Androstenedione, formed in thecal cells under the stimulatory effects of LH, diffuses across the basement membrane where, under the action of FSH, it is converted to estradiol. In the developing follicle LH receptors are only located on the thecal cells and FSH receptors on the granulosa cells. The 'dominant' pre-ovulatory follicle develops LH receptors on the granulosa cells prior to the LH surge. Thecal cells of the pre-ovulatory follicle also develop the capacity to synthesize estradiol (dotted line) and this persists when the cells become incorporated into the corpus luteum.

Endocrinology, An Integrated Approach. Editors: Nussey and Whitehead. 2001

Figure 1-3

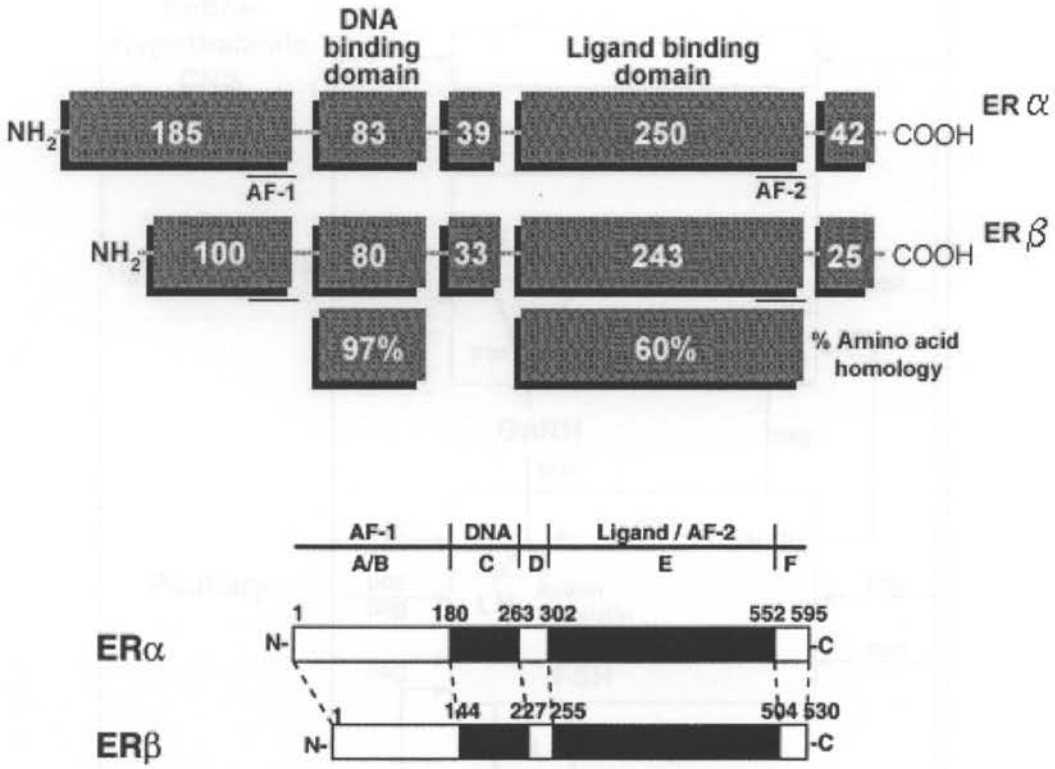


Figure 3
Estrogen receptor alpha and beta are 97% similar in the amino acids of their DNA-binding domain. In contrast, there is only approximately 60% similarity between their ligand-binding domains. Numbers in boxes indicate number of amino acids.

Bottom panel shows the structural and functional domains of the receptors.

Bhavnani BR. Proc Soc Exp Biol Med. 1998 Jan;217(1):6-16
Katzenellenbogen et al. J Ster Biochem Mol Bio. 2000 74(5).

Figure 1-4 HPG feedback

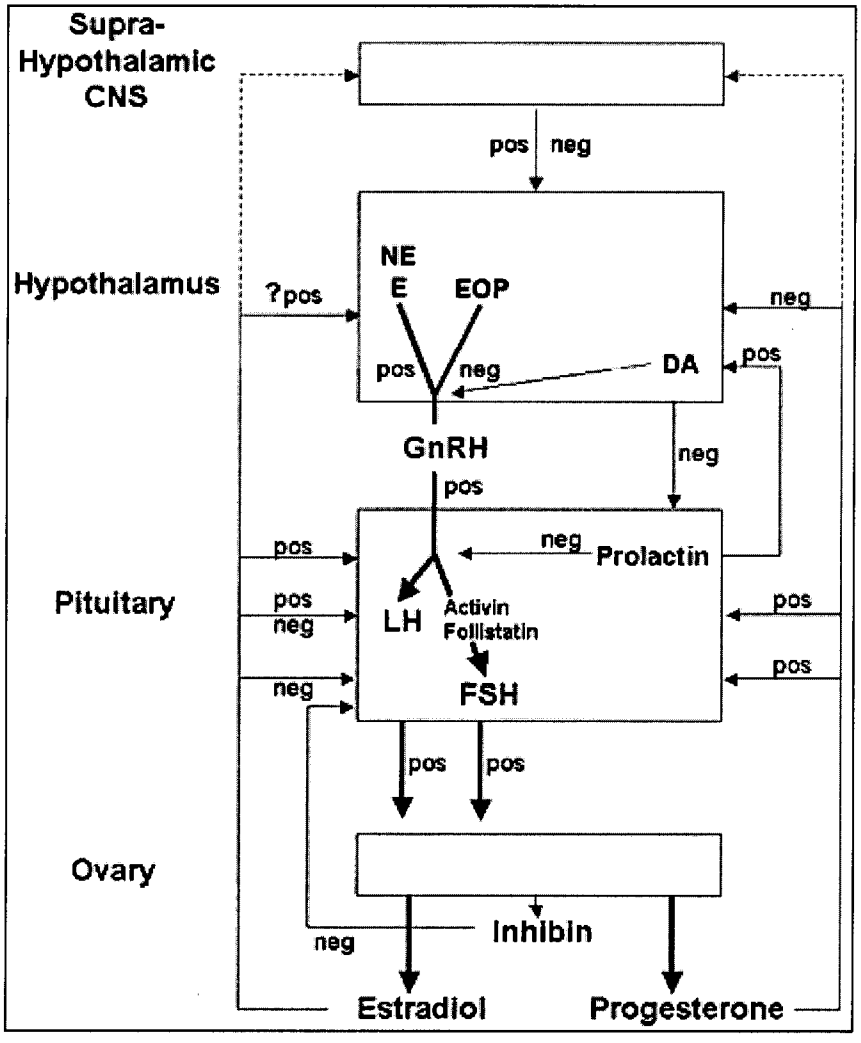


Figure 4

Figure 1-5 Menstural cycle

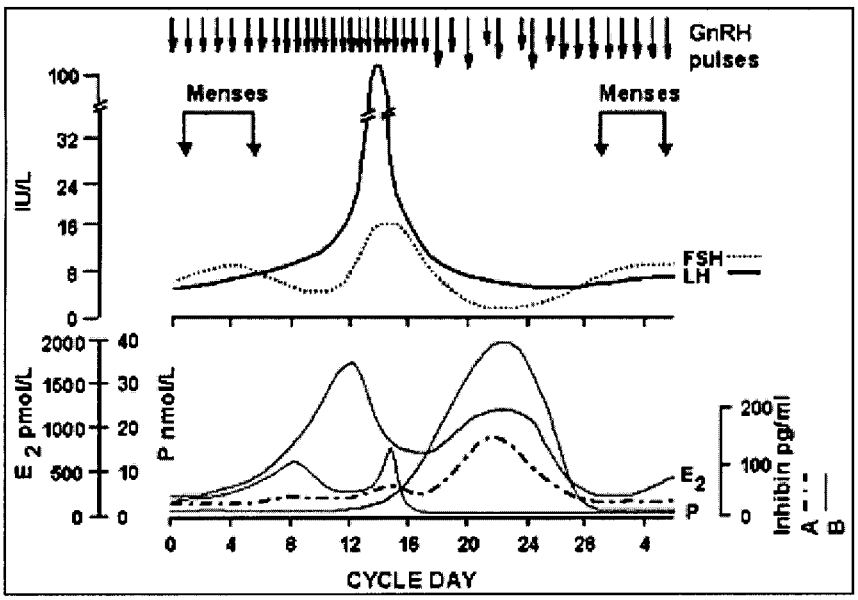


Figure 5

Figure 1-6 The major hypothalamic nuclei in sensing and transducing peripheral metabolic signals

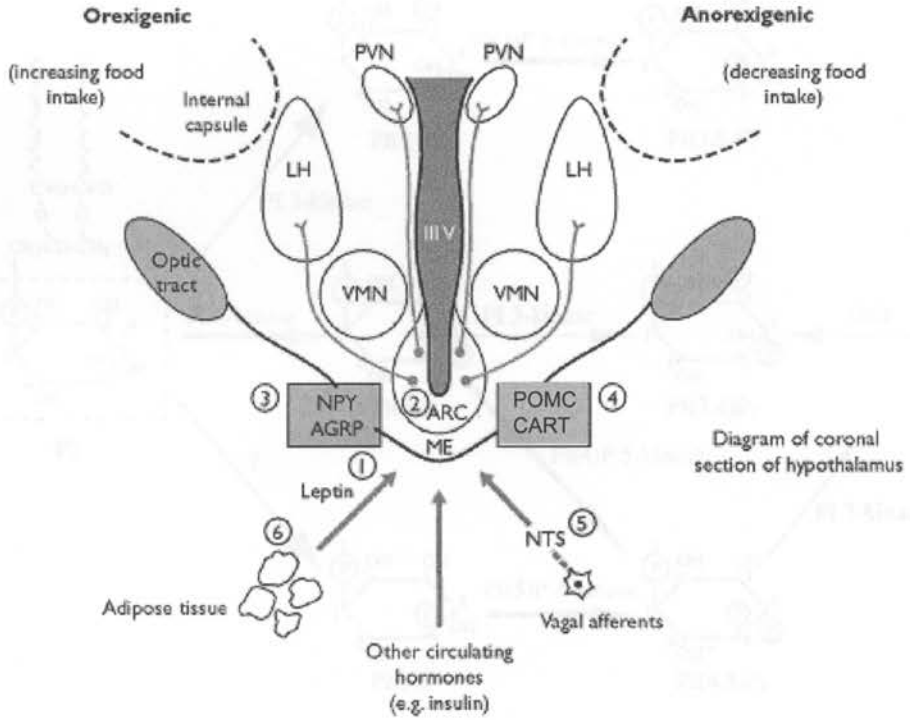


Figure 1-7 Phosphoinositide structures

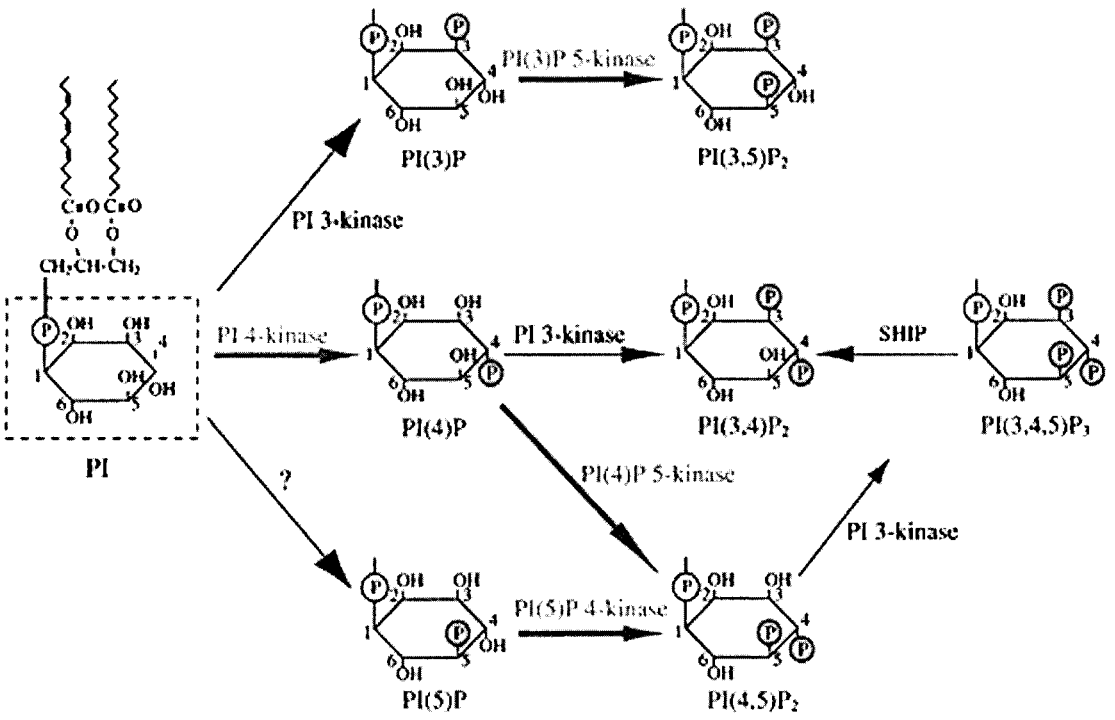
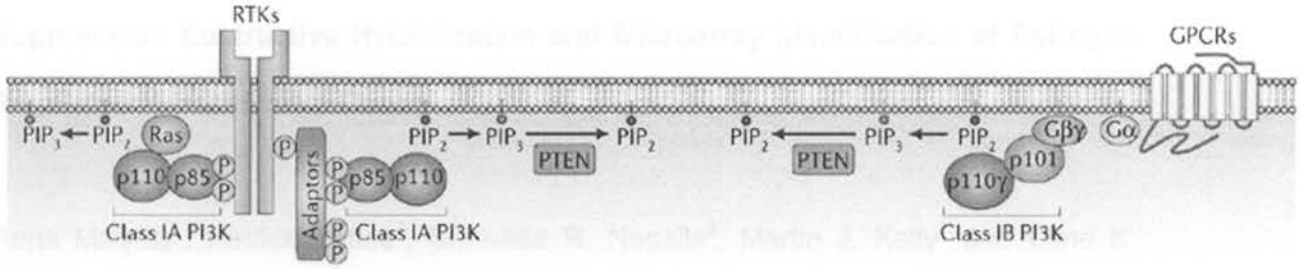


Figure 1-8 PI3K activation and PI3K subunit structures



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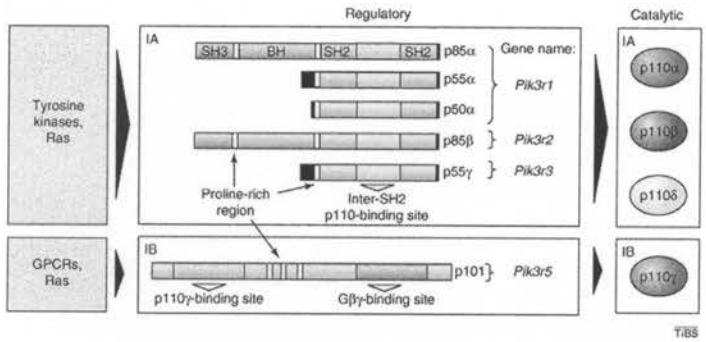

Chapter 2

Suppression Subtractive Hybridization and Microarray Identification of Estrogen Regulated Hypothalamic Genes

Anna Malyala[1], Patrick Pattee[2], Srinivasa R. Nagalla[2], Martin J. Kelly[1] and Oline K. Rønnekleiv[1,3]

(1) Department of Physiology and Pharmacology, (2) Center for Biomarker Discovery, Department of Pediatrics, Oregon Health and Science University, Portland, OR 97239; (3) Division of Neuroscience, Oregon National Primate Research Center, Beaverton, OR 97006

Dr. Nagalla was our collaborator and much of the work in this chapter was performed in the microarray core. Patrick Pattee was instrumental in printing the cDNA chips and taught me the microarray techniques.

ABSTRACT

The gonadal steroid estrogen is a pleiotropic hormone that has multiple effects on numerous cellular functions in every organ system. One of estrogen's major targets is the brain, where the steroid not only affects growth, differentiation and survival of neurons, but regulates cell excitability. Because of estrogen's actions to activate multiple, overlapping pathways, it has been difficult to dissect out the actions of the steroid at the gene transcription level. Although the sensitivity of the molecular biological techniques has improved our level of analysis—e.g., *in situ* hybridization, ribonuclease protection assay, polymerase chain reaction—we still lack a global picture of how different genes interact and are regulated by estrogen. Herein we report the use of suppression subtractive hybridization in combination with microarray analysis of thousands of genes that are regulated during the negative feedback phase of the female reproductive cycle, a period of rapid remodeling of synaptic function in preparation for the preovulatory surge of gonadotropin releasing hormone and subsequently luteinizing hormone. We have found that there are a number of key transcripts that are regulated that contribute to the alteration in synaptic transmission and hence excitability of hypothalamic neurons (e.g., GABA neurons) during negative feedback. These include gec-1, $GABA_B$ R2, PI3 kinase subunit p55γ and a number of proteins containing pleckstrin homology domains that are critical for plasma membrane targeting. Studies are underway to refine our analysis to individual nuclei with PCR and individual cells with *in situ* hybridization. However, what is emerging from this highly sensitive microarray analysis is that estrogen affects neuronal plasticity in hypothalamic neurons not only by transcription of new membrane proteins (e.g., channels) but also by downstream signaling molecules.

INTRODUCTION

It is well established that estrogen controls the mammalian female reproductive cycle by both negative and positive feedback actions on gonadotropin releasing hormone (GnRH) and gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), secretion (see Kalra 1993, Herbison, 1998) (Kalra, 1993; Herbison, 1998). Both GnRH and LH secretion are low during estrogen-induced negative feedback, whereas during positive feedback a GnRH surge occurs which induces a LH surge (Caraty et al., 1989; Pau et al., 1993; Karsch, Bowen, Caraty, Evans, & Moenter, 1997). Although numerous studies have attempted to delineate the mechanisms of estrogen feedback, it remains relatively unclear how estrogen may act in the brain to induce either the suppression of GnRH release or the facilitatory actions that culminate in the GnRH surge.

Estrogen also modulates a number of homeostatic functions controlled by the hypothalamus, such as body temperature, sleep cycles, thirst and appetite by largely unknown mechanisms (Scammell, Elmquist, Griffin, & Saper, 1996; Saper, Chou, & Scammell, 2001; Spiegelman et al., 2001; Gaus, Strecker, Tate, Parker, & Saper, 2002; Freedman & Blacker, 2002; Saper et al., 2002). Hypothalamic GABA neurons are involved in most of these processes, and these neurons are regulated by estrogen in a complex manner (Kelly et al., 2003). GABA neurons express estrogen receptor α (ERα), but also exhibit rapid membrane responses to estrogen, indicating that a number of different signaling mechanisms may exist in these neurons (Herbison, 1997; Herbison, 1998; Wagner et al., 2001). In addition, estrogen can alter synaptic input to hypothalamic neurons, including GABA neurons, via changes in the cellular responsiveness to receptor systems such as $GABA_B$, μ-opioid, and α1-adrenergic (Wagner, Rønnekleiv, & Kelly, 2001; Kelly et al., 2003). Recent evidence also suggests

that estrogen may interact with various intracellular signaling cascades and affect cytoskeletal reorganization through processes involved in neural plasticity and neuroprotection (Driggers & Segars, 2002; Segars & Driggers, 2002; Dudek et al., 2002). These multiple and complex mechanisms by which estrogen may alter cellular functions (McEwen, 2001; Ramirez, Kipp, & Joe, 2001; Behl, 2002; Kelly et al., 2003), require novel approaches to explore the differential downstream effectors in a global manner.

Recent technical advances have allowed comprehensive analysis of estrogen action in cell model systems and in animals. Specifically, differential display PCR and gene arrays, which allow analysis of thousands of genes simultaneously, have been used to study the genomic effects of estrogen receptor activation (Park et al., 2001; Mong, Krebs, & Pfaff, 2002; Mong et al., 2003). In order to further analyze the multifaceted actions of estrogen we have used suppression subtractive hybridization (SSH) (Diatchenko et al., 1996; Gurskaya et al., 1996) combined with microarray technology (Yang, Ross, Kuang, Brown, & Weigel, 1999; Schlingemann et al., 2003) to identify genes that are activated or inhibited by estrogen within the hypothalamus. Although subtractive cloning methods have been used for many years to analyze differences in gene expression (see Carulli et al 1998) (Carulli et al., 1998), the SSH method used in this report was based on the procedure developed specifically to achieve a high level of enrichment of rare as well as abundant transcripts that are differentially expressed (Diatchenko et al., 1996; Gurskaya et al., 1996). Combining this SSH technique with high throughput screening of the harvested clones through the use of cDNA microarrays prior to clone sequencing greatly reduces the likelihood of false positive clones being included in the final set of estrogen responsive genes.

Here we describe the generation of guinea pig specific microarrays comprised of clones derived from SSH libraries from ovariectomized oil- and estrogen-treated female

guinea pigs. SSH was used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress nontarget DNA amplification. Based on the SSH brain-specific cDNA library, guinea pig specific microarray chips were generated and used for analysis of estrogen action in the hypothalamus. Many of the differentially expressed genes identified are involved in signal transduction pathways and synaptic plasticity.

METHODS

Animals and treatments

Female (Topeka) guinea pigs (470-660 gm) were obtained from our institutional breeding facility and maintained under constant temperature and light (on between 06:30 and 20:30 hr) with food and water provided ad libitum. They were ovariectomized under ketamine and xylazine anesthesia (33 and 6 mg/kg, respectively, s.c.) 5 d before experimentation and given either estradiol benzoate (EB; 25 μg, s.c.) or sesame oil vehicle (0.1 ml, s.c.). All animal procedures described in this study were in accordance with institutional guidelines based on National Institutes of Health standards.

Preparation of tissue for SSH

After 24h or 42h treatment (EB or vehicle), animals were sedated with ketamine and euthanized using a small animal guillotine. The brain was removed and placed in a chilled Precision Brain Slicer (EM Corporation, Chestnut Hill, MA). Coronal slices (2-3 mm) were taken, dissected and grouped relative to function as follows: Group I—prefrontal cortex and nucleus accumbens; Group II—preoptic area (POA), basal hypothalamus (BH), amygdala, hippocampus and pituitary; Group III—cortex, striatum and thalamus; Group IV—ventral tegmentum area (VTA), substantia nigra (SN), raphe, pons and medulla; Group V—cerebellum. The dissected tissues were snap frozen in

liquid 2-methyl-butane and stored at –80°C. Total RNA from tissues was extracted and pooled (3 animals per time treatment with EB or vehicle) using the CsCl method (insert ref). Subsequently, poly (A^+) RNA was prepared for the SSH and enriched for Group II (see Fig 1) by weight. RNA quantification was based on OD_{260} and samples were run on a gel to confirm purity.

cDNA Library Construction using SSH

The experimental strategy we used is depicted in Fig 2. Two SSH experiments were performed in forward and reverse directions to generate a brain-specific cDNA library enriched in cDNA sequences corresponding to mRNA species that are preferentially up- or down-regulated by estrogen at 24h or 42h. The PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Palo Alto, CA) was used according to the manufacturer's protocol. Briefly, the tester and driver double-stranded cDNAs underwent digestion with the restriction enzyme RsaI, to obtain shorter, blunt-end fragments. Tester cDNA was then divided into two portions and each ligated with a different double-stranded cDNA adaptor. An excess of driver cDNA was added to each sample of tester cDNA for the first hybridization. Samples were heat denatured and allowed to anneal. The two primary hybridization samples were then mixed together with fresh denatured driver cDNA and a second hybridization performed. The entire population of hybridized and non-hybridized cDNAs was then subjected to two rounds of PCR with nested primers. Products of the PCR reaction were subcloned into pGEM-T vector (Promega, Madison, WI) and 500 colonies isolated from each SSH: 1) 24 h EB-treated tissue as tester, 2) 24 h oil-treated tissue as tester, 3) 42h EB-treated as tester and 4) 42h oil-treated as tester. Each clone was given a unique ID.

Custom-made cDNA Microarrays

The microarray method was carried out as described by the OHSU Center for Biomarker Discovery. Detailed protocols for the printing and processing are available from http://medir.ohsu.edu/_geneview. For these arrays, 2000 clones selected randomly from the SSH library and controls were printed on the arrays. Probes were amplified using universal forward and reverse primers, CTGCAAGGCGATTAAGTTGGGTAA and GTGAGCGGATAACAATTTCACACAGGAAACAGC, respectively, were amino modified with a 5 prime C12 spacer. PCR products were purified using Telechem (Sunnyvale, CA) PCR clean up plates, dried down, resuspended in 20μl of Telechem spotting solution and printed in duplicate on Telechem SuperAldehyde Substrates using a Cartesian Pixsys printer (Cartersian Technologies, Irvine, CA) with quill pins from Telechem. The clones and controls were printed in duplicate on each chip to assess intrachip variation.

Preparation of tissue for microarray analysis

Animals were treated with EB or vehicle (n=3/treatment), sedated and euthanized by a small animal guillotine after 24h. Although clones from both 24h and 42h EB treatments were represented on the microarray chips, here we focused on the 24h EB treatment. The brain was quickly removed, placed in a chilled Precision Brain Slicer (EM Corporation, Chestnut Hill, MA), coronally sliced and dissected for the POA and BH. The 3mm block containing the POA was sectioned in the shape of a trapezoid with the lateral cuts extending in the line of the lateral ventricles to the ventral surface. The dorsal cut was made horizontally at the top of the anterior commissure. In the 4mm block containing the BH, parallel vertical cuts were made from the ventral surface at the edge of the medial amygdala. A horizontal cut was made at the top of the third ventricle. The tissues were snap frozen in 2-methyl-butane and stored at –80°C. Total RNA was isolated from pooled tissue of 3 animals using Trizol and further purified using the

RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quantification was based on OD_{260} and samples were run on a gel to confirm purity.

cDNA Labeling & Hybridization

Seven μg of total RNA were used for indirect biotinylated probe synthesis. Total RNA from POA or BH pooled from 3 animals treated with EB or vehicle was reverse transcribed using 2μg of oligo dT primer (24mer) in the presence of 200mM dNTP mixture (dATP, dTTP, dGTP), 100μM dCTP, 100μM dCTP-Biotin (Perkin Elmer, Boston, MA), and 300 units of Superscript II (Invitrogen) to generate biotin labeled cDNA probes. Following hydrolysis of the original RNA, a Qiagen PCR cleanup kit (Qiagen) was used according to the manufacturer's instructions to purify the cDNA product. The cDNA was dried down and resuspended in 70μl of Ribohybe (40% Formamide, 4X SSC, 1% SDS). Probe was added to the microarray using a lifterslip (Erie Scientific, Portsmouth, NH) and allowed to hybridize in a humidity chamber for 16 h at 50°C. Each probe (EB or vehicle treated cDNA) was individually hybridized to 2 independent chips (single channel hybridization), thus yielding 4 replicate clone spots (clones printed in duplicate on each chip) for microarray analysis.

Microarray Wash and Processing Procedure

Arrays were washed two times for 5 min each in 2XSSC followed by two washes in 0.2X SSC to remove unbound probe. All washes were performed at 50°C with agitation. Arrays were immediately placed in TNB Blocking Buffer (0.1M Tris-HCl, 0.15M NaCl, 0.5% Blocking Reagent (Perkin Elmer), pH7.4) for 30 min then processed in Streptavidin-HRP (Perkin Elmer) by submerging the microarray in 2.5 ml of a 1:600 dilution of streptavidin-HRP to TNB Blocking Buffer for 10 min. Unbound Streptavidin

was removed by two 5 min washes in TNT wash buffer (0.1M Tris-HCl, 0.15M NaCl, 0.05% Tween 20, pH 7.4) and microarrays were immediately submerged in Cy5-tyramide (Perkin Elmer) at a 1:200 dilution of Cy5-tyramide to Amplification Diluent (Perkin Elmer) and allowed to react for 10 min. Two washes were performed in TNT wash buffer for 5 min each, followed by a 5 min wash in 0.02X SSC to remove excess dye and dried by centrifugation.

Scanning and Data Analysis of Microarrays

Arrays were scanned at high and low intensities using a ScanArray sa5000 fluorescent scanner (Perkin Elmer), images collected (.tiff format) and data analyzed by ImaGene software (BioDiscovery, Marina Del Rey, CA). Absolute intensities were measured and data from high and low scans merged to expand the dynamic range using an in house linear regression program. Mean signal intensity was adjusted for local background by subtracting the median background intensity. Data for each array set were exported to ArrayStat™ statistical software (Imaging Research, Inc., Ontario, Canada). The ArrayStat normalization parameters used were "proportional model with offsets and outlier exclusion." The program log transforms (log10) the data and globally centers the transformed data (Nadon & Shoemaker, 2002). Modified ANOVAs (ArrayStat F* tests) and significance of differences between means (z tests) were determined using a pooled error model. Centered expression values and test results were exported to Excel. Normalized means and differences between means were converted from log10 to log2 for ease of comparison with the literature.

Sequencing & Data Mining

After microarray analysis, we sequenced clones that were differentially regulated between EB and vehicle treatment. Selection of clones for sequencing was based on

whether the hybridization signal was above background, the difference was significant ($p < 0.05$) and the difference between treatments was greater than 2 fold. Two hundred eighty-five clones in the POA and 267 clones in the BH met these criteria and were considered for sequencing. Of these, some clones were identified as differentially expressed in both regions. Choosing the clones of greatest statistical significance, we sequenced 152 clones and performed BLAST analysis to look for gene homology.

The genes of interest were further analyzed to look for estrogen response elements EREs) using the Dragon Estrogen Response Element Finder, Version 2 (Bajic et al., 2003). This program is a package for the specific discovery of estrogen response elements in DNA sequences. The consensus ERE is 5'-GGTCAnnnTGACC-3', where n is any nucleotide. To model the ERE, the program uses the Position Weight Matrix method in addition to the probability of pairing the half-sites by the transitional probabilities of the 3' nucleotide of the 5' half-site to the 5' nucleotide of the 3' half-site, ignoring spacer nucleotides. Details can be found at http://sdmc.lit.org.sg/ERE-V2/index.

RESULTS

Animal Model

The female guinea pig was chosen as a model for the human reproductive cycle because the guinea pig has a longer ovulatory cycle (16-18 days) than the rat or mouse (4 days), and estrogen regulation of reproductive functions are more similar to that of primates (Terasawa et al., 1979; Terasawa, Yeoman, & Schultz, 1984; Bethea, Hess, Widmann, & Henningfeld, 1995; King et al., 1998). These similarities to primates are important if the animal model is intended to be used to elucidate the mechanisms of estrogen action that regulate the myriad number of homeostatic functions which are dysregulated in human disease (Bethea, Pecins-Thompson, Schutzer, Gundlah, & Lu,

1998; McEwen, 2001; Brinton, 2002). Our experimental model represents both the negative and positive feedback actions of estrogen at specific time points (Wagner et al., 2001) and hence these time points were used to obtain clones for the SSH experiments. However, in the current study, the SSH clones have only been probed with samples generated from a 24 h estrogen treatment response.

SSH & Microarray Analysis

Data analysis of the microarray hybridization signals suggested the consistency of the data to be high; the variation of clones within the same chip and across independent replicates was less than 2.9%. The reproducibility of intrachip and interchip data gave us confidence that the results obtained for each clone would tell us if it was indeed either up- or down-regulated, even if the magnitude of change might not be exact.

We selected clones for sequencing using the following criteria: hybridization signal above background, statistical significance of expression changes ($p < 0.05$) and the fold change between treatments greater than 2 fold. Of the 2000 clones obtained from the SSH, 275 in the POA and 250 in the BH met the specified criteria for being sequenced. Changing the cut-off value for the fold change to 1.5 increased the number of clones that met consideration for sequencing in the BH to 267 with no change in the number for POA. In the POA, approximately two-thirds of the clones on the chip increased with 24h estrogen treatment while one-third of the clones were decreased. On the other hand, the BH showed an equal distribution of clones that were increased or decreased with estrogen treatment.

Gene Analysis

A selection of control genes known to be regulated by estrogen in the hypothalamus was printed on microarrays (Fig 3). In 24 h estrogen treatmentment samples, progesterone receptor expression was increased by 2.1-fold in the BH and 1.6-fold in the POA; the latter did not reach statistical significance using the stringent criteria specified. Increase in progesterone receptor mRNA and protein is consistent with previous reports in guinea pig (Blaustein, King, Toft, & Turcotte, 1988; Olster & Blaustein, 1990b) and primate (Olster & Blaustein, 1990a). We observed a 1.7-fold decrease in ERα RNA in the BH, consistent with previous findings in the guinea pig (Lagrange, Rønnekleiv, & Kelly, 1997), but this change did not reach statistical significance. Tyrosine hydroxylase (TH) decreased by 1.9-fold in the BH, in contrast to a previous report in the primate where estrogen alone did not produce a change in TH mRNA however, estrogen plus progesterone did decrease TH expression (Kohama & Bethea, 1995). Finally, we found a 4.2-fold decrease of GABA-B receptor 2 in the BH and no change in GAD mRNA expression in agreement with previous data (Wagner et al., 2001).

One hundred fifty-two clones (size ranging between 100-800 bp) were sequenced and analyzed via BLAST searches. Few sequenced clones corresponded to guinea pig specific genes since gene annotation has not been extensive in this species; however, most sequences had high homology to human and mouse genes (see Table 1). Based on literature searches performed for each of the genes, genes were assigned to broad functional categories described by the studies. The genes of particular interest are presented in Table 1. Analysis of the clones sequenced also revealed several redundant genes, mostly of mitochondrial origin (data not shown). Consistent with previous reports of genes regulated by estrogen, nuclear receptor interacting protein (nrip1) mRNA increased 5.9-fold in our study (Thenot, Charpin, Bonnet, & Cavailles,

1999; Inoue et al., 2002) and two POA-specific mRNAs encoding neurobeachin and evectin increased 4.8-fold and 3.5-fold, respectively (Park et al., 2001). These examples and results demonstrate the reliability of the approach we used. Of particular interest was the increased expression of cAMP-guanine nucleotide exchange factor I (cAMP-GEF I), phosphatidylinositol 3 kinase p55 regulatory subunit (PI3Kp55γ), neurobeachin, evectin and centaurinα1; the relevance of these in estrogen signaling are highlighted in the discussion section.

Estrogen exerts its effects via multiple signaling mechanisms (Behl, 2002; Pietras, Nemere, & Szego, 2001) with the classical mode mediated by the binding of the estrogen-cognate receptor complex to a response element which regulates transcription. We searched for EREs and found putative sites in 8 of 32 genes that were evaluated (Table 3). Since estrogen signaling is mediated via a number of mechanisms--e.g. AP-1, Sp1, CRE--other response elements are most likely to mediate transcription of genes without EREs (Kushner et al., 2000; Toran-Allerand, Singh, & Setalo, 1999; Sanchez, Nguyen, Rocha, White, & Mader, 2002b).

DISCUSSION

We have combined SSH and cDNA microarray studies to determine genes regulated by estrogen in the hypothalamus, an area critical to the control of homeostatic functions. A number of the differentially regulated genes identified encode proteins involved in transcriptional regulation, protein trafficking, synaptic transmission and signal transduction. These results allow us to build upon previous work and generate new hypotheses regarding the mechanisms of estrogen action. Microarray analysis is powerful because expression profiling of thousands of genes can be performed in a single experiment. While many advances have been made in terms of sensitivity and

reproducibility of microarrays, a critical factor is the design of the experiment including the animal model. Our approach addresses many of the concerns regarding large scale expression profiling. We looked only at a subset of genes—guinea pig brain specific genes that are rare or differentially expressed, used independent replicates and stringent data analysis methods to produce valid results.

By combining RNA from many brain regions for the SSH, a dilution effect is created such that detection of differentially expressed genes is obscured because of the complexity of the tissue—various cell types with distinct functions. The detection of small but significant changes between treatments or detection of rare transcripts might have been more robust if smaller dissected areas from more animals were used and these individual areas then subtracted by SSH. However, performing follow-up microarray analysis from specific brain regions allowed us to detect genes differentially expressed specifically in that brain region. To minimize technical variability, dissections and RNA extractions were performed by the same person as were microarray procedures. To minimize biological variability, RNA was pooled from three animals. Moreover, our data suggests that independent chip variability was low.

Progesterone receptor (P4) and ERα, genes shown to be regulated in the hypothalamus, were also printed on the microarrays. Progesterone receptor mRNA was increased in the POA by 1.6 fold and in the BH by 2.1 fold, consistent with a number of previous reports (Olster et al., 1990a; Olster et al., 1990b; Bethea & Brown, 1996). We observed a 1.7 fold decrease in ERα RNA in the BH though the change was not significant. The direction of the change however is in agreement with earlier *in situ* hybridization studies showing a decrease in ERα mRNA in the arcuate and VMH in ovariectomized female rats after 24 h and 4 d estrogen treatment (Simerly & Young, 1991; Funabashi et al., 2000). The changes detected in the expression of these well

studied genes using the microarray method suggest that this approach is sensitive but also emphasize the point that a subset of neurons within the dissected area are estrogen-sensitive arguing for even smaller dissections, i.e., punches of hypothalamic areas (Ma, Kelly, & Rønnekleiv, 1990). Although the fold changes observed in known genes indicated that a change of less than 2 fold might be physiologically significant, we selected clones for sequencing based on stringent criteria in order to minimize false positives. Further microarray studies are needed in which individual nuclei are dissected and the number of animals increased. Data from follow-up *in situ* hybridization and quantitative PCR studies should guide the determination of false positive and false negative cut-offs for sequencing the remaining clones.

A number of the sequenced clones were of mitochondrial origin. These genes, far from their known housekeeping role, significantly contribute to neuronal excitability (Miller, 1998). For example, mitochondria regulate calcium homeostasis and are involved in neuronal plasticity (Mattson & Liu, 2003), suggesting that the observed increase in expression might reflect functional changes related to estrogen-induced plasticity.

During negative feedback—i.e., at 24 h EB treatment in our guinea pig model, there is increased GABAergic transmission in the hypothalamus (Herbison, 1998; Wagner et al., 2001; Jackson et al., 2002). Hypothalamic GABAergic neurons express estrogen receptors and undergo ultrastructural changes in response to estrogen leading to a remodeling of inhibitory GABAergic synapses (Leranth, Shanabrough, & Naftolin, 1991; Garcia-Segura, Chowen, Parducz, & Naftolin, 1994; Herbison, 1997). $GABA_B$ receptors are members of the seven-transmembrane G-protein coupled receptors and are functional only when both subunits, R1 and R2, heterodimerize (Hammond, 2001). We found a 4.2 fold decrease of $GABA_BR2$ mRNA, in support of previous electrophysiological data showing that there is a decrease in $GABA_B$ receptor-mediated

response in POA neurons 24 h after estrogen (Wagner et al., 2001). We proposed a model where reduction of autoinhibitory $GABA_B$Rs at the membrane leads to increased firing and increased levels of GABA release. Furthermore, consistent with previous ribonuclease protection assay data, we found no change in GAD mRNA expression at 24 h in this study. We reported that GAD mRNA expression was unchanged at 24 h however, expression was decreased at 42 h, the timepoint corresponding to positive feedback (Wagner et al., 2001). Interestingly, gec1, encoding a $GABA_A$ receptor associated protein (GABARAP), was increased in this study. $GABA_A$ receptors are ligand-gated ion channels and efficient synaptic transmission depends on the precise spatial and temporal localization of these receptors such that they are in close apposition to presynaptic GABA releasing terminals (see Kneusel 2002) (Kneussel, 2002). Based on its localization at intracellular membranes and interactions with the $\gamma 2$ subunit of $GABA_A$ receptor, N-ethylmaleimide-sensitive factor, microtubules and gephyrin, GABARAP appears to play a role in $GABA_A$ R targeting and/or degradation (Nymann-Andersen, Sawyer, & Olsen, 2002; Kittler, McAinsh, & Moss, 2002; Coyle, Qamar, Rajashankar, & Nikolov, 2002). The increased expression of gec1 may result in the targeting of $GABA_A$ receptors to the membrane, contributing to the observed increase in GABAergic transmission during negative feedback (Connolly et al., 1991; Wagner et al., 2001; Herbison, 1998; Jackson et al., 2002). Data show that gec1 is expressed in a number of tissues including the brain and that it is induced by estrogen in guinea-pig endometrial glandular epithelial cells (Vernier-Magnin et al., 2001).

A number of genes found encoded signaling molecules, in particular proteins containing phosphoinositol-binding sites. Phosphoinositides (PtdIns) are minor constituents of cell membranes and act as signal mediators in a spatially and temporally controlled manner. They bind with high-affinity and specificity to a large number of proteins and can initiate downstream signaling cascades leading to cytoskeletal

rearrangements, membrane trafficking, cell growth and differentiation (Toker, 2002). The pleckstrin homology (PH) domain, along with the PDZ-domain, SH2-domain (src homology domain), PX domain and PTB domain (phosphotyrosine binding domain,) have no known catalytic activity and bind phosphatidylinositides (Lemmon, Ferguson, & Abrams, 2002; Fan & Zhang, 2002; Sato, Overduin, & Emr, 2001; Takeuchi et al., 1998). The major role of these domains appears to be recruiting other proteins to a cellular compartment at a particular time where the recruited proteins can then exert their effects. Among the clones sequenced, centaurinα-1, evectin, and neurobeachin contain PH domains while cAMP-GEF1 contains a PDZ domain. Binding of PtdIns to these domains activates the phosphoinositide 3-kinase (PI3K) pathway (Jackson, Kearns, & Theibert, 2000; Krappa, Nguyen, Burrola, Deretic, & Lemke, 1999; Rodgers & Theibert, 2002; Jogl et al., 2002; Quilliam, Rebhun, & Castro, 2002).

PI3K is comprised of an adaptor/regulatory subunit, of which there are a number of isoforms, and a catalytic subunit, p110 (Shin et al., 1998). The PI3K signaling cascade is involved in the promotion of neuritic growth and synaptic plasticity and mediates the cellular effects of insulin, leptin, PDGF and VEGF (Niswender et al., 2003; Vanhaesebroeck et al., 1999). A novel finding is the 2.9 fold increase in the p55γ (PIK) regulatory subunit of PI3K since most studies have focused on estrogen regulation and interaction of the p85α regulatory subunit (Cardona-Gómez et al., 2002; Dudek et al., 2002; Dupont, Karas, & LeRoith, 2000). Estrogen induces the expression of downstream insulin growth factor receptor-1 (IGF-1) signaling molecules such as the p85α subunit upon stimulation of IGF-1 leading to potentiation of tyrosine-phosphorylated insulin receptor substrate 1 (IRS-1) and activation of PI3K, Akt, ERK1 and ERK2. The structure of PIK comprises a unique 30-residue NH2 terminus followed by a proline-rich motif and two Src homology 2 (SH2) domains with significant sequence identify to those in p85α.

The difference in the NH2 terminal region may contribute to differences in subcellular distribution and/or PI3K activation to various growth factor signals. p55γ mRNA is expressed early during development, remains abundant in adult mouse brain and has been shown to form a stable complex with p110 and associate with IRS-1during insulin stimulation, suggesting that p55γ isoform may also be involved in the potentiating effects of estrogen on IGF-1 signaling (Pons et al., 1995). Cross-talk or synergism between the PI3K pathway and estrogen signaling plays a role in synaptic plasticity. Estrogen induces the phosphorylation and peak activation of Akt, a serine/threonine protein kinase with a pleckstrin homology (PH) domain, in the rat hypothalamus during negative feedback (Cardona-Gómez et al., 2002). During this time, there is remodeling of axosomatic inhibitory synapses preceding the gonadotrophin surge (Cardona-Gomez, Trejo, Fernandez, & Garcia-Segura, 2000). These findings highlight the importance of the PI3K pathway and the prominent role phosphatidylinositol binding proteins may play in estrogen signaling.

Neurobeachin is a neuron specific multidomain A-kinase anchor protein (AKAP) that recruits PKA to endomembranes near the *trans*-Golgi network (Wang et al., 2000). Neurobeachin, in addition to having a PH domain, has a C terminal BEACH-WD40 sequence module characteristic of members of the BEACH protein family, of which lysosomal trafficking regulator (LYST) is the prototype. Evidence suggests that there is strong interaction between the BEACH and PH domains forming a prominent groove and hence may be involved in protein-protein interactions (Jogl et al., 2002). That this AKAP seems to be upregulated is interesting since estrogen has been shown to activate PKA and although the mechanism remains unclear, evidence suggests it may be through an indirect mechanism, perhaps via PKC (Watters & Dorsa, 1998; Lagrange et al., 1997). Phosphorylation of cAMP-dependent response element-binding protein (CREB) is often

mediated by PKA and evidence suggests that it may be a target for drugs used in treatment of depression and bipolar disorder, disorders in which many homeostatic functions are abnormal (Duman, 2002; Nestler et al., 2002). Since estrogen can elevate cAMP levels and acute and long-term estrogen treatments increase phosphorylated CREB (pCREB) in the central region of the ventromedial hypothalamus, amygdala, and hippocampus, it has been speculated that the benefits on emotion and cognition seen with estrogen and certain antidepressants (selective serotonin reuptake inhibitors) are mediated through the same mechanisms (Gu, Rojo, Zee, Yu, & Simerly, 1996; Zhou, Watters, & Dorsa, 1996; Carlstrom, Ke, Unnerstall, Cohen, & Pandey, 2001). Regulation of scaffold proteins such as neurobeachin might be relevant for a number of G-protein coupled receptors (GPCRs).

Finally, we show that a number of genes identified have putative EREs suggesting a direct regulation by estrogen. This well characterized mechanism of transactivation involves ligand binding, nuclear receptor dimerization and binding to consensus EREs (see Klinge 2001 for review) (Klinge, 2000). The genes found to contain ERE include transcriptional regulators such as GATA-binding protein, histone methyltransferase and zinc finger homolog 103 and signaling molecules such as adenylyl cyclase5, cAMP-GEF1, protein tyrosine phosphatase and EPH-like receptor protein tyrosine kinase. Of interest is the increased expression of cAMP-GEF1, which is characterized by the presence of cAMP-binding motifs and motifs for Ras superfamily guanine nucleotide exchange factors (GEFs), activators of Ras and Ras-like small G proteins, and presumably couples the cAMP signal transduction system to Ras superfamily cascades (Kawasaki et al 1998). The mRNA expression of cAMP-GEF1 is broadly expressed at low levels in the adult brain but with high levels in select brain regions during development. This suggests that in the adult, areas that undergo synaptic remodeling such as the hypothalamus and hippocampus, there might be a

recapitulation of the developing brain gene expression pattern (Marrs, Green, & Dailey, 2001). Moreover, as described above, the cAMP system has been implicated in modulating synaptic function and involved in mediating the effects of antidepressants. The regulated expression of cAMP-GEF through an ERE in the hypothalamus and presumably other limbic structures suggests that this molecule may be an important mediator of estrogen action.

A number of reports show that estrogen promotes gene transcription independently of the canonical receptor dimerization and subsequent binding to an ERE (Paech et al., 1997). It is clear from our data that many of the differentially expressed genes did not contain EREs arguing that other mechanisms of transcriptional regulation are involved (Kushner et al., 2000; Sanchez, Nguyen, Rocha, White, & Mader, 2002a; Toran-Allerand et al., 1999).

Fig 1 SSH cDNA library; % representation of various brain regions

Fig 2 Experimental strategy flowchart

Fig 3 Known genes

Table 1 Genes of interest table and homology

Table 2 putative EREs

Figure 2-1

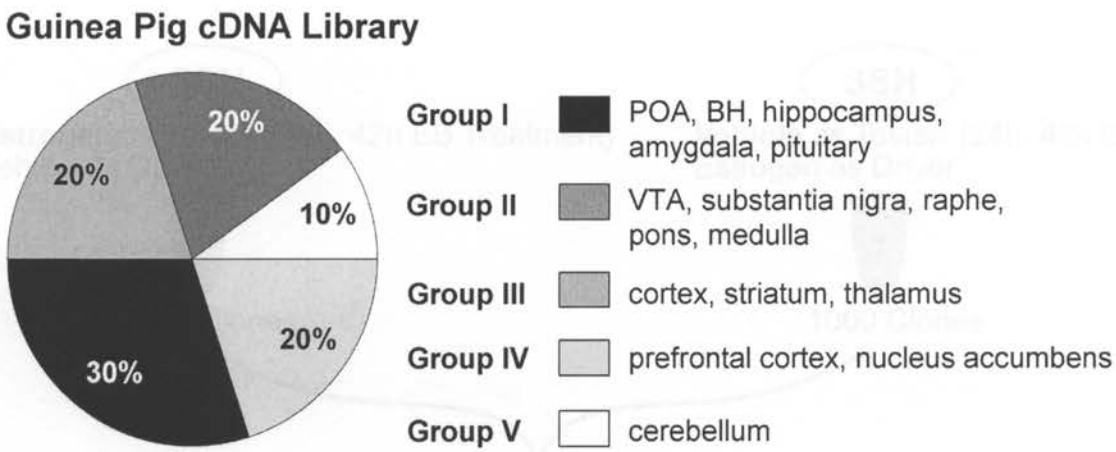
Guinea Pig cDNA Library
20%
20%
10%
20%
30%
Group I
POA, BH, hippocampus, amygdala, pituitary
Group II
VTA, substantia nigra, raphe, pons, medulla
Group III
cortex, striatum, thalamus
Group IV
prefrontal cortex, nucleus accumbens
Group V
cerebellum

Figure 2-2 SSH & microarray strategy

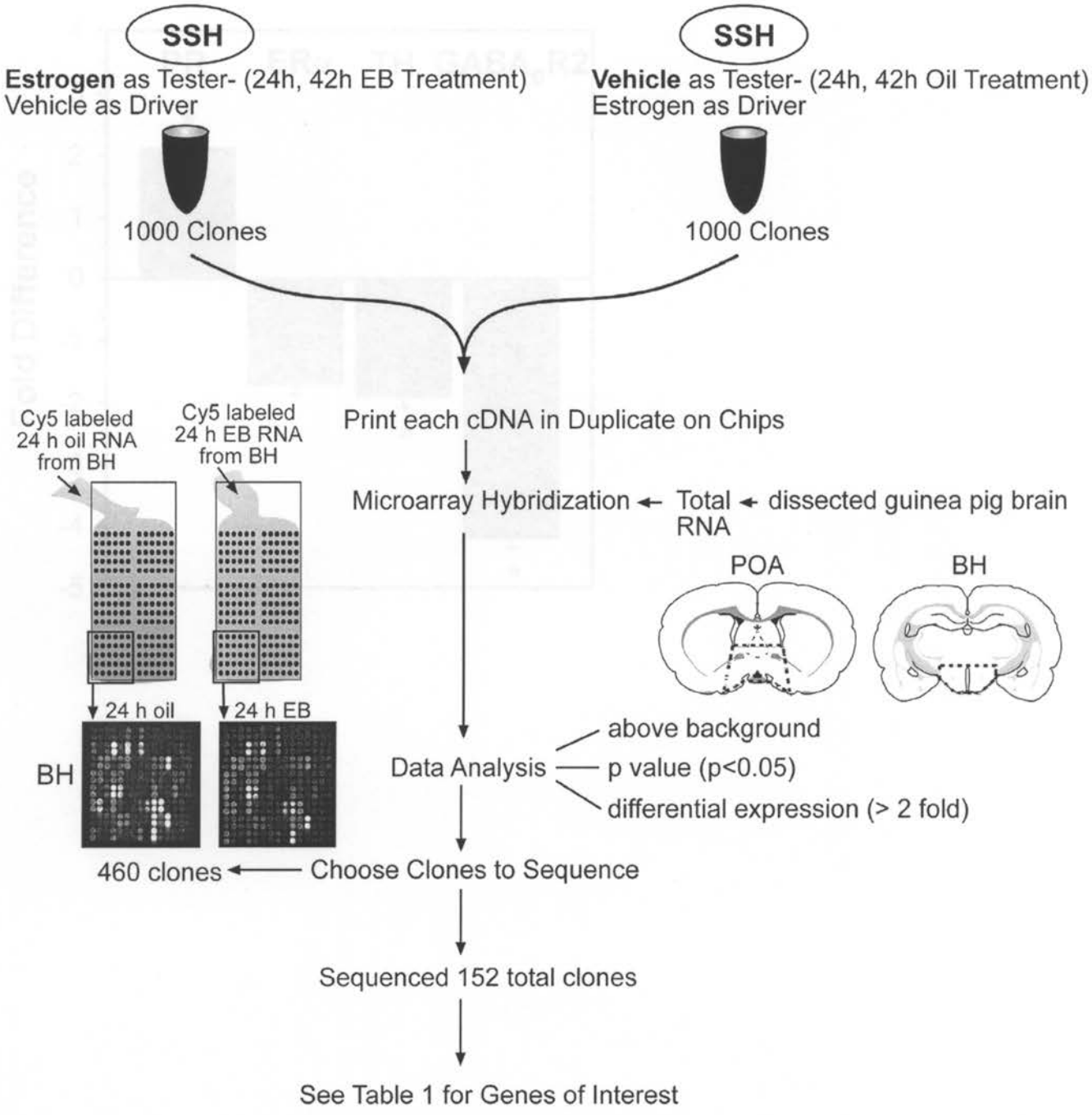

Figure 2-3 Control genes

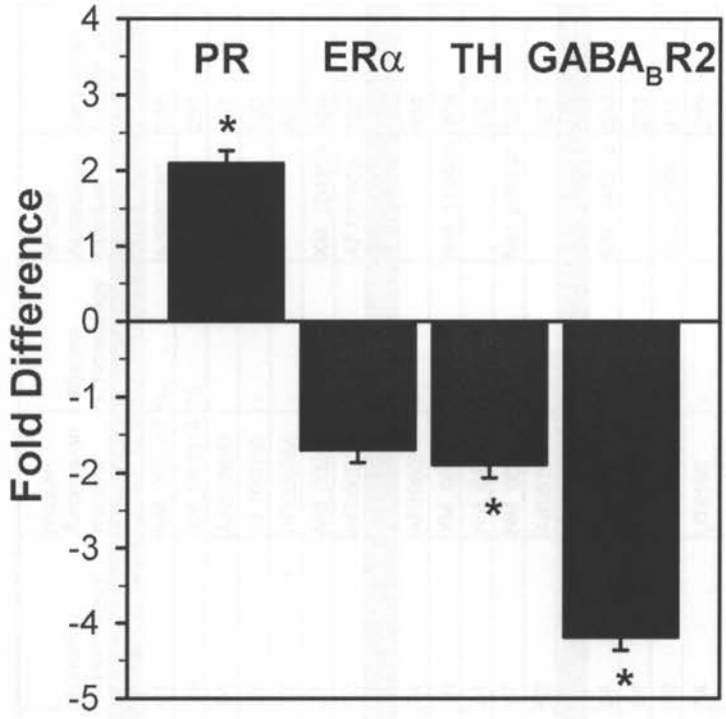

Table 2-1

Table 1: Genes of Interest Genes up- or down-regulated with 24 h estrogen treatment in the preoptic area (POA) and basal hypothalamus (BH). Fold change, clone length and percent homology to the human and mouse genes are presented where available.

Gene	POA	BH	Human % Homology	Human Accession number	Mouse % Homology	Mouse Accession number	Sequence Length (bp)
Protein Trafficking							
glucose regulated protein 78 kDa (GRP78/BIP)	+ 2.6		91	NM_005347	90	AJ002387	335
heat shock 90kD protein	+ 4.0		75	XM_084514	75	BC049124	251
GEC-1 (gec-1)	+ 2.7		94	AF312680	-		137
deubiquitinating enzyme (UNPH4)	-5.4		93	AF106069	-		337
ubiquitin conjugating enzyme E2G1, UBE2G	-3.2		77	BC026288	-		639
proteasome 26s subunit, ATPase2, (PSMC2)	-2.6		87	NM_002803	87	XM_204224	484
rab11a GTPase		+ 3.3	68	AF000231	63	AF127669	557
Transcriptional Regulation							
GATA-binding protein & histone deacetylase-like protein	+ 3.1	+ 2.4	74	AF196971	-		664
nuclear receptor interacting protein (Nrip1)	+ 5.9		90	XM_009699	87	NM_173440	279
transcriptional regulator interacting with PHD-bromodomain2 (TRIP-Br2)	+ 4.9		81	NM_014755	-		782
zinc finger protein 103 homolog	+ 5.2		87	NM_005667	88	NM_009543	397
KIAA1717 (SET domain-containing protein7, histone methyltransferase)	-2.9		63	AB051504	-		657
Signal Transduction							
evectin (pleckstrin homology domain containing member (PLEKH B2))	+ 3.5		84	NM_017958	82	NM_145516	630
similar to Probable G protein-coupled receptor GPR12	+ 4.3		91	XM_208711	-		283
RAN, member RAS oncogene family		+ 2.7	89	BC000852	85	NM_009391	411
cAMP-regulated guanine nucleotide exchange factor I (cAMP-GEF I)	+ 3.0		54	U78168	-		322
hSLK STE20-like kinase	+ 3.0		98	AB002804	-		188
protein tyrosine phosphatase, receptor	+ 2.3	+ 2.4	-		93	NM_178180	211
neurobeachin	+ 4.8		92	NM_015678	91	XM_130885	201
phosphatidyl inositol 3 kinase, regulatory subunit	+ 2.9		85	NM_181504	78	AK051694	700
serine/threonine protein kinase Kp78	+ 3.7		99	AF159295	-		313
centaurin α-1-PI3 binding protein	-3.2		89	NM_006869	87	AK076768	459
oxidored-nitro domain containing protein (NOR1)	-2.7		80	NM_145047	79	BC045150	752
protein kinase inhibitor gamma (PKIG)	-3.9		74	AF182032	72	NM_011106	505
protein-tyrosine kinase (EPH-like receptor PTK)	-2.6		42	L36645	-		780
Channels							
potassium channel TWIK-2 (KCNK6)		+ 4.5	94	AY075098	-		329
Synaptic Transmission							
vesicle-associated membrane protein 2 (Vamp2)		+ 3.4	-		50 % rat	NM_012663	477
Other							
Huntingtin interacting protein	-2.7		90	XM_290829	85	AK013006	347
myelin proteolipid protein mRNA		-2.5	92	BC002665	90	M14674	493

Table 2-2

Table 2: Homologs of Estrogen Responsive Guinea Pig Genes containing EREs The Dragon Finder program was used to search for EREs of homologs to the guinea pig sequences. Listed are the sequence name, accession number, sequence length and predicted position of ERE relative to the 5' end of the input sequence.

Accession Number	Sequence Name	Sequence Length (bp)	Predicted Position of ERE Forward Strand	Predicted Position of ERE Reverse Complement Strand
NM_005667.1	Homo sapiens zinc finger protein 103 homolog	3423	+297 GC-GGTCG-GCG-GGGCC-TG	-2759 CA-GGCCA-AGT-TAACC-AA
AB051504.1	Homo sapiens KIAA1717(SET domain-containing protein7, histone methyltransferase)	6992	+1913 AA-AGTCA-TTT-CACCC-CT +2820 TG-AGTCA-GTT-TGGTC-TG	
NM_178180.1	Mus musculus protein tyrosine phosphatase, receptor type, Z polypeptide 1 (Ptprz1)	3784		-2875 AA-GGGCA-GGG-CGCCC-TG
AY075098.1	Cavia porcellus potassium channel TWIK-2 (KCNK6) gene	2987		-2295 CA-AGACA-AGG-TGACC-AA
U78168.1	Homo sapiens cAMP-regulated guanine nucleotide exchange factor I (cAMP-GEFI)	3394	+1365 AG-GGTCT-TCA-TGCCC-AG +2136 CA-GGTGG-AGC-TGATC-CA	

Chapter 3

PI3K Signaling Effects in Hypothalamic Neurons Mediated by Estrogen

Anna Malyala[1], Chunguang Zhang[1], Damani Bryant[1], Martin Kelly[1] and Oline Rønnekleiv[1,2]

[1]Department of Physiology & Pharmacology, Oregon Health & Science University, Portland, Oregon 97239
[2]Division of Neuroscience, Oregon National Primate Research Center, Beaverton, Oregon 97006

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Abstract

Multiple mechanisms mediate the effects of estrogen in the central nervous system including signal transduction pathways such as protein kinase A, protein kinase C, and phosphatidylinositol 3-kinase (PI3K) pathways. Previously, we demonstrated that a number of PI3K-related genes are regulated by estrogen in the hypothalamus including the PI3K p55γ regulatory subunit. We hypothesized that PI3K activation is critical for estrogen signaling and that the p55γ subunit may be more prevalent than the p85α regulatory subunit in the hypothalamus. Therefore, in the present study we compared the mRNA distribution of the p55γ and p85α regulatory subunits using *in situ* hybridization in guinea pig. Expression level of p55γ mRNA was greater than p85α in most hypothalamic nuclei. Twenty-four hour estrogen treatment increased p55γ mRNA expression in the paraventricular, suprachiasmatic, arcuate and ventromedial nuclei while little to no change was observed for p85α mRNA. Quantitative real-time PCR confirmed the *in situ* hybridization results. We then investigated the role of PI3K signaling in estrogen-mediated changes of arcuate proopiomelanocortin (POMC) neuronal excitability using whole cell recording. One cellular mechanism by which estrogen increases neuronal excitability is to desensitize (uncouple) $GABA_B$ receptors from their G protein-gated inwardly-rectifying K^+ channels in hypothalamic neurons. We found that the PI3K inhibitors wortmannin and LY294002 significantly reduced the estrogen-mediated $GABA_B$ receptor desensitization in POMC arcuate neurons, suggesting that PI3K signaling is a critical downstream mediator of the estrogen-mediated rapid effects. Collectively, these data suggest that the interplay between estrogen and PI3K occurs at multiple levels and includes transcriptional and membrane-initiated signaling events that ultimately lead to changes in homeostatic function.

Abbreviations

AC	anterior commissure
AD	anterodorsal nucleus thalamus
AHNa	anterior hypothalamic nucleus, anterior area
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus hypothalamus
BNST	bed nuclei stria terminalis
CM	centromedial nucleus of the thalamus
CTX	cerebral cortex
DB	diagonal band of Broca
DBh	diagonal band of Broca, horizontal limb
DBv	diagonal band of Broca, vertical limb
DMH	dorsomedial nucleus hypothalamus
fx	columns of the fornix
HIP	hippocampus
LH	lateral hypothalamus
LS	lateral septal nucleus
lv	lateral ventricle
ME	median eminence
MEPO	median preoptic nucleus
mPOA	medial preoptic area
MH	medial habenula
MS	medial septal nucleus
mtt	mammillothalamic tract
OC	optic chiasm
PH	posterior hypothalamic nucleus
PMd	dorsal premammillary nucleus
PMv	ventral premammillary nucleus
PVH	paraventricular nucleus hypothalamus
PVT	paraventricular nucleus thalamus
S	septum
SCN	suprachiasmatic nucleus
sm	stria medullaris
SON	supraoptic nucleus
RT	reticular nucleus thalamus
VMH	ventromedial nucleus hypothalamus
VMHdm	ventromedial nucleus hypothalamus, dorsomedial part
VMHvl	ventromedial nucleus hypothalamus, ventrolateral part
ZI	zona incerta
3v	third ventricle

Introduction

The hypothalamus is a critical center for the regulation of a number of autonomic functions, many of which are also modulated by estrogen. Neurons from the various hypothalamic nuclei receive numerous sensory and metabolic inputs, integrate these signals and respond accordingly to regulate homeostatic functions such as reproduction, energy balance, thermoregulation and stress responses. The heterogeneity of the hypothalamus differentiates this structure from other brain regions. For example, in addition to the conventional neurotransmitters, a number of neuropeptides are expressed within discrete neuronal populations that are primarily involved in neurosecretion. The morphological, physiological and neurochemical changes that take place in response to estrogen further increase complexity within the hypothalamus.

To begin the task of unraveling signaling events and genes regulated by estrogen in the hypothalamus, we have used a genetic approach that involved suppression subtractive hybridization and microarray analysis (Malyala, Pattee, Nagalla, Kelly, & Rønnekleiv, 2004). Our initial results showed that a number of estrogen-regulated genes encode molecules related to the phosphatidylinositol 3-kinase (PI3K) pathway including the PI3K p55γ regulatory subunit. Activation of PI3K in the hypothalamus regulates energy homeostasis (Xu et al., 2005; Plum et al., 2006) and reproductive behavior (Etgen et al., 2003), and is required for insulin and leptin signaling in the arcuate nucleus (Niswender et al., 2001a; Niswender et al., 2003; Xu et al., 2005; Spanswick, Smith, Groppi, Logan, & Ashford, 1997). Interestingly, males and females are differentially sensitive to the catabolic actions of leptin and insulin that is likely due to gender differences in circulating estrogen levels and their subsequent effects on the hypothalamus (Clegg, Brown, Woods, & Benoit, 2006; Clegg et al., 2006). The differential gender sensitivity to leptin and insulin, and the convergence of these

peripheral hormones on PI3K, prompted us to investigate the estrogen regulation of p55γ subunit transcription and PI3K signaling in hypothalamus.

PI3K consists of a regulatory and catalytic subunit and is activated when the src (Rous sarcoma virus oncogene) homology 2 (SH2) domain of the regulatory subunit binds to the phosphorylated tyrosine residues of the activated receptor tyrosine kinases (see (Caterina et al., 2006) for review). This effect both relieves the inhibition of the catalytic subunit and targets it to the plasma membranes where the lipid substrate of catalytic enzyme is located. The 5 mammalian regulatory subunit isoforms can act as positive or negative regulators of PI3K activity depending on the cell type and stimulus (Vanhaesebroeck, Ali, Bilancio, Geering, & Foukas, 2006). The structure of each regulatory isoform is distinct such that the individual domains could have multiple functions that contribute to protein-protein interactions and more importantly, to overall cell signaling. For example, the unique N-terminal sequence of the p55γ regulatory subunit binds to tubulin (Inukai et al., 2000)and Rb (Xia, Cheng, Akinmade, & Hamburger, 2003) with much higher affinity and may transduce signals different than that of the p85α regulatory subunit. Thus, changes in expression levels of the regulatory subunits may be a mechanism to augment or dampen PI3K signaling (Ueki et al., 2003; Geering, Cutillas, Nock, Gharbi, & Vanhaesebroeck, 2007). We hypothesized that altering PI3K regulatory subunit expression levels is one mechanism by which estrogen modulates PI3K signal transduction. Interestingly, there have been very few studies on the neuronal distribution and regulation of PI3K regulatory subunit expression (Pons et al., 1995; Shin et al., 1998; Horsch & Kahn, 1999; Trejo & Pons, 2001) and virtually no reports of gonadal steroid hormone influence on PI3K subunit expression. Presently, we have characterized the distribution and estrogen regulation of p55γ and p85α regulatory subunits in the female guinea pig forebrain. In addition, we show that PI3K plays a

critical role in the estrogen-mediated desensitization of $GABA_B$ receptors in POMC neurons, which ultimately leads to enhanced excitability. Data from these investigations strongly suggest both rapid signaling via PI3K and regulation of PI3K regulatory subunit expression are involved in the cellular effects of estrogen in hypothalamic neurons.

Methods

Animals

All animal treatments described in this study are in accordance with institutional guidelines based on National Institutes of Health standards and were performed with institutional Animal Care and Use Committee approval at the Oregon Health and Science University. Female Topeka guinea pigs, bred in our institutional breeding facility, and multicolor guinea pigs (Elm Hill Breeding Labs, Chelmsford, MA) were used in these experiments as previously described (Qiu et al., 2003). Adult female guinea pigs were maintained under constant temperature and light (light on between 06:30 hr and 20:30 hr local time) with food and water provided ad libitum. The animals were ovariectomized under ketamine/xylazine anesthesia (33/6 mg/kg subcutaneously) 5-6 days before experimentation and given a subcutaneous injection of either 17β-estradiol (E_2) in the form of 17β-estradiol benzoate (EB; 25 μg in 0.1 ml) or sesame oil vehicle alone (0.1 ml). This is a physiological dose of EB in the guinea pig and induces both negative and positive feedback on the hypothalamic-pituitary-gonadal (HPG) axis (Wagner et al., 2001). Twenty-four hours later, the animals were killed by decapitation after sedation with ketamine and blood samples were collected. For the electrophysiology experiments, transgenic female mice expressing enhanced green fluorescent protein (EGFP) under the control of the POMC promoter (EGFP-POMC) were used in these studies (Bosch, Qiu, Kelly, & Rønnekleiv, 2003; Ibrahim et al., 2003). Animals were group-housed until surgery at which time they were housed individually. All animals were maintained under controlled temperature and photoperiod (light on at 06:00 hr and off at 18:00 hr) and given free access to food and water. Adult females were ovariectomized under isoflurane anesthesia 5-7 days prior to experimentation and sacrificed between 10:00 hr and 11:00 hr.

Tissue preparation

For *in situ* hybridization, 2 mm coronal blocks through the preoptic area (POA) and basal hypothalamus (BH) were fixed in 4% paraformaldehyde for 6hr, soaked in 20% buffered-sucrose solution (pH 7.4), embedded in O.C.T. (Sakura Finetek, Torrance, CA), and frozen in isopentane at -55°C. Coronal sections (20 μm) were cut on a cryostat and thaw-mounted onto Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). The sections were stored at -80°C. For quantitative real-time PCR analysis, a brain slicer (EM Corporation, Chestnut Hill, MA) was used to produce 1 mm frontal slices. These were placed in RNAlater (Ambion, Austin, TX) and the various brain regions were microdissected under a dissecting microscope (See Fig. 6). Microdissected tissues were rapidly frozen and stored at -80°C.

Cloning of the guinea pig p55γ and p85α regulatory subunits

A 258 bp fragment of the guinea pig p55γ subunit gene and a 243 bp fragment of the guinea pig p85α subunit gene were cloned using reverse transcription (RT) and PCR. Oligonucleotide primers were designed based on the cDNA clone sequence for p55γ using Clone Manager 5 software. The 5' primer (5'-CTCATACCTGGCGCTACTG-3') was 100% homologous to the human (gi|40254447 [GenBank]: 708-726 bp) phosphoinositide-3-kinase, regulatory subunit 3 (p55γ) sequence. The 3' primer (5'-GGAAGAACCAGGCTCTACGG-3') was 100% homologous to the human (gi|40254447 [GenBank]: 947-966 bp) phosphoinositide-3-kinase, regulatory subunit 3 (p55γ) sequence. Oligonucleotide primers were designed based on homology to the human sequence for p85α using Clone Manager 5 software (Sci Ed Software, Cary, NC). The 5' primer (5'-AAGCAGGCAGCTGAGTAT-3') was 100% homologous to the human

(gi|32455247 [GenBank]: 903-921 bp) phosphoinositide-3-kinase, regulatory subunit 1 (p85α) sequence. The 3' primer (5'-TTGGAAGCAGCAACCGAA-3') was 100% homologous to the human (gi|32455247 [GenBank]: 1128-1146 bp) phosphoinositide-3-kinase, regulatory subunit 1 (p85α) sequence. Primer synthesis by Promega included at the 3'-end of both primers a 3'-adenosine ligated to linearized vector DNA that has been cleaved at an EcoR V site and had a single 3'-terminal thymidine added to both ends used with the pGEM-T Easy Vector System PCR cloning kit (Promega).

The p55γ and p85α cDNA was amplified from 200 ng of total RNA extracted from the guinea pig hypothalamus using RT-PCR (GeneAmp kit; PerkinElmer, Foster City, CA). An oligo-dT primer was used for the cDNA first-strand synthesis. Reverse transcription was performed for 15 min at 42°C. PCR was conducted for 45 cycles of denaturation (94°C; 45 s), annealing (56°C; 45 s), and extension (72°C; 1 min 10 s), with a 7 min final extension. The resulting 258 bp and 243 bp products for p55γ and p85α respectively, were gel-purified, subcloned into the pAMP10 vector by using the CloneAmp System and sequenced.

In situ hybridization

Radioactive antisense cRNA probes were transcribed *in vitro* with T7 or SP6 RNA polymerase from guinea pig PI3K p55γ and PI3K p85α constructs, respectively. The sense probes were prepared by using SP6 or T7 polymerase in the presence of ^{35}S-uridine 5'(α-thio) triphosphate (^{35}S-UTP). Residual DNA was digested with 10 U DNase I (Roche Diagnostics, Indianapolis, IN). The antisense and sense RNA probes were separated on G-50 Sephadex column (Amersham Biosciences, Piscataway, NJ). Slides from oil- and estrogen-treated females were simultaneously reacted. These slides were post-fixed in fresh 4% paraformaldehyde in Sorensen's phosphate buffer (0.03 M, pH

7.4) for 15 min, rinsed in Sorensen's buffer and treated with proteinase K (1.0 μg/ml) for 3-6 min at room temperature. Sections were then treated with 0.1 M triethanolamine (3-5 min), followed by 0.25% acetic anhydride in 0.1 M triethanolamine (10 min), and rinsed briefly in 2X standard saline citrate (SSC). Sections were prehybridized for 1 hr at 58°C with hybridization buffer (50% formamide, 1X Denhardt's solution, 10% dextran sulfate, 100 μM dithiothreitol (DTT), 200mM sodium chloride, 10 mM Tris-HCL, pH 8.0, 1 mM EDTA, pH 8.0, 125 μg/ml tRNA; Sigma) and then quickly rinsed in 2X SSC buffer. The ^{35}S-labeled antisense and sense riboprobes were heat-denatured, diluted with hybridization buffer and used at a final concentration of 1.2 x 10^4 cpm/μl. Subsequently, the sections were covered with glass coverslips, sealed, and hybridized in a moist chamber for at least 20 hr at 59°C. After hybridization, the slides were rinsed in 2X SSC buffer, reacted with RNAse (20 μg/ml) for 45-60 minutes at room temperature and washed in decreasing concentrations of SSC (2X, 1X, 0.5X, 0.1X) at 55°C with a final wash for 1 hr in 0.1 X SSC at 65°C. The slides from oil- and estrogen-treated females were dehydrated in ethanol, placed side by side on a flat surface together with autoradiographic ^{14}C microscales and exposed to Hyperfilm-β_{max} (Amersham Biosciences, Piscataway, NJ) for 6 days at 4°C. The slides were then dipped in Kodak NTB-2 nuclear track emulsion and exposed for 20-28 days at 4°C. Thereafter, slides were developed in D19 developer, fixed in Kodak fixer, counterstained with hematoxylin, dehydrated and coverslipped. Quantification of film images was performed using a Macintosh G4 computer equipped with NIH Image 1.61 program. Film images were scanned (HP scanjet 7400c) and digitized images processed in Adobe Photoshop (Mountain View, CA) and Macromedia FreeHand (San Francisco, CA) software programs. Contrast and brightness were adjusted in scanned images of films to compensate for uneven illumination in brightfield images. Film images of 4-9 sections

for each brain region from matched oil-control and EB-treated females were analyzed and an average density value was obtained from each brain region after subtracting background, which was used for further analysis. The ^{14}C microscale was used as a standard to calculate the density of mRNA signals and the optical density was converted to nanocuries (nCi) per gram. Comparisons between the two groups were performed using a two-tailed Student's t-test. Differences were considered statistically significant if the probability of error was less than 5%.

Images of emulsion-coated slides of PI3K p55γ were analyzed using a Nikon E800 microscope. Darkfield and brightfield images were captured using a Nikon DS-L1 digital camera and illustrated using Adobe Photoshop and Macromedia FreeHand software programs. Contrast and brightness were adjusted in scanned images to match the original image seen in the microscope.

Quantitative Real-time PCR

Total RNA was extracted from microdissected tissue samples using the RNaqueous Micro kit (Ambion) and quantified on a spectrophotometer (Nanodrop Technologies, Wilmington, DE). Total RNA was DNase I treated (DNAfree; Ambion) at 37°C for 30 min, and the DNase I was heat inactivated at 65°C for 10 min. Two-hundred ng of total RNA was reverse-transcribed into cDNA using 100 ng random hexamers (Promega), 10 mM DTT, 0.2 μM dNTPs, 10mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2mM $MgCl_2$, 15 U RNasin (Promega) and 50 U MuLV Reverse Transcriptase (RT; Applied Biosystems, Foster City, CA) for 1 hr at 42°C, denatured at 99°C for 5 min and then cooled on ice for 5 min. A negative control reaction was run for each RNA sample (minus RT) to confirm the absence of genomic DNA contamination in subsequent PCR reactions. Sample cDNA was diluted 1:20 with nuclease-free water (Ambion).

Quantitative real-time PCR (qRT-PCR) for guinea pig glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PI3K p55γ, and PI3K p85α transcripts was accomplished with primers designed using Clone Manager 5 software and the use of SYBR green chemistry (Applied Biosystems) on the 7500 ABI real-time PCR system (Applied Biosystems).

The primer sequences were as follows: guinea pig GAPDH (242 bp) forward (5'-CATCCACTGGTGCTGCCA-3') and reverse (5'-GTCCTCGGTGTAGCCCAAG-3'); PI3K p55γ (108 bp) forward (5'-GGTGGCTTTGGTGGAAGAG-3') reverse (5'-GAGTATGGACCGCGATGAC-3'); and PI3K p85α (190 bp) (5'-GCCAGACCTCATTCAGCTAAG-3') and reverse (5'-GTTGCTGCTGCCAACATTC-3'). Forward and reverse primers were used at a final concentration of 500 nM in a reaction that also contained 10 μl 2X mastermix, 4 μl cDNA and nuclease-free water to a final volume of 20 μl. Samples were run in duplicate on the ABI 7500 under the following thermal cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a dissociation step for melting curve analysis. To exclude the possibility of contamination, sterile water controls were run in parallel to every qRT-PCR run. Amplification efficiency for all primers was assessed using serial dilutions (1:5, 1:10, 1:50, 1:100, 1:500, 1:1000) of basal hypothalamic cDNA. A standard curve was constructed and the efficiency was calculated according to the following formula: $E = 10^{(-1/m)} - 1$, where m = slope (Livak & Schmittgen, 2001). Primer efficiencies were as follows: GAPDH, 98%; p55γ, 100%; p85α, 93%. The similar efficiencies allowed us to use the ΔΔCT method to determine differences in our treatment groups (Livak et al., 2001; Pfaffl, 2001). Melting curve analysis confirmed primer specificity in amplification of a single product for each primer pair. GAPDH was used as an internal reference for normalization after we determined that GAPDH does not change by EB treatment in the

brain regions analyzed. The expression value of each gene was quantified on the basis of the interpolated cycle number in which the fluorescence reached a threshold value (Ct value), using background fluorescence in cycles 1-15 as baseline. The p55γ and p85α transcripts were normalized to the amount of GAPDH in that sample to calculate a relative amount of transcript present for each gene. The normalized expression values for all control and EB treated samples were averaged and an average fold change was calculated using $2^{(-\Delta\Delta Ct)}$ where ΔCT = target CT – control CT, ΔΔCT = ΔCT target - ΔCT calibrator (Livak et al., 2001). A Student's t-test was conducted between the normalized relative expression values for each individual control and EB-treated samples to determine statistical relevance. The relative abundance of p55γ and p85α transcripts in various brain regions was measured using the ΔCT values and normalized to the diagonal band of Broca from both groups of animals (e.g., used as calibrator).

Single-cell RT-PCR

To further determine if p55γ and p85α are expressed in neurons, we harvested arcuate neurons from intact male guinea pigs. These experiments were performed using intact male guinea pigs in order to avoid effects that might otherwise be observed in ovariectomized or cycling females. Cell harvesting was performed as previously described Cell harvesting was performed as previously described (Qiu et al., 2003). Briefly, 200-300 μm arcuate slices were cut on a vibratome and placed in an auxiliary chamber containing oxygenated aCSF. The slices were allowed to recover for 1-2 hrs in the oxygenated chamber before dispersion. The arcuate nucleus was microdissected and incubated in 5-10 ml aCSF (124 mM NaCl, 5 mM KCl, 2.6 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM Hepes, 10 mM D-glucose, in DEPC-treated water, pH 7.3, 300 mOsm) containing 1 mg/ml protease for ~15 min at 37°C.

The tissue was washed four times in one volume of low calcium aCSF and two times in aCSF. The cells were isolated by trituration with a flame-polished Pasteur pipette. The cells were dispersed onto a 35 mm petri dish, visualized under a Leitz inverted microscope, then patched and harvested into the patch pipette by applying negative pressure. The contents of the pipette were expelled into a siliconized microcentrifuge tube containing 0.5 µl 10X buffer (100 mM Tris-HCl, 500 mM KCl, 1% Triton-X), 15 U Rnasin, 0.5 µl 100 mM DTT and DEPC-treated water in a 5 µl volume. The tubes were immediately frozen at -80°C until the reverse transcription procedure. The harvested cells were reverse transcribed as described previously (Ibrahim et al., 2003; Qiu et al., 2003). Briefly, the harvested cell solution and 25 ng of hypothalamic total RNA in 1 µl were denatured for 5 min at 65°C then cooled on ice for 5 min. Single stranded cDNA was synthesized from cellular RNA by adding 50 U MuLV-RT, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 µM dNTPs, 15 U RNasin, 10 mM DTT and 100 ng random hexamers in DEPC-treated water for a final volume of 20 µl. Cells and tissue RNA used as negative controls, were processed as described above but without RT. The reaction mixtures were incubated at 42°C for 60 min, denatured at 99°C for 5 min and cooled on ice for 5 min.

Specific primers for guinea pig PI3K p55γ (255 bp) forward (5'-GCTTTAGCTCCAGCCACTTG-3') reverse (5'-GCACAGCAATGTCTGAACAC-3'), p85α (283 bp) forward (5'-CCACTACCGGAATGARTCTC-3') reverse (5'-GGTCTGGCAYTGTTCTTCAA-3') and GAPDH (212 bp) forward (5'-CATCCACTGGTGCTGCCAAG-3') reverse (5'-GTCCTCGGTGTAGCCCAAGA-3') were designed using Clone Manager software. Each primer pair was tested to determine the optimal conditions (i.e., magnesium concentration, annealing temperature and the amount of cDNA template) for single-cell PCR. PCR was performed using 3 µl of cDNA

template from each RT reaction in a 30 µl PCR volume containing: 3 µl 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM dNTPs, 2 Units Taq DNA polymerase (Promega), 0.22 µg TaqStart Antibody (Clontech) and 0.2 µM each of forward and reverse oligonucleotides. Taq DNA polymerase and TaqStart Antibody were combined and incubated at room temperature for 5 min then the remainder of the reaction contents was added to the tube. Fifty cycles of amplification were performed using a MJ Research PTC-100 thermocycler in 0.5 ml thin walled PCR tubes according to one of the following protocols: 94°C, 2 min, 50 cycles of 94°C, 1 min; 58°C, 1 min; 72°C, 1 min, with a final 72°C extension for 5 min. Ten µl of the PCR products were visualized with ethidium bromide on a 2% agarose gel. In addition to the controls described above, harvested aCSF in the vicinity of the dispersed cells was used as a control in the RT-PCR.

Electrophysiology Recordings

Electrophysiology experiments were carried out in transgenic mice expressing enhanced green fluorescent protein (EGFP)-labeled POMC neurons to allow targeting of these anorexigenic neurons (Ibrahim et al., 2003). Adult (6-8 wk) females were ovariectomized and allowed to recover for one week at which time they were anesthetized with halothane and decapitated. The brain was rapidly removed and a block containing the basal hypothalamus (BH) was immediately dissected and submerged in 4°C oxygenated (95% O_2, 5% CO_2) high sucrose aCSF: 208 mM sucrose, 2 mM KCl, 26 mM $NaHCO_3$, 10 mM glucose, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4. Coronal slices (250 µm) from the BH were cut on a vibratome during which time (10 min) the slices were bathed in high sucrose CSF at 4°C. The slices were then transferred to an auxiliary chamber where they were kept at room temperature (25°C) in normal aCSF until recording (recovery for 2 h). A single slice was

transferred to the recording chamber at a time, and was kept viable by continually perfusing with warm (35°C), oxygenated normal aCSF at 1.5 ml/min.

Whole-cell patch recordings were made under a Zeiss Axioskop FS out-fitted with epifluorescence (FITC filter set) and IR-DIC video microscopy (Ibrahim et al., 2003). The area containing POMC neurons was initially identified under low power (5X objective) with UV illumination. Then the EGFP-POMC neurons were visualized through a 40x water immersion objective (Achroplan, Zeiss, Jena, Germany), and their position was marked on a monitor and compared carefully to its position under IR-DIC imaging. Patch pipettes (A-M Systems, Seattle; 1.5 mm O.D. borosilicate glass) were pulled on a Brown/Flaming puller (Sutter Instrument Co., Model P-97) and were filled with the following solution: 128 mM potassium gluconate, 10 mM NaCl, 1 mM $MgCl_2$, 11 mM EGTA, 10 mM Hepes, 2 mM ATP, 0.25 mM GTP; adjusted to pH 7.3 with KOH; 295 mOsm. Pipette resistances were 4-6 MΩ when filled with above pipette solution. In whole cell configuration, access resistance of the pipette was 20-40 MΩ. The membrane capacitance of cells was measured by manual compensation. The input resistance was calculated by measuring the slope of the current-voltage relationship curve between -70 and -50 mV. The targeted POMC neurons were visualized with IR-DIC for positioning the patch-pipette and standard whole-cell patch recording procedures and pharmacological testing were followed as previously described (Ibrahim et al., 2003; Qiu et al., 2003). An EC_{50} concentration of the $GABA_B$ agonist baclofen (5 μM) was used for the whole-cell recording experiments. After seals were formed and the whole-cell confirmation obtained, the slices were perfused with TTX (1 μM) for 5 min. The first $GABA_B$ response was generated by perfusing baclofen (5 μM) until a steady-state current was obtained (first response, R1). After drug washout, when the current returned to pre-drug resting levels, the cells were perfused with E_2 (100 nM) alone or in combination with PI3K inhibitor wortmannin (100 nM) or LY294002 (10 μM), and a second response was

measured to baclofen (R2). The effects of E_2 with and without the PI3K inhibitors were expressed as a percentage of R2 over R1 (Fig. 11A). Comparisons between groups were made using an unpaired t-test. Differences were considered significant if the p value was < 0.05.

Results

PI3K p55γ and p85α subunit clones

To study the mRNA expression of the p55γ and p85α subunits in the guinea pig brain, we used PCR to clone guinea pig-specific cDNA sequences that were 258 bp and 243 bp in length, respectively. The guinea pig PCR product of the p55γ subunit was 94%, 91% and 89% homologous to the corresponding human, mouse and rat sequences, respectively. The guinea pig PCR product of the p85α subunit was 91%, 87% and 87% homologous to the corresponding human, mouse and rat sequences. Therefore, the riboprobes synthesized from these clones were specific for the PI3K p55γ and p85α subunits.

Distribution and estrogen regulation of PI3K p55γ and p85α subunit mRNA expression using in situ hybridization

The brain distribution of PI3K p55γ and p85α has not been previously characterized in the guinea pig. Therefore, we used *in situ* hybridization with ^{35}S-labeled probes against the PI3K p55γ and p85α regulatory subunits to characterize their mRNA brain distribution in the guinea pig forebrain. *In situ* hybridization film autoradiography revealed a striking difference in brain distribution between these two subunits. For example, p55γ subunit mRNA was observed mainly in diencephalic regions while p85α subunit mRNA was localized primarily in the cortex (Fig.1, 2, 3). Specifically, p55γ

mRNA was observed in the diagonal band of Broca (DB), septum, hippocampus as well as a number of thalamic and hypothalamic nuclei (Fig. 1 A-H). Within the thalamus, nuclei such as the paraventricular, reticular and anterodorsal exhibited dense expression of p55γ, as did the medial habenula and zona incerta (Fig. 1D-H). Within the hypothalamus, dense labeling was found in the majority of nuclei including the anteroventralperiventricular (AVPV), median preoptic (MEPO), medial preoptic (mPOA), suprachiasmatic (SCN), paraventricular (PVH), arcuate (ARC), ventromedial (VMH) and dorsomedial (DMH) nuclei (Figs 1A-H, 2). Under brightfield illumination, the p55γ mRNA expression was observed over cells with large pale nuclei suggesting neuronal localization (Fig. 2L). It should be noted that essentially no p55γ mRNA was observed throughout the rostral-caudal extent of the cerebral cortex (Fig. 1A-H). Sections reacted with the corresponding sense probe exhibited no specific label (data not shown).

In contrast to p55γ mRNA, the p85α subunit mRNA was primarily distributed in the cortex but expression was also observed in other brain regions including the hippocampus, PVT, zona incerta and some hypothalamic nuclei (Fig. 1A'-H'). Within the hypothalamus, p85α subunit mRNA was found in regions such as the supraoptic, PVH, VMH, ARC, and DMH (Fig. 1A'-H'). The dense cortical labeling was particularly obvious under brightfield illumination of emulsion-coated slides in which dense clusters of grains were detected over cortical neurons (Fig. 3). No specific labeling was detected in tissue sections with radiolabeled sense p85α probe (data not shown).

Since previous data from microarray analysis suggested that p55γ mRNA expression is regulated by estrogen in the hypothalamus (Malyala et al., 2004), we sought to determine whether estrogen altered p55γ mRNA expression in specific hypothalamic nuclei using *in situ* hybridization autoradiography. We found increased p55γ mRNA expression in a number of hypothalamic regions in EB-treated compared to oil-treated

guinea pigs (Fig. 4, 5A). Densitometry measurements of film images revealed that animals treated with an LH surge-inducing dose of EB exhibited significantly higher mRNA levels of the p55γ subunit in the SCN, PVH, ARC and VMHvl as compared to the oil-treated animals (Fig. 4, 5A, $p < 0.05$; $n = 4$). No significant expression differences were found in the AVPV, mPOA, and DMH. In contrast to the p55γ subunit, densitometry measurements of film images showed no significant changes in p85α subunit mRNA expression between 24 h EB- and oil-treated animals (Fig. 5B).

Distribution and estrogen regulation of PI3K p55γ and p85α subunit transcripts using quantitative real-time PCR

To further substantiate the differential mRNA distribution and E_2 regulation of the two PI3K subunits within the hypothalamus, we measured p55γ and p85α mRNA transcript levels from microdissected hypothalamic nuclei of EB- and oil-treated ovariectomized female guinea pigs using qRT-PCR (Figs. 6, 7). We determined using cDNA serial dilutions that the primer efficiencies for p55γ and p85α were 100% and 93%, respectively, which allowed us to use the ΔΔCt method for quantification (Pfaffl, 2001; Livak et al., 2001), and compare the tissue expression of the two transcripts (Fig. 7). We also illustrated the amplification of a specific single product for p55γ and p85α through single-product melting curves, which further validated the assay (Fig. 7B, D). qRT-PCR results confirmed the autoradiography film densitometry *in situ* hybridization data. PI3K p55γ mRNA showed highest expression in the SCN and PVH, moderate to high expression in the ARC, VMHdm, VMHvl, DMH and LH, and lowest expression in the CTX (Fig. 8A). Similar expression of p85α was observed in the ARC to that of p55γ mRNA (Fig. 8B). The other nuclei examined revealed low p85α mRNA expression and

similar to the *in* situ hybridization data, the expression of p85α mRNA was many-fold higher in the cerebral cortex (Fig. 8B).

When comparing EB- and oil-treatment, the PI3K p55γ mRNA expression was significantly increased in the SCN, PVH, ARC and VMHvl of EB-treated versus oil-treated animals, which confirmed the *in situ* hybridization findings (Fig. 9A, $p < 0.05 - 0.01$; $n = 6$). The VMHdm region showed a trend of increased p55γ mRNA with estrogen treatment, however the apparent difference was not significant. The p85α mRNA expression was not affected by EB treatment in most hypothalamic nuclei, which is consistent with the *in situ* hybridization data. In contrast to the *in situ* hybridization results, quantitative RT-PCR revealed a significant EB-induced increase in p85α mRNA expression in the VMHdm (Fig. 9B, $p < 0.05$, $n = 6$).

PI3K p55γ mRNA expression in arcuate nucleus neurons using single cell RT-PCR

We used single cell RT-PCR analysis to further determine that p55γ and p85α are expressed in arcuate nucleus neurons. Harvested single cells were reverse transcribed and the cDNA subsequently utilized for PCR analysis. Examples of single cell PCR data are shown in Figure 10A. Data from 61 dispersed cells obtained from 3 animals that included POMC neurons revealed that p55γ mRNA was expressed in 15 cells (25%) and p85α mRNA was expressed in 24 cells (39%) (Fig.10, $p = 0.12$, $n = 3$). The non-significant difference in the number of cells expressing each subunit is in agreement with tissue qRT-PCR and densitometry data.

Estrogen attenuates the $GABA_B$ receptor agonist response in POMC neurons of the arcuate nucleus

Previous data from our laboratory has established that μ-opioid and $GABA_B$ receptors are coupled to the same population of G protein-coupled inwardly rectifying K^+ (GIRK) channels in hypothalamic neurons and that estrogen rapidly uncouples (desensitizes) these receptors in hypothalamic neurons (Loose, Rønnekleiv, & Kelly, 1991; Kelly, Loose, & Rønnekleiv, 1992; Lagrange, Wagner, Rønnekleiv, & Kelly, 1996; Parent & Hazrati, 1995; Qiu et al., 2003; Qiu et al., 2006b). Therefore, we were interested in whether PI3K signaling is a component of this estrogen-mediated desensitization of $GABA_B$ receptors in arcuate POMC neurons. We used whole-cell recordings to measure the rapid effects of E_2 on the activation of the GIRK channel conductance by the $GABA_B$ receptor agonist baclofen in a transgenic mouse model in which we could easily visualize (EGFP)-labeled POMC neurons. For the electrophysiology analysis, only cells with gigaohm or better seals were included in the study. The mean resting membrane potential was -59.0 ± 1.3 mV at a 0 pA holding current, and the mean input resistance was 1.15 ± 0.15 GΩ, similar to previously published results (Ibrahim et al., 2003). To measure E_2 modulation of the $GABA_B$ response, we used an EC_{50} concentration (5 μM) of baclofen and the protocol depicted in Fig. 11A. We measured a robust outward current in response to baclofen that subsided after washout (Fig. 11B). The application of baclofen 20 min later elicited the same response, suggesting that desensitization and rundown did not occur in response to successive applications of 5 μM baclofen. However, if E_2 (100 nM) was applied during the interim period (i.e., after the washout of the first application of baclofen), there was a significant ($p < 0.001$; $n = 7$ cells) decrease of 58 ± 5.9% in the response to the second application of baclofen (Fig. 11C). Current-voltage relationships generated before and during the application of 100 nM E_2 showed that it did not change the reversal potential, which was at the predicted Nernst equilibrium potential for K^+, for the baclofen-mediated response.

PI3K inhibition abrogates estrogen attenuation of the $GABA_B$ response in POMC neurons

Next, we examined whether PI3K signaling is involved in the estrogen-attenuation of baclofen-induced hyperpolarization. If PI3K signaling is involved, then the effects of estrogen on the $GABA_B$ response should be antagonized by PI3K inhibitors. Indeed, whole-cell electrophysiology recordings from POMC neurons showed that the estrogen-mediated attenuation (desensitization) of the $GABA_B$ response was blocked by co-perfusion with wortmannin (100 nM) (Figure 11). Wortmannin alone had no effect (n = 2, data not shown). Likewise, the more selective PI3K inhibitor LY294002 also abrogated the estrogen-mediated inhibition of the $GABA_B$ response. Therefore, these data suggest that PI3K is an integral component of rapid estrogen signaling in hypothalamic neurons leading to increased POMC cell excitability.

Discussion

The *in situ* hybridization and single cell PCR results show PI3K p55γ regulatory subunit mRNA expression in hypothalamic neurons and is regulated by estrogen in the SCN, PVN, ARC and VMHvl nuclei. In contrast to the p55γ regulatory subunit, the PI3K p85α subunit mRNA is heavily expressed in the cortex with lower expression levels in hypothalamic regions. Estrogen regulation of p85α mRNA in the hypothalamus is only seen in the VMHdm nucleus. Furthermore, PI3K signaling is a component of the membrane-mediated estrogen desensitization of the $GABA_B$ agonist-induced hyperpolarization of POMC neurons in the arcuate nucleus. These data demonstrate that estrogen regulates hypothalamic PI3K p55γ mRNA expression and rapidly activates PI3K in POMC neurons, thus PI3K may be an integral component of acute and more sustained actions of estrogen in the hypothalamus.

PI3K p55γ and p85α subunit distribution

Based on our previous data, we were interested in determining whether the expression of specific PI3K regulatory subunits is altered by estrogen. Therefore, the present study investigated the distribution and estrogen regulation of PI3K p55γ and p85α regulatory subunits in the guinea pig CNS, which have not been previously reported. *In situ* hybridization with specific probes for the p55γ and p85α subunits demonstrated that these subunits are differentially distributed in the guinea pig brain and that the distribution pattern was similar for ovariectomized oil- and estrogen-treated animals. Our *in situ* hybridization data showed that the p55γ subunit was distributed primarily in the preoptic area, basal hypothalamus and hippocampus as well as in the septum and thalamus. The p85α subunit was highly expressed in most cortical regions but less abundant in subcortical areas such as the hypothalamus and thalamus. These results differ from previously published data where p55γ mRNA was localized mainly in the cerebellum, olfactory bulb and pituitary and p85α was more diffusely distributed throughout the brain (Horsch et al., 1999; Horsch et al., 1999). The reason for this discrepancy is not clear; however, it may be due to differences in probe size, *in situ* hybridization protocol or species. The distribution and expression differences of these regulatory subunits in our studies suggest that they may have non-redundant roles in PI3K signaling within the CNS. The distribution and function of the p85α subunit and its related isoforms have been most thoroughly characterized in relation to their roles in insulin signaling in the periphery (Ueki et al., 2003; Terauchi et al., 1999; Mauvais-Jarvis et al., 2002; Ueki et al., 2002; Barbour et al., 2004; Cornier, Bessesen, Gurevich, Leitner, & Draznin, 2006). Although PI3K subunits were originally isolated from brain cDNA libraries (Otsu et al., 1991; Hiles et al., 1992), few studies have focused on their

distribution and roles in the central nervous system (Trejo et al., 2001; Benomar et al., 2005; Veerasingham, Yamazato, Berecek, Wyss, & Raizada, 2005; Wang, Ou, & Chan, 2005; Bock et al., 2003; Pons et al., 1995; Shin et al., 1998; Horsch et al., 1999; Mannella & Brinton, 2006). Even fewer studies have specifically analyzed the p55γ subunit in the CNS (Pons et al., 1995); (Trejo et al., 2001; Shin et al., 1998) and these studies were incomplete since they focused on expression specifically in the cerebellum (Trejo et al., 2001; Shin et al., 1998) or in whole brain homogenates (Pons et al., 1995). Hence, the present study in which we describe that p55γ is primarily and extensively distributed throughout the diencephalon is novel and suggests that this subunit may have an important but undetermined role in this region. It should be noted that several antisera were tested using western blot to detect p55γ protein expression with inconsistent (negative) results.

Estrogen regulation of PI3K p55γ and p85α subunit expression in the hypothalamus

Using autoradiography film densitometry and qRT-PCR, we identified several hypothalamic nuclei including the SCN, PVN, ARC and VMHvl in which p55γ mRNA expression increased in estrogen as compared to oil-treated animals. The hypothalamus is clearly a target for estrogen and contains estrogen receptors α and β (Lauber, Mobbs, Muramatsu, & Pfaff, 1991; Pelletier, Liao, Follea, & Govindan, 1988; Simerly et al., 1990; Blaustein & Turcotte, 1989; Blaustein, Lehman, Turcotte, & Greene, 1992; DonCarlos et al., 1991; Warembourg, Jolivet, & Milgrom, 1989; Gundlah et al., 2000; Shughrue et al., 1997; Shughrue & Merchenthaler, 2001; Warembourg et al., 2004). Because many of the regions in which p55γ mRNA was regulated also express ERα, we searched for estrogen response elements (EREs) in the p55γ gene using the

Dragon ERE Finder software (Bajic et al., 2003). We found two putative ERE patterns in the human genome with consensus sequences of 5'-GA-GGTGA-TGA-TGCCC-TA-3' and 5'-GG-GGTCC-CAC-TGGTC-TG-3'. Verification of these EREs by other methods will need to be investigated. We also searched for EREs in the p85α gene however we did not find any ERE patterns in the human genome. Interestingly, a recent study shows a putative non-conserved sterol-regulatory element binding protein (SREBP) response element (SRE) site in the promoter region of the p55γ gene (Kallin et al., 2007). SREBPs are ubiquitous transcription factors, key regulators of lipid metabolism *in vivo* and activation of SREBP-1 by growth factors or insulin upregulates p55γ expression (Kallin et al., 2007).

Estrogen increased the expression of p55γ mRNA in the SCN. The SCN orchestrates circadian and seasonal rhythms to regulate physiology and behavior (Kalsbeek et al., 2006). Increased that included POMC neurons mRNA expression in the SCN by estrogen is interesting in light of a recent study identifying a novel protein that mediates neuronal survival partially through PI3K signaling (Kiyama, Isojima, & Nagai, 2006). This novel protein, period1 interacting protein of the suprachiasmatic nucleus (PIPS), is primarily expressed in the hypothalamus with particularly high expression in the SCN (Matsuki, Kiyama, Kawabuchi, Okada, & Nagai, 2001) and may play a role in the output signaling of the circadian clock. Our data suggest that estrogen may act directly or indirectly on neurons of the biological clock through estrogen receptors to influence PI3K signaling by altering subunits levels. In this respect, several studies have illustrated ER α and/or β mRNA expression in the SCN of rat, mouse (Shughrue et al., 1997; Shughrue, Scrimo, Lane, Askew, & Merchenthaler, 1997; Merchenthaler, Lane, Numan, & Dellovade, 2004; Laflamme et al., 1998) and human (Kruijver, Balesar, Espila, Unmehopa, & Swaab, 2002; Kruijver, Balesar, Espila, Unmehopa, & Swaab, 2003; Kruijver & Swaab, 2002). However, the specific role of p55γ and functional consequences of estrogen-regulated PI3K signaling in the SCN await further investigation.

We also found that estrogen significantly increased p55γ mRNA in the ARC, PVN and VMHvl. The ARC has reciprocal connections with a number of hypothalamic nuclei including the PVN, VMH and DMH (Berthoud, 2002; Berthoud, 2007), all of which express ERα in guinea pig (Warembourg et al., 2004). The ARC contains various populations of neurons including NPY/AgRP neurons that potently stimulate food intake and POMC/CART neurons that induce an anorectic response (Funahashi et al., 2003).

Neurons of the ARC are key targets for the actions of leptin and insulin (Porte, Jr., Baskin, & Schwartz, 2002). In addition to energy balance, these neuronal populations are also involved in reproduction (Schneider, 2004). Intracerebroventricular administration of PI3K inhibitors blocks the anorexigenic effects of leptin and insulin on food intake and demonstrates the importance of hypothalamic nuclei including the ARC and VMH in mediating the effects (Niswender et al., 2003; Niswender et al., 2001b). Furthermore, intracerebroventricular infusion of PI3K inhibitors partially attenuates lordosis in ovariectomized rats following estrogen priming (Etgen et al., 2003). These data highlight the direct or indirect role of PI3K signaling in homeostatic behaviors. Our finding of increased p55γ subunit mRNA in the ARC with E_2 treatment suggests a mechanism by which estrogen regulates PI3K signaling, and in turn PI3K-mediated behavior.

The VMH also participates in a broad array of homeostatic functions including ingestive, sexual and affective behaviors (Borszcz, 2006; Kow, Brown, & Pfaff, 1994; Davidowa, Li, & Plagemann, 2002). For example, disruption of the VMH leads to obesity (Majdic et al., 2002) and electrolytic lesioning of the ventrolateral portion of the VMH (VMHvl) inhibits lordosis behavior in rats (Pfaff & Sakuma, 1979; Emery & Moss, 1984). Although the role of PI3K signaling in the VMH to mediate these behaviors has not been tested directly, the presence of insulin receptors (Numan & Russell, 1999) and glucose-sensing neurons (Kang, Routh, Kuzhikandathil, Gaspers, & Levin, 2004; Song & Routh, 2005; Song, Levin, McArdle, Bakhos, & Routh, 2001) in this region suggests that PI3K may indeed play a role. It should be noted that ERα is highly expressed in the VMHvl and this area projects to other brain regions that also express estrogen receptors (Turcotte & Blaustein, 1999). Within the VMH, leptin receptors are expressed in the VMHdm and excluded from the VMHvl (McClellan, Parker, & Tobet, 2006) and because

PI3K is an important component of leptin signaling, the regulation of p85α by estrogen may play a role in mediating leptin's effects.

In addition to its critical role in the hypothalamo-pituitary-adrenal (HPA) axis, the PVH is a key autonomic center involved in the central regulation of arterial blood pressure by the binding of angiotensin II hormone to type 1-angiotensin receptors (Pilowsky & Goodchild, 2002). Activation of these Gq-coupled receptors triggers a number of intracellular signaling pathways including PI3K that results in modulation of catecholamine neurotransmission, primarily by inducing transcription of the genes for tyrosine hydroxylase, dopamine β-hydroxylase, and the noradrenaline transporter (reviewed in (Sumners, Fleegal, & Zhu, 2002)). Interestingly, the PVH expresses ERβ but little to no ERα (Laflamme et al., 1998; Shughrue et al., 1997) suggesting that ERβ may play a role in the transcription of p55γ in this brain region. The consequences of PI3K subunit alteration in the PVH by estrogen needs to be further investigated.

Role of PI3Kinase in mediating the rapid, membrane initiated signaling of estrogen

Our data indicate that PI3K is important for membrane-localized rapid responses to E_2. The importance of phosphoinositides in cellular signaling is becoming increasingly clear. Membrane phosphoinositides are differentiated by the combinatorial phosphorylation of their inositol head group at the 3-, 4- and 5-hydroxyl positions, each of which are recognized by specific domains of proteins (Balla, 2006; Balla, 2005). The different phosphoinositide forms rapidly interconvert via specific lipid kinases and phosphatases, but can also be broken down by phospholipases. The continual change in the relative populations of these membrane lipids profoundly affects cellular activities (Suh & Hille, 2002).

$GABA_B$ and μ-opioid receptors are Gαi-coupled GPCRs, and GTP binding leads to dissociation of Gα and Gβγ subunits. Upon ligand binding, the Gβγ subunit activates GIRK channels, which leads to membrane hyperpolarization via an increased K^+ conductance (Kofuji, Davidson, & Lester, 1995). However, activation of GIRK channels requires permissive levels of membrane phosphatidylinositol (4,5) bisphosphate (PIP_2) and increased channel activity results from Gβγ-mediated stabilization of PIP_2-GIRK binding (Huang, Feng, & Hilgemann, 1998; Zhang, He, Yan, Mirshashi, & Logothetis, 1999). In fact, the regulation of channel activities by Gβγ, $PI(4,5)P_2$, or phosphorylation occurs on the time scale of a few seconds which can be resolved by electrophysiological techniques (Suh et al., 2005).

Recent studies from our lab have shown that estrogen, via a membrane associated G protein-coupled receptor, can rapidly desensitize $GABA_B$ and μ-opioid receptors via a PLC-PKC-PKA pathway (Qiu et al., 2003; Qiu et al., 2006b). Additionally, in the present studies, we have found that PI3K plays a critical role in facilitating the rapid membrane response to estrogen, as elucidated by the use of PI3K inhibitors. There are at least two possible explanations for the ability of PI3K inhibitors to blunt the rapid effects of estrogen in POMC neurons: (1) activation of PI3K by the mER is integral part of the rapid estrogen signaling leading to a desensitization of $GABA_B$ (and μ-opioid) receptors; or (2) the PI3K signaling pathway is not coupled to the mER, but plays a permissive role in modulating GIRK activity as described above—i.e., PI3K inhibitors block the basal PI3K activity and increase the expression of PIP_2 in the membrane thereby augmenting GIRK channel activity and countering the effects of the mER signaling pathway. In either case, the upregulation of PI3K p55γ regulatory subunit and kinase activity (Inukai et al., 2000) could contribute to the rapid membrane signaling of estrogen.

The question arises as to what is the physiological significance of the estrogen-driven upregulation of PI3K and plasma membrane signaling cascades? The simplest answer

is that estrogen has a self-priming effect on hypothalamic neuronal activity. In our model of estrogen feedback regulation of the hypothalamus, estrogen causes new gene transcription during the early follicular phase of the ovulatory cycle (Kelly & Lagrange, 1998; Wagner et al., 2001; Kelly & Rønnekleiv, 2002; Qiu et al., 2003; Qiu et al., 2006a). The resultant upregulation of signaling molecules, including PI3K and Gec-1, and ion channels such as Cav3.1 (Wong, Le, Zsarnovszky, & Belcher, 2003; Malyala, Kelly, & Rønnekleiv, 2005; Qiu et al., 2006a) could set the stage for the actions of multiple plasma membrane receptors including ionotropic (GABA) receptors (via Gec-1), Gαs-coupled (noradrenergic) receptor regulation (via neurobeachin) of ion (calcium) channels and hormones/growth factor receptors (via PI3K). For example, we have reported on the reduced sensitivity of preoptic GABA neurons to the auto-regulatory inhibition of GABA during estrogen feedback (Wagner et al., 2001). This depends on the uncoupling of the $GABA_B$ (Gαi,o) receptors from GIRK channels via PLC and PI3K activation (Qiu et al., 2003). In addition, desensitization of Gαi,o coupling of $GABA_B$ and μ-opioid receptors to GIRK channels via mER-mediated PLC and PI3K activity in arcuate POMC (β-endorphin) neurons would lead to enhanced opioid/melanocortin tone to govern energy homeostasis, stress responses, etc. to ensure successful reproductive behavior (Qiu et al., 2003; Qiu et al., 2006b; Rønnekleiv & Kelly, 2005). Although the regulation of PI3K p55γ needs to be elucidated within individual neurons in this complex circuitry, the discovery of enhanced expression of this PI3K regulatory subunit (p55γ) highlights the complexity of estrogen signaling in the hypothalamus and how this gonadal hormone serves as a central mediator of homeostatic functions.

Fig. 1. Comparison of PI3K p55γ and p85α mRNA brain distribution. Film images of p55γ and p85α subunit mRNA in the guinea pig forebrain. Brightfield view of film autoradiograms from coronal sections comparing the distribution of p55γ (**A-H**) and p85α (**A'-H'**) mRNA from rostral (**A,A'**) to caudal (**H,H'**) in a representative E_2-treated female guinea pig. The figure illustrates the distribution of p55γ and p85α subunit mRNA in adjacent sections using *in situ* hybridization film autoradiography with specific ^{35}S-labeled probes. Films were exposed for 6 days. The darkness of the image represents the density of mRNA expression. Expression of p55γ mRNA is absent in the cortex whereas the highest expression of p85α mRNA is in the cortex. For abbreviations see list.

Fig. 2. Expression and distribution of PI3K p55γ mRNA. Emulsion autoradiograms of PI3K p55γ subunit mRNA in guinea pig forebrain by *in situ* hybridization. Darkfield photomicrographs illustrating p55γ mRNA signal (white is signal) in coronal sections through the (**A**) DB, (**B**) POA, (**C**) SCN and (**D-F**) basal hypothalamus. Higher magnification darkfield images showing p55γ mRNA hybridization clusters in different nuclei including (**G**) PVH, (**G,H**) VMHdm, (**I**) VMHvl, (**G,H,K**) ARC, (**J**) ZI. Boxed area from the arcuate nucleus (**K**) of p55γ mRNA darkfield image is shown as brightfield image (**L**) illustrating p55γ mRNA grains over some (arrow) but not all (arrowhead) Nissl-stained nuclei. For abbreviations see list. Scale bars = 400 μm, A-F; 100 μm in G; 50 μm in H-K; 25 μm in L.

Fig. 3. Expression and distribution of PI3K p85α mRNA. Darkfield photomicrograph from coronal section illustrating p85α mRNA signal (white is signal) throughout the (**A**)

cortex. Boxed area from the cortex (**A**) of p85α mRNA is shown as brightfield image (**B**) illustrating clusters of p85α mRNA grains (black). Scale bars = 400 μm in A, 25 μm in B.

Fig. 4. PI3K p55γ mRNA expression differences in oil and estrogen treated animals. Film images of p55γ mRNA expression in ovariectomized (**A-E**) oil- or (**F-J**) EB-treated guinea pigs. Images of film autoradiograms illustrate the distribution of p55γ mRNA in matched coronal sections from rostral (**A,F**) to caudal (**E,J**) in oil and EB-treated animals. The darkness of the image represents the density of mRNA expression. For abbreviations see list.

Fig. 5. PI3K p55γ and p85α mRNA expression levels in various brain regions. Distribution and quantitative analysis of (**A**) p55γ and (**B**) p85α mRNA in tissue sections through the preoptic and basal hypothalamic regions obtained from oil and E_2-treated animals (n = 4). Values represent the mean density ± SEM of (**A**) p55γ or (**B**) p85α mRNA levels (nanocuries per gram of tissue) in film images from the different brain regions. (* $p < 0.05$, two-tailed student t-test). Note that PI3K p85α was highly expressed in the cortex (160.30 ± 15.46 nCi/g).

Fig. 6. Preoptic and basal hypothalamic tissue microdissections for RNA extraction and subsequent qRT-PCR analysis. Figures are modified from the rat atlas (Swanson) to represent coronal sections in the female guinea pig. Tissues were dissected from female guinea pig coronal brain blocks (1 mm thick) as indicated by the dashed outlines. The regions dissected included diagonal band of Broca (DB), medial preoptic area (mPOA), suprachiasmatic nucleus (SCN), paraventricular nucleus of the hypothalamus (PVN), arcuate nucleus (ARC), ventromedial nucleus-dorsomedial region (VMHdm),

ventromedial nucleus-ventrolateral region (VMHvl), lateral hypothalamus (LH), and dorsomedial nucleus (DMH).

Fig. 7. Primer efficiencies and quantitative real-time PCR (qRT-PCR) assays for p55γ and p85α mRNA transcripts. Top panel illustrates cDNA serial dilutions and the corresponding cycle number when fluorescent signal was detected for (**A**) p55γ and (**C**) p85α. Panels **B** and **D** depict representative dissociation curves, showing single-product melting at 78°C for (**B**) p55γ and 80.3°C for (**D**) p85α. Single peaks illustrate that only one product is formed and lack of any other peaks illustrates that the primers did not form primer dimers. Insets in **B** and **D** show slope of the standard curve using serial dilution data and the primer efficiencies translate to 100% for (**B**) p55γ and 93% for (**D**) p85α. These efficiencies allowed us to use the ΔΔCt method for quantification purposes.

Fig. 8. PI3K p55γ and p85α mRNA expression levels in various brain areas. Relative mRNA abundance of (**A**) p55γ and (**B**) p85α in guinea pig brain regions using qRT-PCR. Values represent the relative abundance of p55γ and p85α mRNA in microdissected brain regions (n = 6) and gross dissection of cortex (n = 1). For each subunit, relative abundance was quantified as the Ct $2^{-\Delta Ct}$, where ΔCt = Ct (subunit) – Ct (GAPDH) and normalized to value of Diagonal Band of Broca region .

Fig. 9. PI3K p55γ and p85α mRNA expression levels in various brain areas of oil and estrogen treated animals. Quantitative real-time PCR measurements of (**A**) p55γ and (**B**) p85α mRNA in microdissected brain nuclei of oil- and EB-treated guinea pigs. (**A**) Values represent the mean fold-change ± SEM of p55γ mRNA levels in different brain regions (n = 5-6). (* $p < 0.05$, ** $p < 0.01$, two-tailed student t-test). (**B**) Values represent

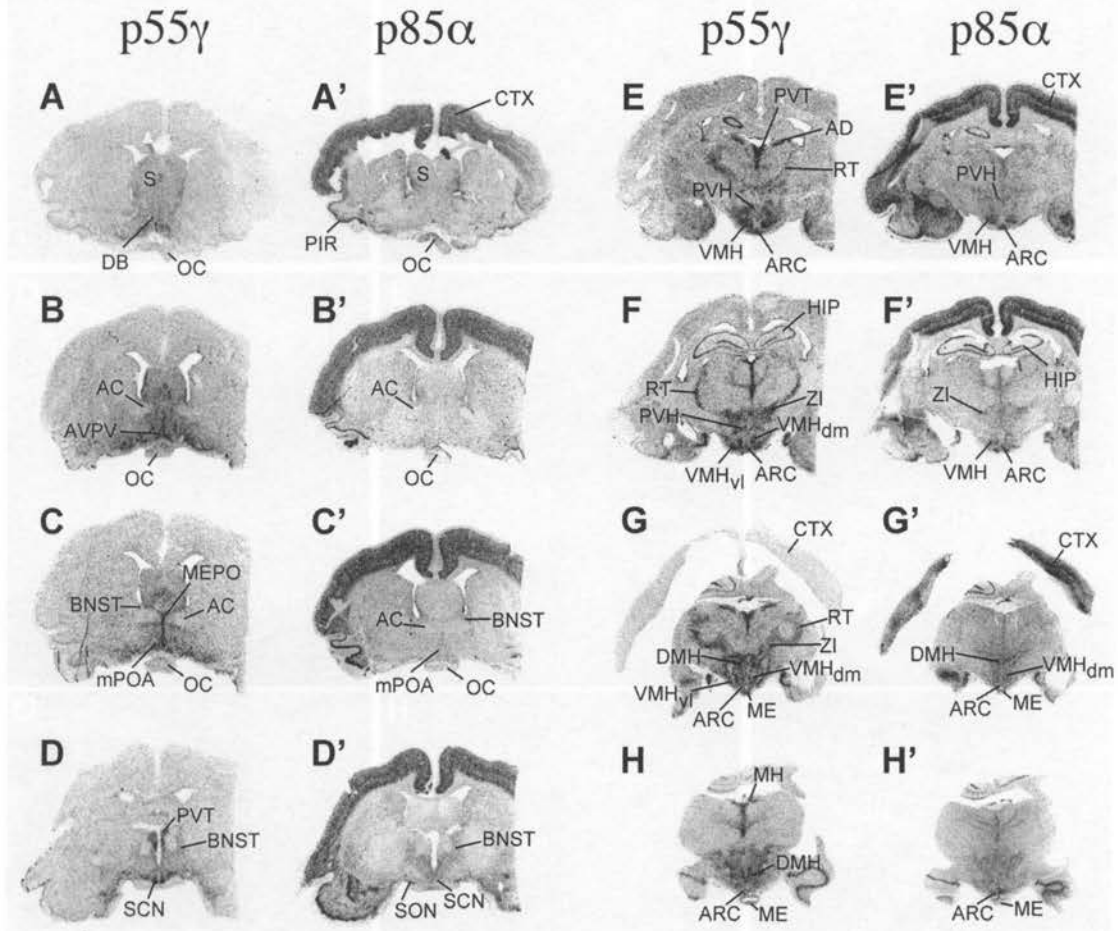
the mean fold-change ± SEM of p85α mRNA levels in different brain regions (n = 5-6). (* $p < 0.05$).

Fig. 10. Expression of p55γ and p85α mRNA in single arcuate nucleus neurons from intact male animals. Representative gels illustrating that single arcuate neurons express mRNA for p55γ, p85α, POMC and GAPDH (**A**). The expected band sizes are 255 bp for p55γ, 283 bp for p85α, 206 bp for POMC and 212 bp for GAPDH. Quantification of the number of neurons expressing p55γ (25%) or p85α (39%) mRNA transcripts did not show significant statistical difference (**B**).

Fig. 11. Functional assay using electrophysiology to assess the role of PI3K signaling to mediate rapid estrogen responses in arcuate POMC neurons. (**A**) Description of whole-cell electrophysiology drug application protocol. Whole-cell recordings were made from ~250 μm thick coronal hypothalamic slices according to established methods as described previously (Qiu et al., 2003). For the whole-cell recording experiments, an EC_{50} concentration of the $GABA_B$ agonist baclofen (5 μM) was used. After seals were formed and the whole-cell confirmation obtained, the slices were perfused with TTX (1 μM) for 5 min. The first $GABA_B$ response was generated by perfusing baclofen (5 μM) until a steady-state current was obtained (R1). After drug washout, when the current returned to pre-drug resting levels, the cells were perfused with E_2 (100 nM) alone or in combination with PI3K inhibitor wortmannin (100 nM) or LY294002 (10 μM), and a second response was measured to baclofen (R2). (**B**) Estrogen rapidly attenuates the $GABA_B$ response. Representative traces of the $GABA_B$ responses (**B, panel 1**) with no steroid or drug treatment (control), (**B, panel 2**) before and after E_2 treatment, (**B, panel 3**) before and after E_2 + wortmannin (100 nM), and (**B,**

panel 4) before and after E_2 + LY294002 (10 μM). (**C**) Estrogen attenuation of the $GABA_B$ response is abolished by PI3K inhibition. Bar graphs summarizing the effects of PI3K inhibitors wortmannin (100 nM) or LY294002 (10 μM) on the estrogen-mediated attenuation of the $GABA_B$ response. Bars represent the mean ± S.E.M. of 4-8 cells (***$p < 0.001$ for E_2 versus control; **$p < 0.01$ for E_2 versus E_2 + LY294002; *$p < 0.02$ for E_2 versus E_2 + Wortmannin).

Fig.1


p55γ
p85α
p55γ
p85α
A
A'
E
E'
S
CTX
PVT
AD
RT
PVH
DB
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PIR
OC
VMH
ARC
VMH
ARC
B
B'
F
F'
AC
AC
HIP
RT
ZI
PVH
AVPV
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OC
OC
VMHvl
ARC
ZI
HIP
VMH
ARC
C
C'
G
G'
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AC
AC
BNST
CTX
CTX
RT
DMH
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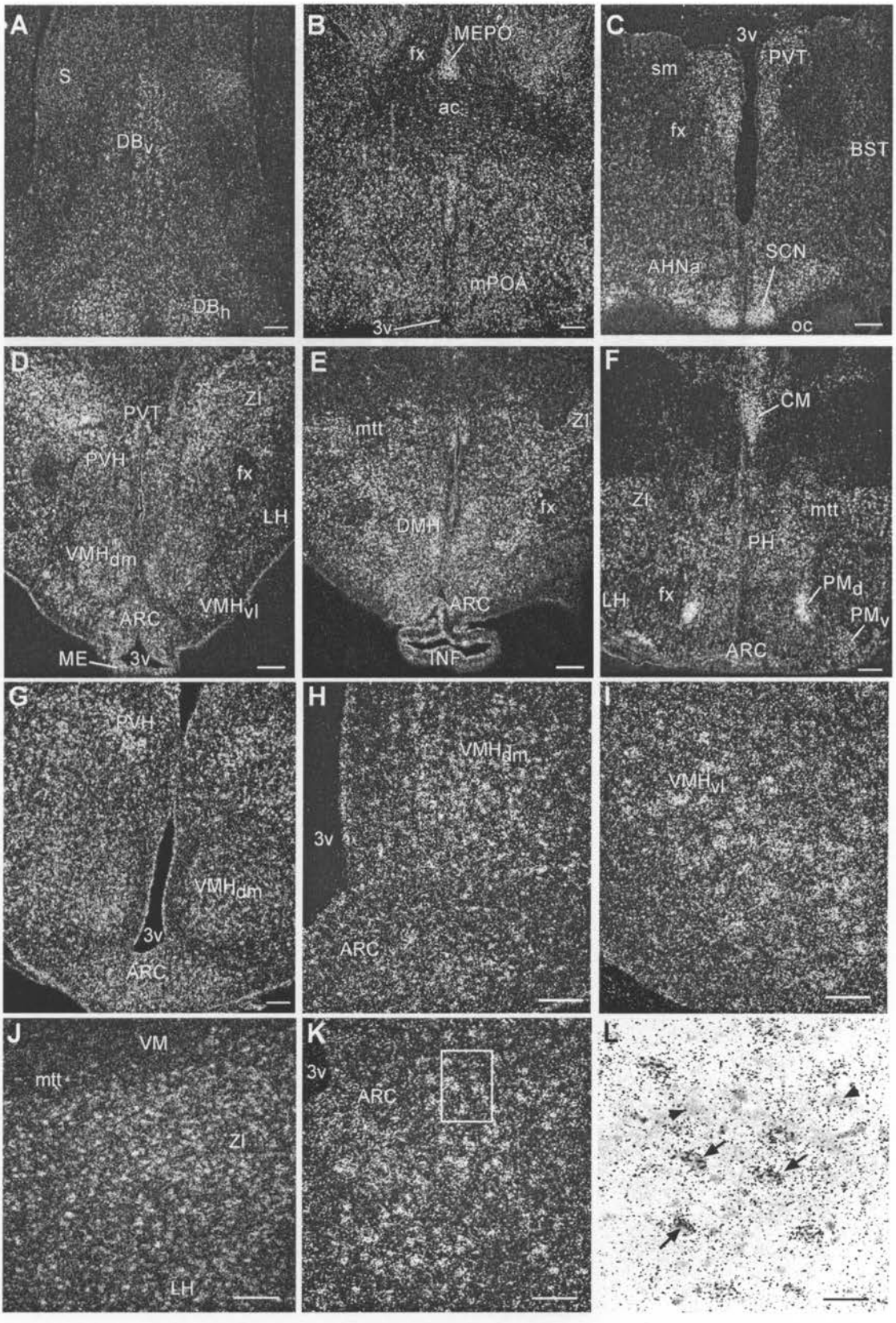
VMHdm
VMHvl
ARC
ME
DMH
VMHdm
ARC
ME
mPOA
OC
mPOA
OC
D
D'
H
H'
PVT
BNST
BNST
MH
DMH
SCN
SON
SCN
ARC
ME
ARC
ME

Fig.2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S
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DBh
B
MEPO
fx
ac
mPOA
3v
C
3v
PVT
sm
fx
BST
AHNa
SCN
oc
D
PVT
ZI
PVH
fx
LH
VMHdm
ARC
VMHvl
ME
3v
E
mtt
ZI
fx
DMH
ARC
INF
F
CM
ZI
mtt
PH
LH
fx
PMd
PMv
ARC
G
PVH
VMHdm
3v
ARC
H
VMHdm
3v
ARC
I
VMHvl
J
VM
mtt
ZI
LH
K
3v
ARC
L

Fig.3

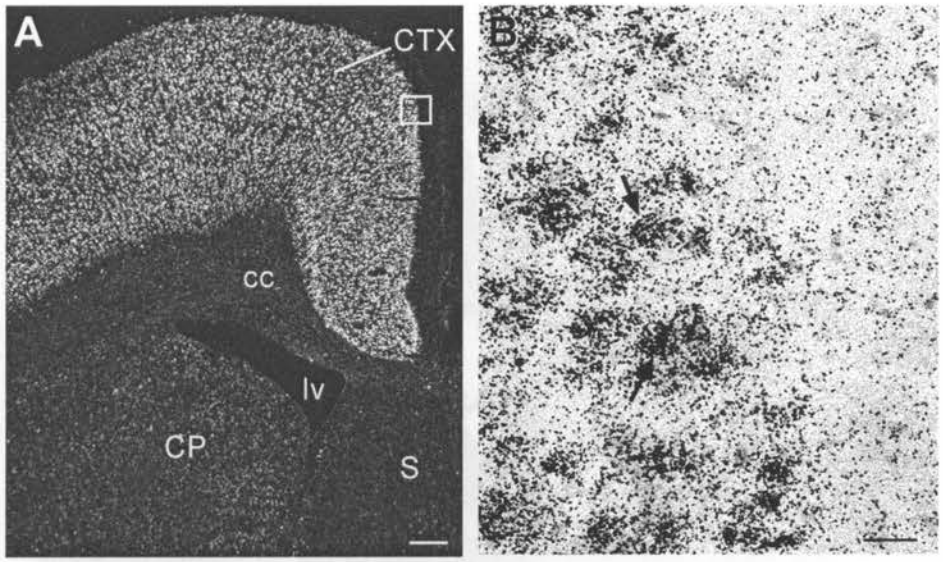
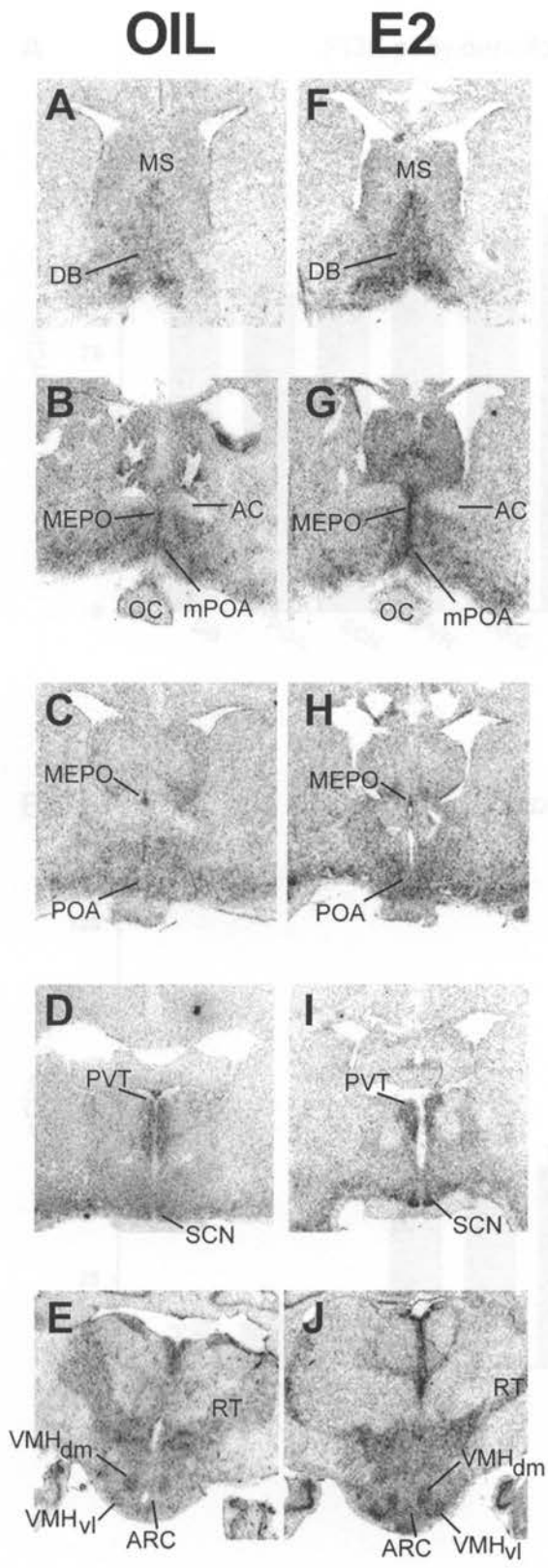


Fig.4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E2
A
MS
DB
F
MS
DB
B
MEPO
AC
OC
mPOA
G
MEPO
AC
OC
mPOA
C
MEPO
POA
H
MEPO
POA
D
PVT
SCN
I
PVT
SCN
E
RT
VMHdm
VMHvl
ARC
J
RT
VMHdm
ARC
VMHvl

Fig.5

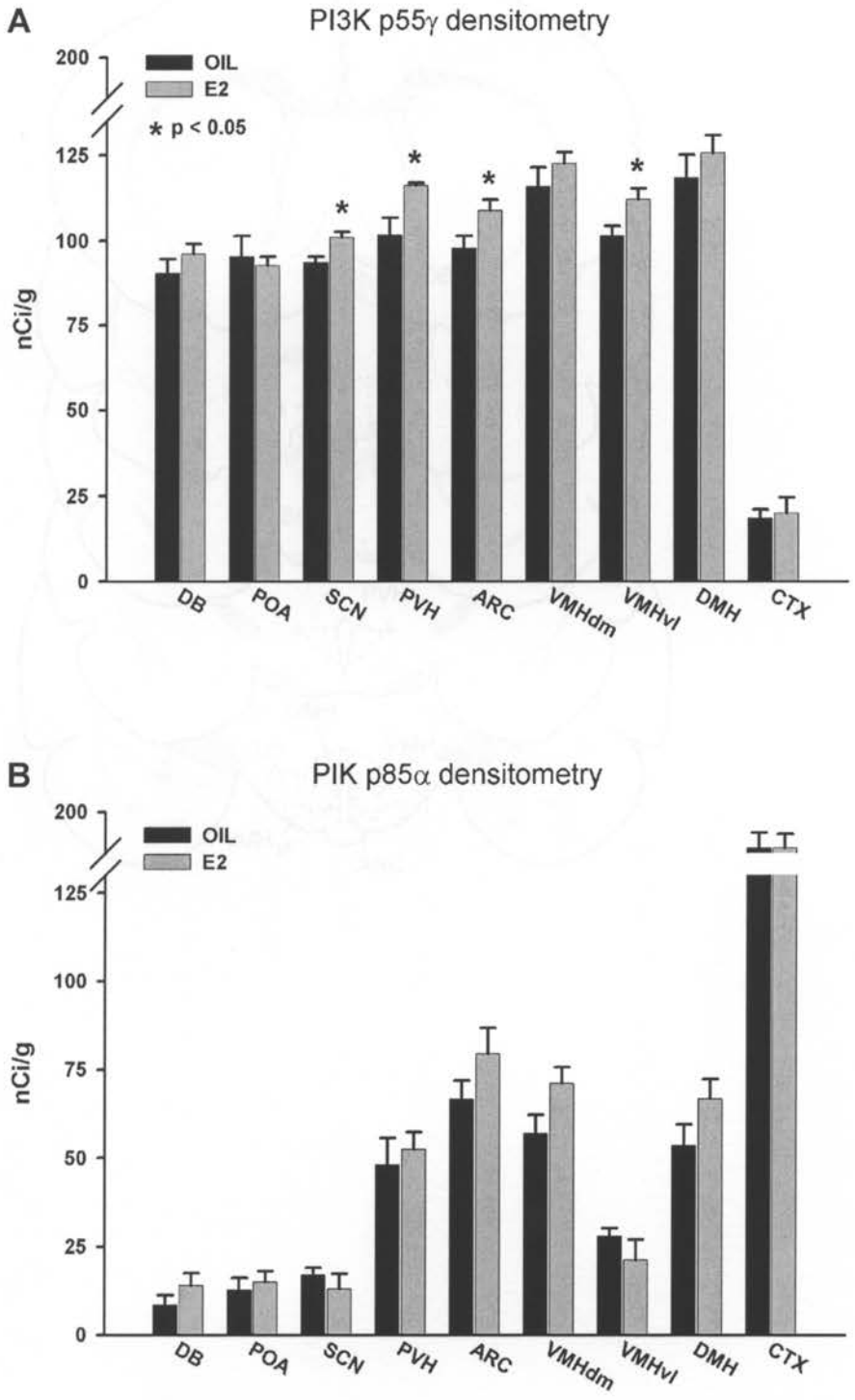

A
PI3K p55γ densitometry
OIL
E2
* p < 0.05
nCi/g
200
125
100
75
50
25
0
DB
POA
SCN
PVH
ARC
VMHdm
VMHvl
DMH
CTX
B
PIK p85α densitometry
OIL
E2
nCi/g
200
125
100
75
50
25
0
DB
POA
SCN
PVH
ARC
VMHdm
VMHvl
DMH
CTX

Fig.6

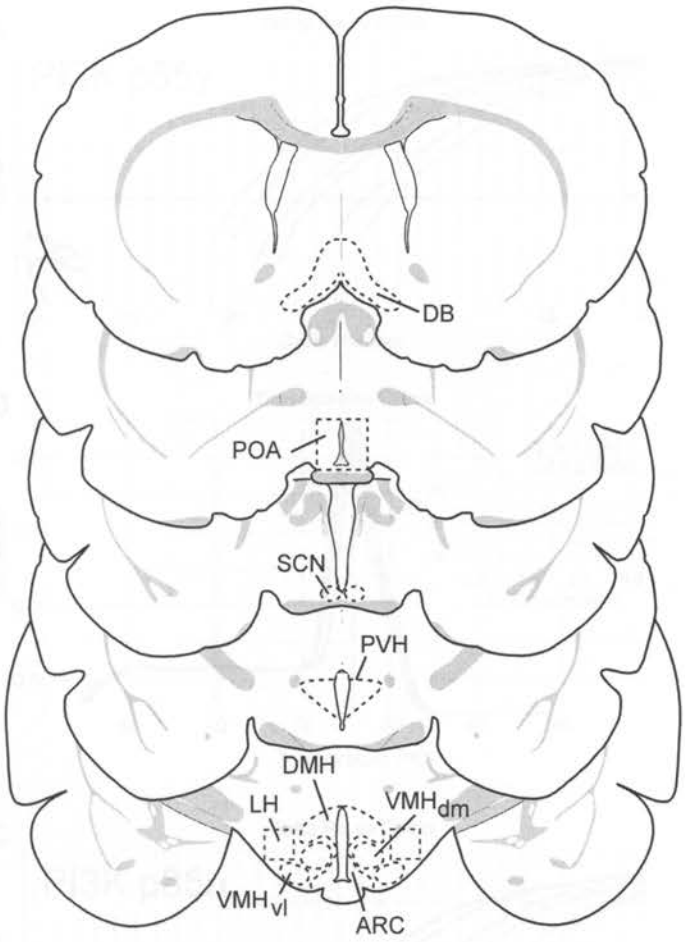
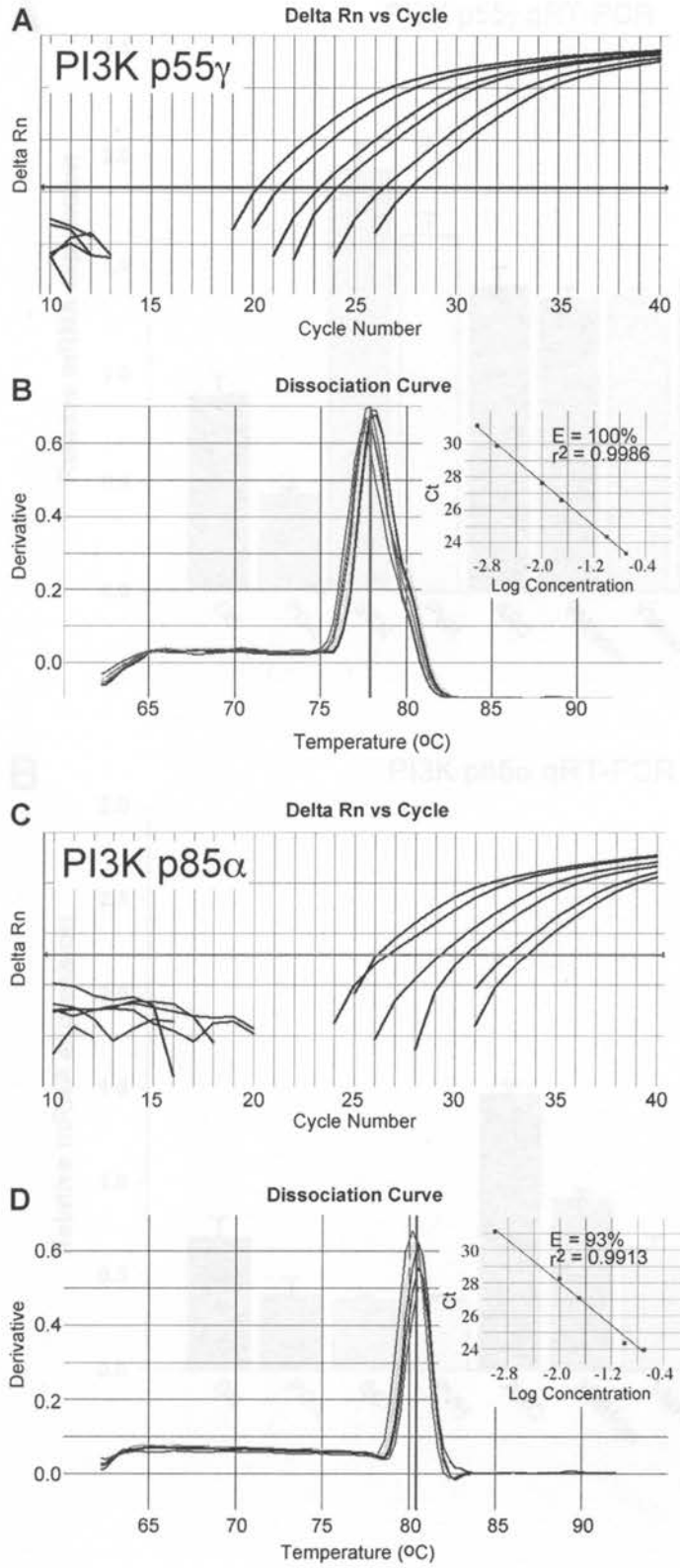


Fig.7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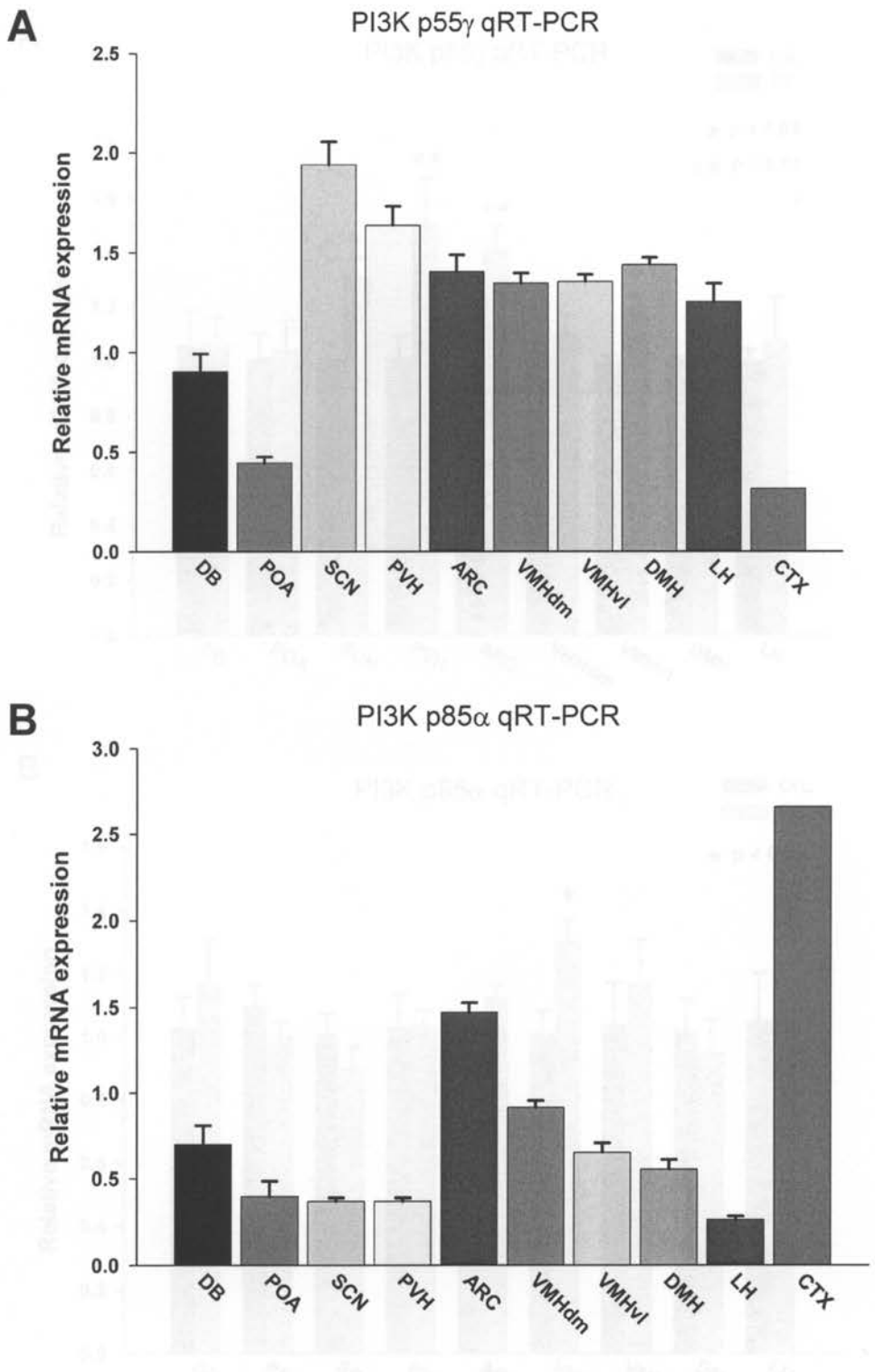
Delta Rn vs Cycle
PI3K p55γ
Delta Rn
10
15
20
25
30
35
40
Cycle Number
B
Dissociation Curve
Derivative
0.0
0.2
0.4
0.6
65
70
75
80
85
90
Temperature (°C)
Ct
30
28
26
24
E = 100%
r2 = 0.9986
-2.8
-2.0
-1.2
-0.4
Log Concentration
C
Delta Rn vs Cycle
PI3K p85α
Delta Rn
10
15
20
25
30
35
40
Cycle Number
D
Dissociation Curve
Derivative
0.0
0.2
0.4
0.6
65
70
75
80
85
90
Temperature (°C)
Ct
30
28
26
24
E = 93%
r2 = 0.9913
-2.8
-2.0
-1.2
-0.4
Log Concentration

Fig.8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PI3K p55γ qRT-PCR
Relative mRNA expression
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2.0
1.5
1.0
0.5
0.0
DB
POA
SCN
PVH
ARC
VMHdm
VMHvl
DMH
LH
CTX
B
PI3K p85α qRT-PCR
Relative mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DB
POA
SCN
PVH
ARC
VMHdm
VMHvl
DMH
LH
CTX

Fig.9

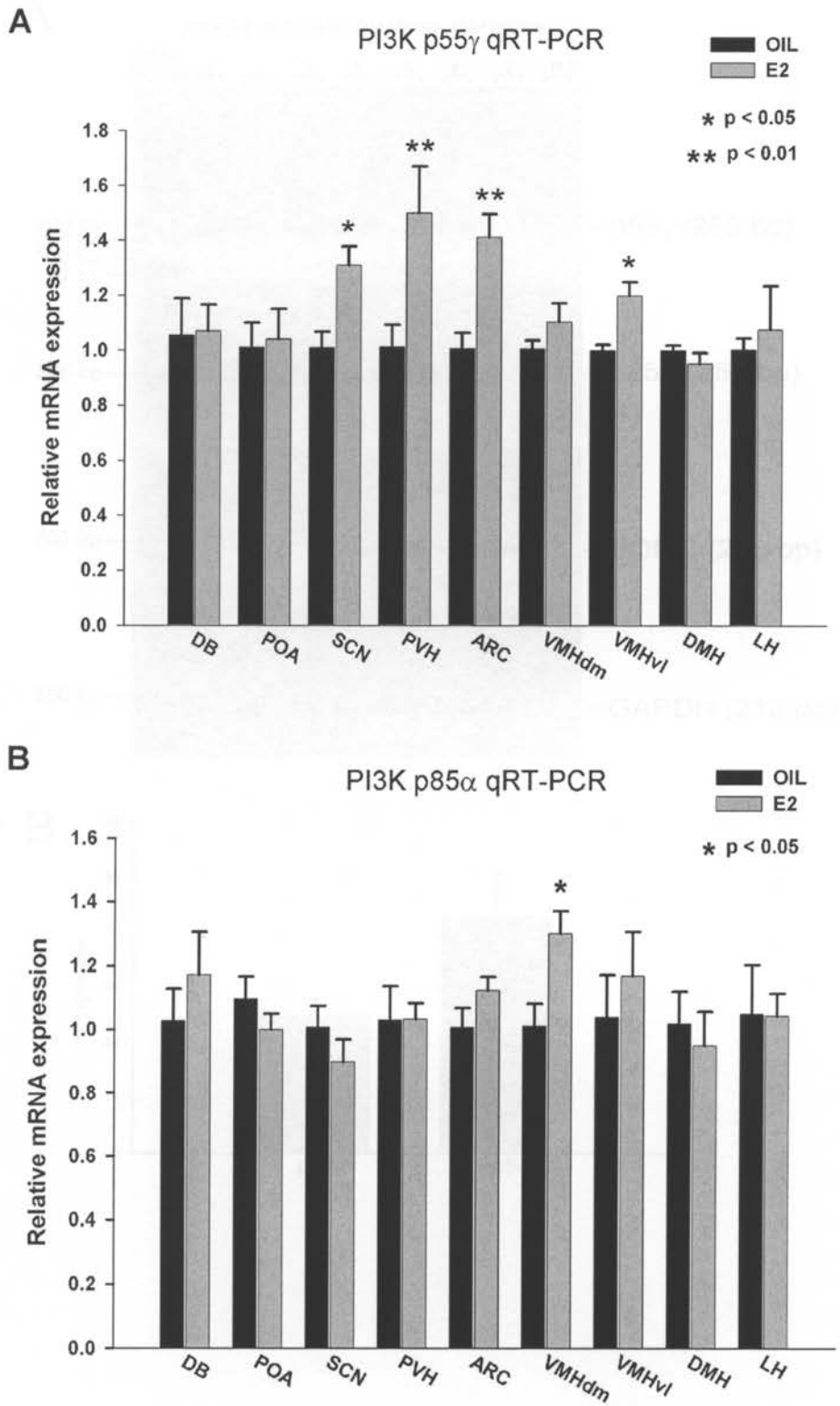

A
PI3K p55γ qRT-PCR
OIL
E2
* p < 0.05
** p < 0.01
Relative mRNA expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
**
*
DB
POA
SCN
PVH
ARC
VMHdm
VMHvl
DMH
LH
B
PI3K p85α qRT-PCR
OIL
E2
* p < 0.05
Relative mRNA expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
DB
POA
SCN
PVH
ARC
VMHdm
VMHvl
DMH
LH

Fig.10

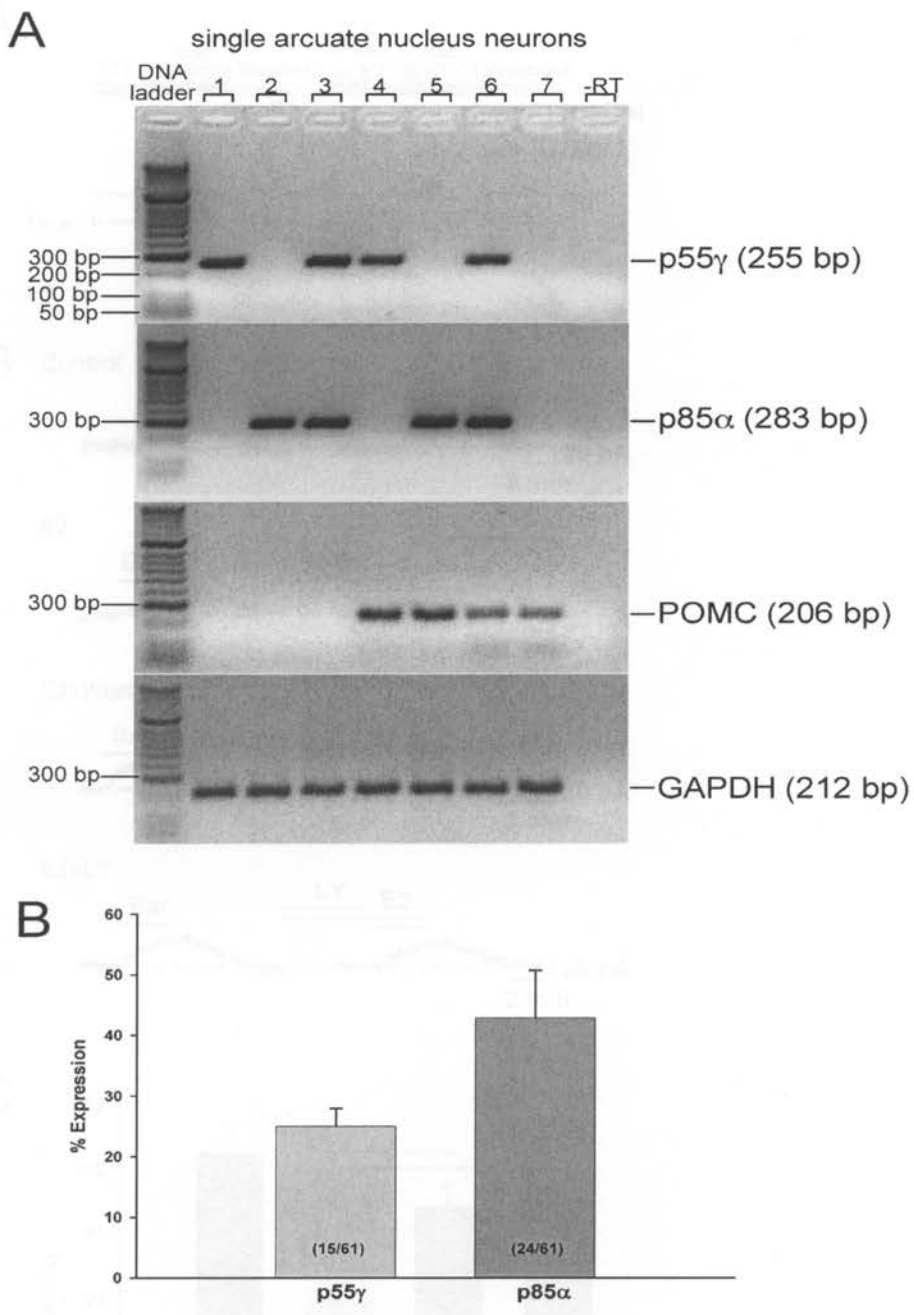
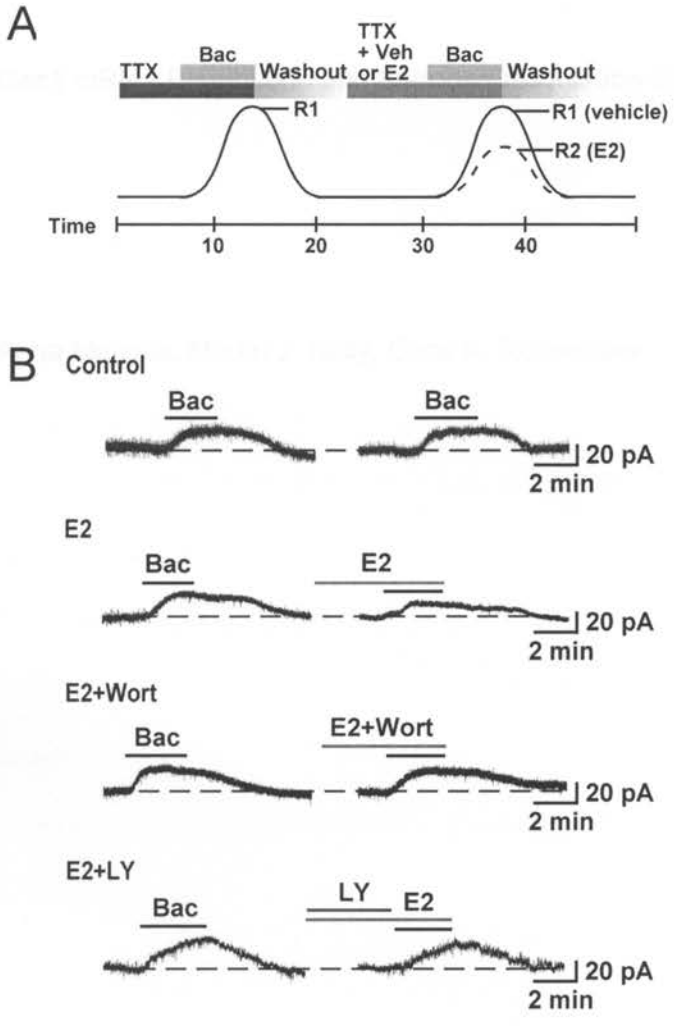

A
single arcuate nucleus neurons
DNA ladder
1
2
3
4
5
6
7
-RT
300 bp
200 bp
100 bp
50 bp
—p55γ (255 bp)
300 bp
—p85α (283 bp)
300 bp
—POMC (206 bp)
300 bp
—GAPDH (212 bp)
B
% Expression
60
50
40
30
20
10
0
(15/61)
(24/61)
p55γ
p85α

Fig.11


A
TTX
Bac
Washout
TTX
+ Veh
or E2
Bac
Washout
R1
R1 (vehicle)
R2 (E2)
Time
10
20
30
40
B
Control
Bac
Bac
20 pA
2 min
E2
Bac
E2
20 pA
2 min
E2+Wort
Bac
E2+Wort
20 pA
2 min
E2+LY
Bac
LY
E2
20 pA
2 min
C
R2/R1 (%)
120
100
80
60
40
20
0
***
*
**
(8)
(7)
(4)
(6)
CONTROL
E2
E2+WORT
E2+LY

Chapter 4

Gec1 mRNA Distribution and Estrogen Regulation in Guinea Pig Brain

Anna Malyala, Martin J. Kelly, Oline K. Ronnekleiv

Abstract

Gec1 is a $GABA_A$ receptor associated protein that interacts with $GABA_A$ receptors, opioid receptors and tubulin. GABA and opioid neurotransmission is a critical component of estrogen modulation to regulate homeostatic functions. In this study, we used in situ hybridization to localize gec1 mRNA in guinea pig brain and quantitative real-time reverse transcription PCR of microdissected hypothalamic nuclei of vehicle- and estrogen-treated animals to assess gec1 mRNA regulation by estrogen. The data revealed that gec1 mRNA is widely expressed in the brain, including hypothalamic regions. Within the hypothalamus, estrogen increased gec1 mRNA expression in arcuate, preoptic, and other nuclei after 24 h. These data suggest that inhibitory tone of estrogen during negative feedback may in part be due to receptor trafficking by regulation by gec1.

Introduction

GABA is an inhibitory amino acid that is widely distributed in the mammalian central nervous system, including the hypothalamus, and GABAergic neurotransmission plays a critical role in the control of reproduction. For example, GABA regulates the activity of GnRH neurons in the preoptic area (POA) and mediobasal hypothalamus (MBH) of the guinea pig and primate to control the pulsatile secretion of gonadotropins (Silverman, Krey, & Zimmerman, 1979; Goldsmith, Thind, Song, Kim, & Boggan, 1990; Goldsmith, Thind, & Boggan, 1990; Witkin, Ferin, Popilskis, & Silverman, 1991; King et al., 1998). Inhibitory GABA synapses are present on GnRH neurons and there is evidence that GABA directly inhibits GnRH neurons (Leranth, MacLusky, Sakamoto, Shanabrough, & Naftolin, 1985; Lagrange et al., 1995). While GABAergic neurotransmission regulates neural circuits that control gonadal steroid production, gonadal steroids in turn affect GABA transmission. Specifically, gonadal steroids have been shown to regulate $GABA_A$ and $GABA_B$ receptor subunit mRNA expression and receptor function (Fenelon, Sieghart, & Herbison, 1995; Brussaard et al., 1997; Smith et al., 1998; Wagner et al., 2001). In addition, hypothalamic GABA neurons undergo ultrastructural changes in response to estrogen that lead to remodeling of inhibitory synapses (Herbison, 1997; Garcia-Segura et al., 1994). Furthermore, estrogen regulates opioid receptor mRNA and opioid neurotranmission (Kelly, Lagrange, & Rønnekleiv, 1995; Acosta-Martinez & Etgen, 2002; Brown, Pasi, & Etgen, 1996; Cunningham, Fang, Selley, & Kelly, 1998; Joshi, Billiar, & Miller, 1993; Quiñones-Jenab, Jenab, Ogawa, Inturrisi, & Pfaff, 1997). These data demonstrate that GABA and opioid neurotransmission are key components of estrogen-influenced neuronal signaling.

Efficient neurotransmission relies on clustering of postsynaptic receptors. $GABA_A$ and other receptor targeting to the plasma membrane is facilitated by adaptor

proteins that bridge the receptors and the cytoskeleton. $GABA_A$ receptor associated protein-like 1 (GABARAP, also known as gec1) and GABARAP were recently identified as potential linkers between $GABA_A$ and opioid receptors and microtubules (Pfaff et al., 1979) (Wang, Bedford, Brandon, Moss, & Olsen, 1999) (Karp, Maissel, & Livneh, 2007) (Nelson, 2004). This was of considerable interest since previous microarray findings show that gec1 is regulated by estrogen in the hypothalamus (Malyala et al., 2004). Here, we investigated the distribution and regulation by estrogen of gec1 mRNA in the guinea pig brain.

Methods

Animals

Adult female (Topeka) guinea pigs were maintained under constant temperature and light (light on between 06:30 and 20:30 h local time) with food and water provided ad libitum. Animal care and use were approved by an institutional committee and were according to NIH and International guidelines. The animals were ovariectomized under ketamine/xylazine anesthesia (33/6 mg/kg subcutaneously) 5-6 days before experimentation and given a subcutaneous injection of either 17β-estradiol (E2) in the form of 17β-estradiol benzoate (here referred to as E2; 25 μg in 0.1 ml) or sesame oil vehicle alone (0.1 ml). Twenty-four hours later, the animals were killed by decapitation after sedation with ketamine and blood samples were collected. Brains were removed quickly and rinsed in chilled phosphate buffer solution.

Tissue preparation

For in situ hybridization, 2-3 mm coronal blocks through the POA and BH were fixed in 4% paraformaldehyde for 6hr, soaked in 20% buffered-sucrose solution (pH 7.4),

embedded in O.C.T. (Sakura Finetek, Torrance, CA), and frozen in isopentane at -55°C. Coronal sections (20 mm) were cut on a cryostat and thaw-mounted onto Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). The sections were stored at -80°C.

For quantitative real-time PCR analysis, a brain slicer (EM Corporation, Chestnut Hill, MA) was used to produce 1 mm frontal slices. The 1 mm slices were placed in RNAlater and the various hypothalamic regions were microdissected under a light microscope. The slices included the preoptic area, arcuate nucleus, paraventricular nucleus, ventromedial nucleus, and lateral hypothalamus. Microdissected tissues were rapidly frozen and stored at -80°C. Total RNA was extracted using RNaqueous-Micro Kit (Ambion) according to manufacturer's directions, and the concentration was determined by using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA samples were stored at -80°C until further use.

Cloning

A 241 bp fragment of the guinea pig gec1 was cloned using reverse transcription (RT) and PCR. Oligonucleotide primers were designed based on the cDNA clone sequence for gec1 using Clone Manager 5 software. The 5' primer (5'-AACACCTCTTCCGAGAGA -3') was 100% homologous to the guinea pig (gi|16950547 [GenBank]: 58-76 bp) gec1 sequence. The 3' primer (5'-GCACCGGATGTCACTACT -3') was 100% homologous to the guinea pig (gi|16950547 [GenBank]: 280-298 bp) gec1 sequence. Primer synthesis by Promega included at the 3'-end of both primers a 3'-A ligated to linearized vector DNA that has been cleaved at an EcoR V site and had a single 3'-terminal thymidine added to both ends used with the pGEM-T Easy Vector System PCR cloning kit (Promega).

The gec1 cDNA was amplified from 200 ng of total RNA extracted from the guinea pig hypothalamus using RT-PCR (GeneAmp kit; PerkinElmer, Foster City, CA). An Oligo-dT

primer was used for the cDNA first-strand synthesis. Reverse transcription was performed for 15 min at 42°C. PCR was conducted for 45 cycles of denaturation (94°C; 45 s), annealing (56°C; 45 s), and extension (72°C; 1 min 10 s), with a 7 min final extension. The resulting 241bp product was gel-purified, subcloned into the pAMP10 vector by using the CloneAmp System, and sequenced.

In situ hybridization

Radioactive antisense cRNA probe was transcribed in vitro with SP6 RNA polymerase from guinea pig gec1 constructs, and the sense probe was prepared by using T7 polymerase in the presence of [^{35}S]-uridine 5'(α-thio) triphosphate (^{35}S-UTP). Residual DNA was digested with 10 U DNase I (Roche Diagnostics, Indianapolis, IN). The antisense and sense RNA probes were separated on G-50 Sephadex column (Amersham Biosciences, Piscataway, NJ). Slides from oil and estrogen treated females were always reacted together. These slides were post fixed in fresh 4% paraformaldehyde in Sorensen's phosphate buffer (0.03 M, pH 7.4) for 15 min, rinsed in Sorensen's buffer, and treated with proteinase K (1.0 mg/ml) for 3-6 min at room temperature. Sections were then treated with 0.1 M triethanolamine (3-5 min), followed by 0.25% acetic anhydride in 0.1 M triethanolamine (10 min), and rinsed briefly in 2X standard saline citrate (SSC). Sections were prehybridized for 1 hour at 58°C with hybridization buffer (50% formamide, 1X Denhardt's solution, 10% dextran sulfate, 100 mM dithiothreitol (DTT), 200mM sodium chloride, 10 mM Tris-HCL, pH 8.0, 1 mM EDTA, pH 8.0, 125 mg/ml tRNA; Sigma) and then quickly rinsed in 2X SSC buffer. The 35S-labeled antisense and sense riboprobes were heat-denatured, diluted with hybridization buffer, and used at a final concentration of 1.2 X 104 cpm/ml. Subsequently, the sections were covered with glass coverslips, sealed, and hybridized in a moist chamber for at least 20 hours at 59°C. After hybridization, the slides were rinsed in 2X SSC buffer,

reacted with RNAse (20 µg/ml) for 45-60 minutes at room temperature, and washed in decreasing concentrations of SSC (2X, 1X, 0.5X, 0.1X) at 55°C with a final wash for 1 hour in 0.1 X SSC at 65°C. The slides from oil- and estrogen-treated females were dehydrated in ethanol, placed side by side on a flat surface together with autoradiographic 14C microscales and were exposed to Hyperfilm-βmax (Amersham Biosciences, Piscataway, NJ) for 5 days at 4°C. The slides were then dipped in Kodak NTB-2 nuclear track emulsion and exposed for 21 days at 4°C. Thereafter, slides were developed in D19 developer, fixed in Kodak fixer, counterstained with hematoxylin, dehydrated and coverslipped. Quantification of film images was performed using a Macintosh G4 computer equipped with NIH Image 1.61 program. Film images of 4-5 sections for each brain region from matched oil-control and E_2-treated females were analyzed, and an average density value was obtained from each brain region, which was used for further analysis. The ^{14}C microscale was used as a standard to calculate the density of mRNA signals, and the optical density was converted to nanocuries (nCi) per gram. Comparisons between the two groups were performed using a two-tailed Student's t-test. Differences were considered statistically significant if the probability of error was less than 5%.

Images of emulsion coated slides of gec1 were analyzed using a Nikon E800 microscope. On some slides, immunohistochemistry was performed using an antibody to β-end after *in situ* hybridization. Darkfield images were captured using Nikon digital camera and illustrated using Adobe Photoshop (Mountain View, CA) and Macromedia FreeHand (San Francisco, CA) software programs. Contrast and brightness were adjusted in scanned images to match the original image seen in the microscope.

Quantitative Real-time PCR

Total RNA was subjected to DNase treatment (DNAfree; Ambion, Austin, TX) according to manufacturer's directions. Two hundred ng of RNA was reverse-transcribed into cDNA, and qRT-PCR using SYBR green chemistry (Applied Biosystems) was performed on the 7500 ABI real-time PCR system (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference for normalization after we determined that GAPDH does not change by E2 treatment in the brain regions we analyzed. The expression value of each gene was normalized to the amount of GAPDH in that sample to calculate a relative amount of transcript present for each gene. The normalized expression values for all control and treated samples were averaged, and an average fold change was determined using the $\Delta\Delta$Ct method (Benomar et al., 2005). A Student's t test was conducted between the normalized relative expression values for each individual control and treated samples to determine statistical relevance.

Single-cell RT-PCR

Cell harvesting was performed as previously described (Qiu et al., 2003). Briefly, two - three 300 µm arcuate slices were cut on a vibratome and placed in an auxiliary chamber containing oxygenated aCSF. The slices were allowed to recover for 1-2 hrs in the oxygenated chamber before dispersion. A discrete region of the mediobasal hypothalamus was microdissected and incubated in 2-3 ml Hank's balanced salt solution (HBSS) (in DEPC-treated water, pH 7.3, 300mOsm) containing 1 mg/ml protease for ~ 15 min at 37°C. The tissue was washed four times in one volume low calcium aCSF and two times in HBSS. The cells were isolated by trituration with a flame-polished pasteur pipette. The cells were dispersed onto a 35 mm petri dish, visualized under a Leitz inverted microscope, then patched and harvested into the patch pipette by applying negative pressure. The contents of the pipette were expelled into a siliconized

microcentrifuge tube containing 5 μl of 1X RT buffer (Promega) with RNasin and DTT. The tubes were immediately frozen at -80°C until the reverse transcription procedure. Specific primers for guinea pig gec1, POMC, GAD and GAPDH were designed using Clone Manager software. Each primer pair was tested to determine the optimal conditions (i.e., magnesium concentration, annealing temperature and the amount of cDNA template) for single-cell PCR. Harvested cells were denatured and single-stranded cDNA was synthesized from cellular mRNA. Forty to fifty cycles of amplification were performed using a MJ Research PTC-100 thermocycler in 0.5 ml thin walled PCR tubes according the following protocol: 94°C for 2 min, 40-50 cycles of 94°C for 45 sec; 58-60°C for 45 sec; 72°C for 1 min, with a final 72°C extension for 5 min. PCR products were visualized with ethidium bromide on a 2% agarose gel. Controls for RT-PCR included harvested aCSF from the surrounding tissue slice, water blank, and extracted RNA and single cells that were reacted without reverse transcriptase.

Results

Our aim was to determine hypothalamic gec1 mRNA distribution and regulation since gec1 protein may be pivotal in modulating GABA and opioid neurotransmission, both of which are also affected by estrogen. We used PCR to clone a guinea pig-specific cDNA sequence that was 241 bp in length. The PCR product is 100% homologous to the published guinea pig sequence.

Gec1 distribution using in situ hybridization

Distribution of gec1 using in situ hybridization has not been previously characterized. We used in situ hybridization with a [^{35}S]-labeled probe against gec1 to characterize the mRNA distribution in the guinea pig brain. Data from in situ film autoradiography revealed widespread expression of gec1 in the hypothalamus and all

other brain regions examined (Figure 1 a-e). Particularly high expression was noted in the diagonal band of Broca (DB), preoptic area (POA), arcuate nucleus (ARC), ventromedial nucleus (VMH), hippocampus and some thalamic nuclei. Moderate expression was seen in the cortex and most other brain regions. No specific labeling was detected with the sense probe (data not shown). These data are consistent with previously published data that show extensive gec1 mRNA expression using northern blots and qRT-PCR of various dissected brain regions of the rat.

We were mainly interested in the hypothalamic expression of gec1 and therefore used densitometry measurements of key hypothalamic nuclei to determine if estrogen altered gec1 mRNA expression in these regions. Densitometry data revealed significantly increased expression in the ARC but not in VMH or DMH (Figure 2, $p<0.05$; n=4-6).

Gec1 mRNA regulation by estrogen in microdissected hypothalamic nuclei using qRT-PCR

To verify changes observed with microarray and *in situ* hybridization densitometry analyses, we measured gec1 mRNA from microdissected regions of 24 h estrogen- and vehicle-treated ovariectomized guinea pigs using qRT-PCR. Gec1 mRNA was significantly increased in the POA and ARC (Figure 3, $p<0.05$; n=5-6). These results complemented the *in situ* hybridization data.

Localization of gec1 mRNA in POMC neurons of the arcuate nucleus by in situ hybridization coupled with immunocytochemistry, and single-cell RT-PCR

To determine whether gec1 mRNA was expressed in POMC neurons, we used in situ hybridization coupled with immunocytochemistry and found gec1 colocalization to β-endorphin stained neurons (Figure 4). Furthermore, gec1 colocalization with POMC

neurons was also seen using single-cell PCR (Figure 5). Our data revealed that gec1 was present in neurons other than POMC as well. These data suggest that gec1 may play role in GABA and/or opioid receptor trafficking in POMC neurons.

Discussion

The current study investigated the distribution and regulation of gec1 mRNA in the guinea pig hypothalamus. Consistent with previous studies, our results show that gec1 mRNA is extensively expressed in the brain (Nemos et al., 2003; Cvetkovic et al., 2003). What is novel here is the gec1 regulation by estrogen and the localization of gec1 specifically to POMC neurons.

The gec1 cDNA clone was first isolated as an early estrogen-upregulated molecule from the cDNA library of the cultured guinea-pig endometrial glandular epithelial cells (Benomar et al., 2006). They show a two-fold expression increase of gec1 in these cells after 17β-estradiol treatment for 2 h in the presence of cycloheximide, which prolongs and superinduces early genes. The gec1 gene was subsequently identified by screening a guinea-pig genomic library (Vernier-Magnin et al., 2001). Soon thereafter, the human gec1 cDNA was cloned from placenta and was named $GABA_A$ receptor-associated protein-like 1 (GABARAPL1) due to its high homology with the cDNA encoding $GABA_A$ receptor associated protein (GABARAP) (79% identity in nucleotide sequence) (Pilowsky et al., 2002). Importantly, a functional estrogen response element (ERE) was recently identified in the first exon of the guinea-pig gec1 gene (Matsuki et al., 2001).

The data presented here agrees with previous reports and gec1 mRNA is widely distributed in tissues, as shown by northern blot analysis with the brain showing one of the highest levels of expression (Pilowsky et al., 2002) (Vernier-Magnin et al., 2001). Interestingly, the level of gec1 mRNA was further shown to be higher in human central

nervous system than that of GABARAP mRNA (Nemos et al., 2003). Interestingly, Mansuy-Schlick et al recently used quantitative real-time PCR to study the expression of the various GABARAP-like proteins in subregions of the rat brain and show gec1 is differentially expressed throughout the rat brain with the highest mRNA levels in the pons and the diencephalon (Cvetkovic et al., 2003). Our in situ data support the high level of gec1 mRNA expression in the diencephalon which includes the hypothalamus and thalamus.

The gec1 protein is a small protein of 117 amino acids and the identical homology of amino acid sequences across species indicates it is evolutionarily conserved Liu- (Maswood, Cosmi, Alfinito, Leventhal, & Deecher, 2006). Gec1 belongs to a family of microtubule-associated proteins, which include the mammalian proteins GABARAP and Golgi-associated ATPase enhancer of 16 kDa (GATE-16), the latter is the mammalian homologue of a yeast ubiquitin-like protein involved in autophagy. The amino acid sequence of gec1 is ~87% similar to GABARAP and ~61% similar to GATE-16. GABARAP interacts with the $\gamma 2$ subunit of the GABAA receptor, which promotes clustering of the receptor and alters its channel kinetics (Wang et al 1999 and Chen et al 2000). More importantly, it has been specifically shown that GABARAP enhances $GABA_A$ receptors trafficking to the plasma membrane in neurons (Zhang et al., 2005). Recently, Mansuy et al. reported that gec1 interacts with tubulin and promotes its assembly in vitro and also show gec1 interaction with the $\gamma 2$ subunit of $GABA_A$ receptor suggesting that gec1 might function in a manner similar to GABARAP (Karp et al., 2007). The Liu-Chen group investigated the distribution and ultrastructural localization of gec1 in the hypothalamus by electron microscopy (Maswood et al., 2006). They show significant gec1 immunoreactivity in hypothalamic nuclei including the supraoptic, paraventricular and arcuate nuclei and the median eminence. While both gec1 and

GABARAP may play similar roles, the present data suggest that gec1 may be important for estrogen-regulated neuronal activity. Figure 4 depicts a model in which gec1 interaction with $GABA_A$ may mediate estrogen effects in hypothalamic neurons. Overall, these data indicate a role for gec1 in hypothalamic control of GABA and opioid neurotransmission and suggest that gec1 may be a mediator of estrogen-regulated inhibitory tone.

Chapter 4

Figure 4-1

Film images of gec1 mRNA distribution. Bright-field view of film autoradiograms illustrating gec1 mRNA distribution in guinea pig forebrain.

Figure 4-2

Gec1 mRNA Expression. Darkfield photomicrograph of coronal section through guinea pig brain illustrating the mRNA expression of gec1 in the arcuate nucleus (ARC) and median eminence (ME) (A). Photomicrographs of same tissue section on which in situ hybridization was performed for gec1 mRNA followed by POMC immunohistochemistry. Gec1 mRNA grains shown in white (B), POMC neurons shown in red (C), composite image of Gec1 mRNA

Figure 4-3

Gec1 mRNA colocalization. More examples of gec1-POMC colocalization.

Figure 4-4

Primer efficiency for gec1. Standard curve for gec1 showing primer efficiency and dissociation curve showing single-product melting at 81.2°C.

Figure 4-5

Quantitative real-time PCR. Quantitative real-time PCR measurements of gec1 mRNA in microdissected brain nuclei. Values represent the mean fold-change ± SEM of gec1 mRNA levels in different brain regions (n=5-6). (* $p<0.01$, two-tailed student t-test).

Figure 4-6

Cellular model of gec1. Model illustrating the effects of estrogen in hypothalamic neurons. Estrogen binds to membrane-estrogen receptor to activate phospholipase C to produce IP3 and DAG. PKC activation by DAG stimulates adenylyl cyclase, which then activates PKA. PKA activation inhibits receptor coupling of $GABA_B$ and μ-opioid receptors to their effector systems, e.g., GIRK. Furthermore, PKC can also activate PI3K signaling that leads to cytoskeletal remodeling and gene transcription. Estrogen-mediated increase of GEC1, a $GABA_A$ receptor associated protein that interacts with

$GABA_A$ receptors and tubulin, may lead to increased cell surface expression of $GABA_A$ receptors and facilitate hyperpolarization of the neuron. Furthermore, changes in PI3K subunit expression and subsequent Akt activation could also lead to increased $GABA_A$ receptor trafficking to the membrane.expression and POMC immunohistochemistry (D). Example of a high power view of gec1-POMC colocalization (E). Abbreviation: 3V, third ventricle. Bar, 100mm (A) and 25mm (B, C, and D).

Figure 4-1

Gec1 *in situ* hybridization images from diagonal band of Broca region to arcuate nucleus region

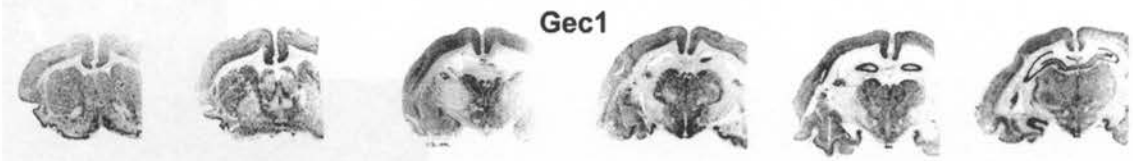


Figure 4-2

A Gec *in situ* hybridization darkfield image in the arecuate nucleus and median eminence B, C, D, E combined with immunohistochemistry of POMC cells

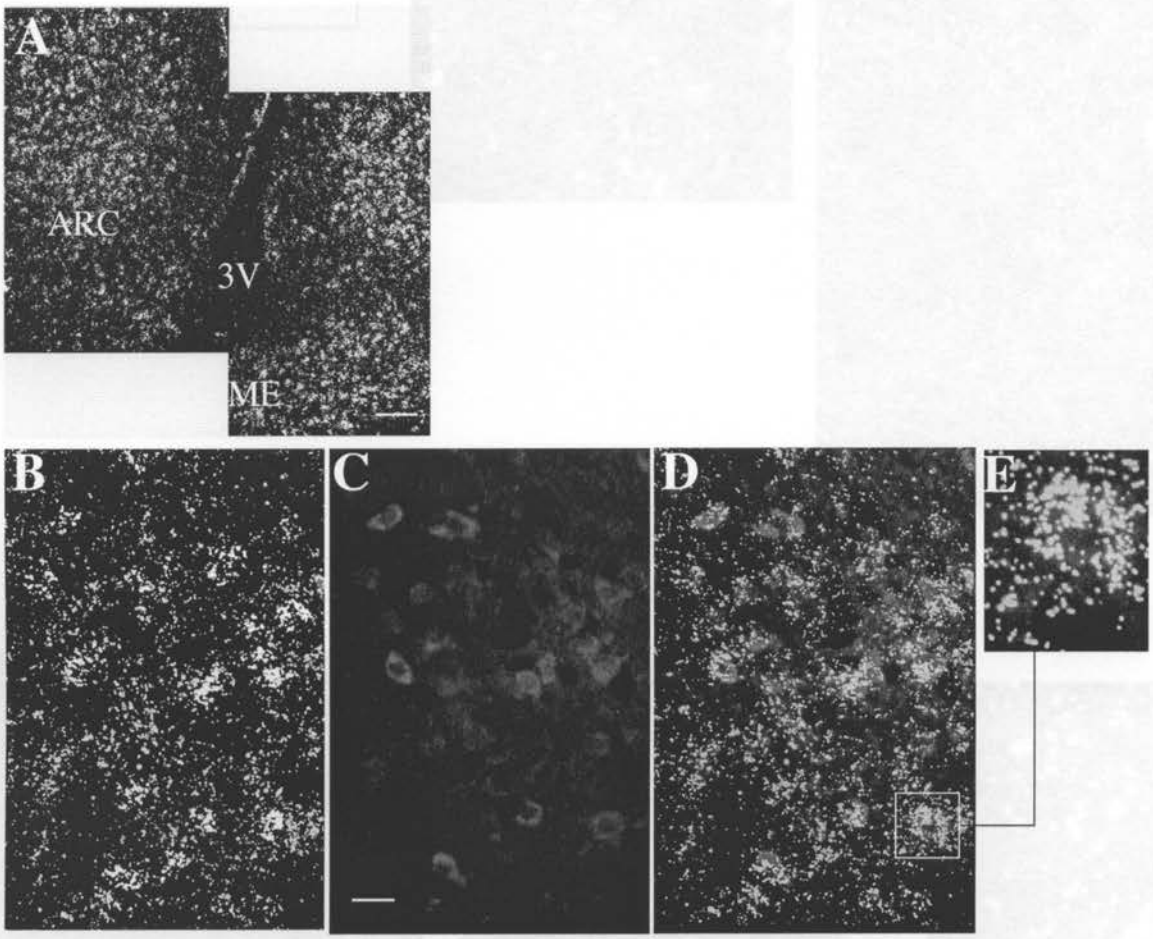
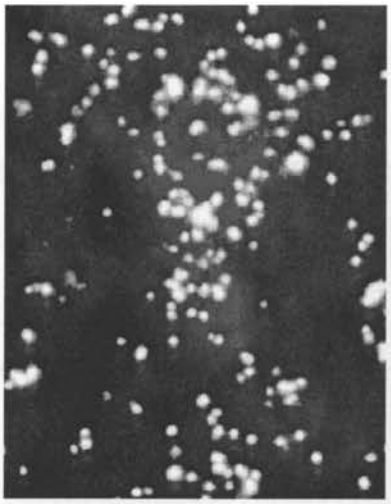
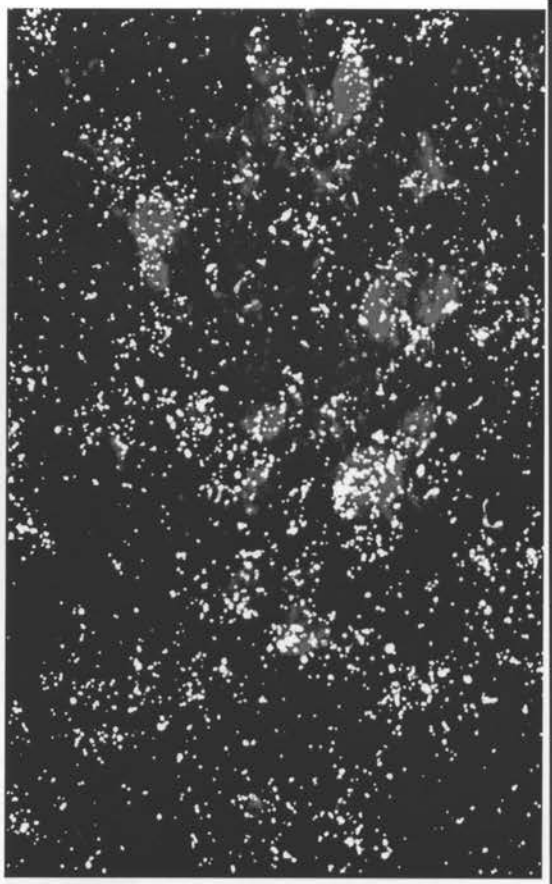
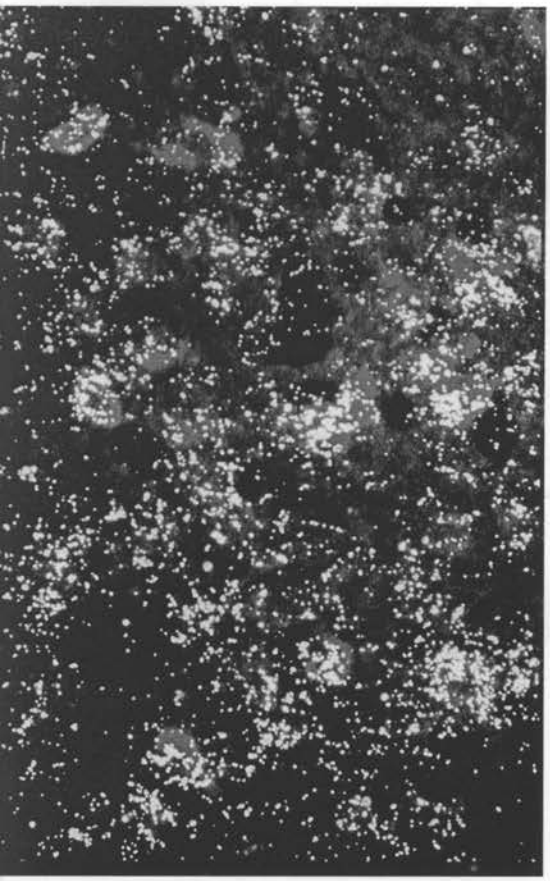


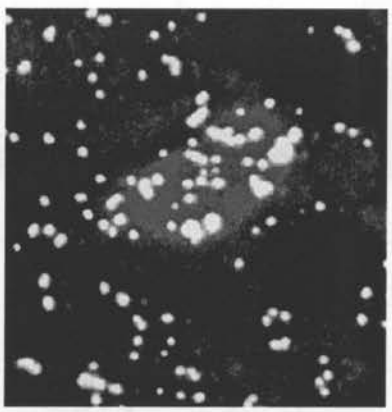
Figure 4-3

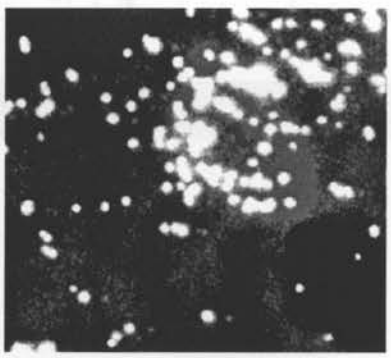
Examples of colocalization











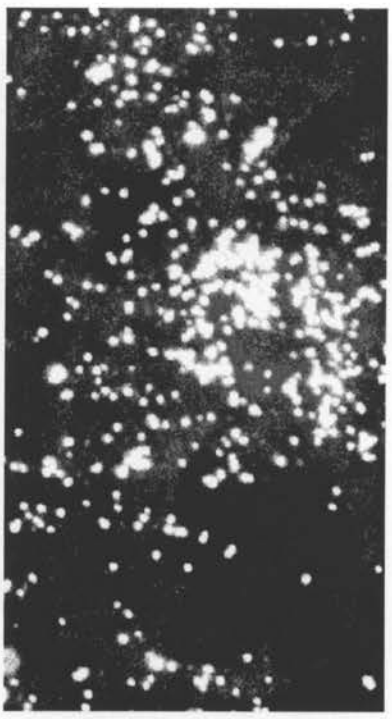


Figure 4-4 Gec1 standard curves and primer efficiency

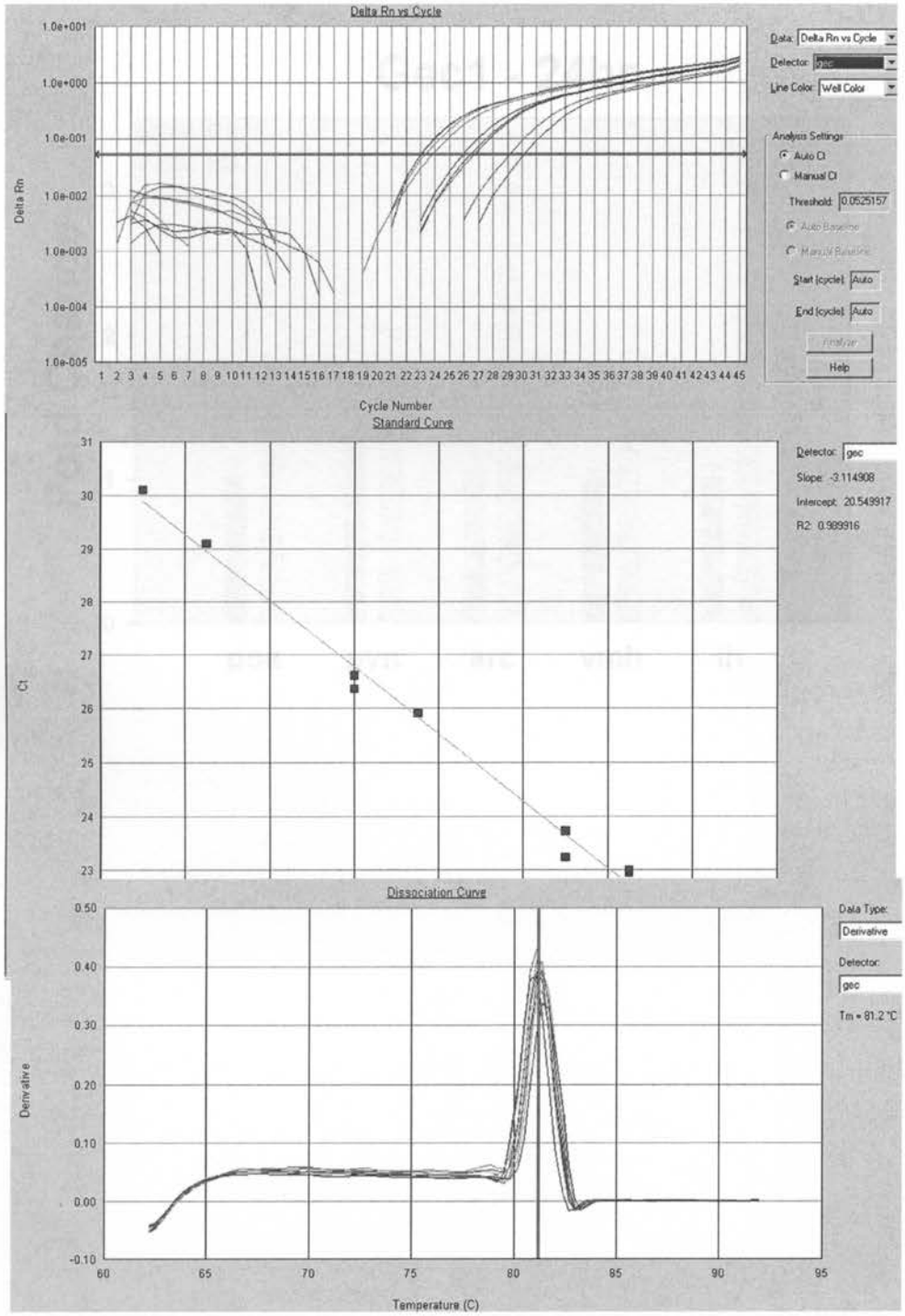


Figure 4-5 Gec qRT-PCR data

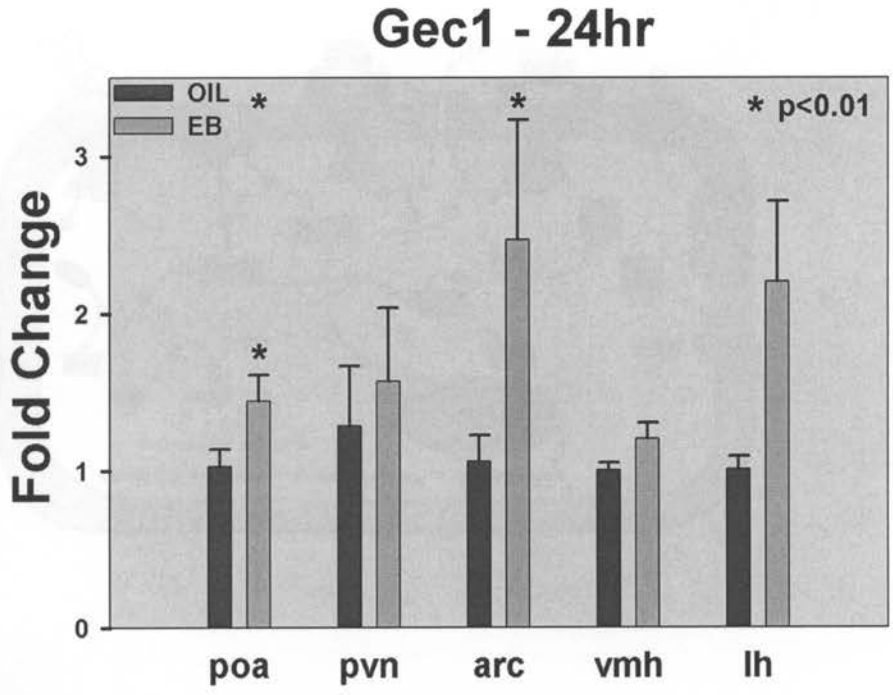
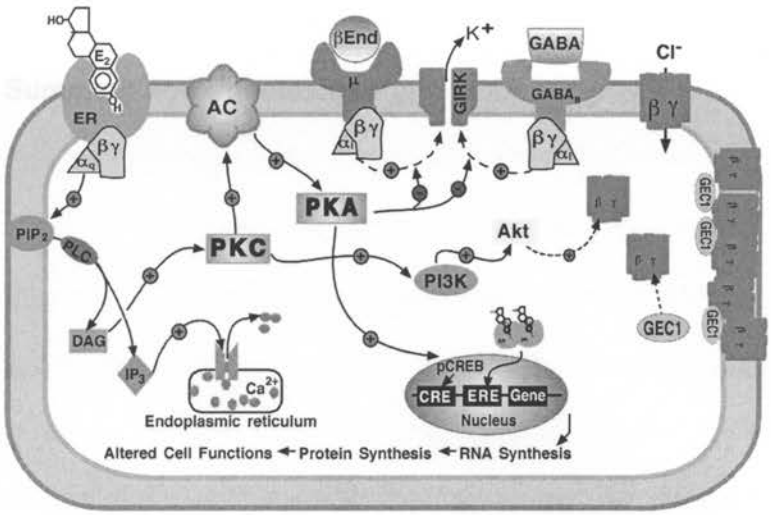


Figure 4-6 Gec cell model



Chapter 5

Summary and Conclusions

Summary and Conclusions

In summary, using a combination of techniques to examine the molecular mechanisms leading to physiological changes induced by estrogen, we find that both rapid effects and transcriptional changes alter excitability of hypothalamic neurons. Taking advantage of an animal model we developed to investigate estrogen's feedback actions on secretion of gonadotropin-releasing hormone and the advances in high-throughput technology, we used suppressive subtractive hybridization combined with microarray analysis to study the transcriptional changes induced by estrogen and observed mRNA expression changes in transcripts encoding proteins critical for neurotransmitter release and receptor dynamics (Malyala, Bosch, Kelly, & Rønnekleiv, 2004). Please refer to Figure 1 of this chaper for cell model of regulated genes.

The data here suggest that PI3K p55γ and gec1 are components of estrogen signaling in the hypothalamus. In particular, alterations in GABA and PI3K signaling can be mediated by rapid or long-term effects of estrogen. The estrogen-induced increase in p55γ subunit but not p85α in many hypothalamic regions suggests a mechanism for increased PI3K signaling that may further be augmented by the synergistic effects of growth factors. The expression, increase and co-localization of p55γ to arcuate neurons such as POMC and NPY argue for a functional role in mediating physiological effects of estrogen. Using the PI3K inhibitor wortmannin, we find that PI3K signaling may also in part mediate the rapid effects of estrogen to uncouple GABAB (and μ-opioid) receptors from their cognate inwardly rectifying K^+ channels. However, the specific mechanism of this effect at the membrane is complicated and requires further studies to elucidate the molecular actions. Furthermore, we find an increase in gec, a member of the $GABA_A$ receptor associated protein family, and correlate that with the increased inhibitory tone impinging on GnRH neurons during the negative feedback phase of the reproductive

cycle. For many years, the dogma in the field was that ERα and ERβ were the only mediators of estrogen effects; however, many of the genes regulated in these studies did not appear to have estrogen response elements suggesting that other promoter sites may be just as important for the transcriptional and rapid effects of estrogen. Finally, these data suggest that both rapid and long-term effects of estrogen are important in regulating homeostasis and future investigations will need to design experiments that can unravel or tease the effects apart. But, the caveat is that the short and long-term effects of estrogen action may actually be due to a combination involving parallel or complementary pathways.

High throughput assays to assess estrogen-induced neuronal gene expression changes

Although a number of transcripts that are regulated by estrogen have been identified (O'Lone, Frith, Karlsson, & Hansen, 2004), the full scope of estrogen's action is not known. Therefore, new approaches such as differential display and gene microarray (Mong et al., 2002) are being used in order to ascertain a more global picture of E2-regulated genes and how these changes subsequently affect complex physiological processes such as reproduction, stress responses, feeding and cognition. The biggest challenge in using such approaches for studying neuronal effects by estrogen, or any other molecule, is the tissue complexity and cellular heterogeneity of the central nervous system (Jung, Watson, & Simpkins, 2005; Peters et al., 2000). The complexity of neuronal microarray studies can be gleaned from the percent of published articles to date—only 15% of microarray papers listed using the PubMed search engine are related to the brain.

One problem is detecting small changes in gene expression that may be drowned out or diluted by using large tissue dissections; the best example of this in the hypothalamus

may be the population of GnRH neurons. This population is not very large (~1000 cells) and these neurons are diffusely scattered in the preoptic and mediobasal hypothalamic areas (Grove-Strawser et al., 2002; Rønnekleiv & Kelly, 1986). Furthermore, the detection of changes is also daunting when the complexity of neuronal interactions is taken into consideration. For example, certain signaling molecules may be differentially expressed in opposite directions in adjacent inhibitory and excitatory neurons or pathways, canceling each other out at the whole tissue level in a microarray experiment. A case for this scenario may be made for the POMC and NPY neurons of the arcuate nucleus that are both activated by leptin however, the direction of regulation depends on other factors (Xu et al., 2005; Christian & Moenter, 2007).

We used a couple of strategies to maximize the quality of the data generated using microarray analysis of estrogen-regulated genes within the hypothalamus. First, we employed suppression subtractive hybridization (in both forward and reverse directions) to eliminate the population of mRNAs that are equivalent between estrogen- and vehicle-treated samples. Additionally, we created a brain specific (and model specific, e.g., guinea pig) cDNA library from the resulting clones of the SSH experiment to print cDNA chips. Finally, we used RNA extracted from small dissections of hypothalamic regions to label and hybridize to the cDNA chips. Based on subsequent studies (from our lab and others) since our initial microarray experiments, it is evident that even more rigorous approaches need to be taken. This includes the technical advances made to harvest regional and specific cellular samples. For example, recent approaches to achieve increased specificity include using specific regions or cells, using functional imaging methods to aid in tissue selection, microdissection of specific nuclei or brain areas, and separation and sorting of individual cells based on fluorescent signals or other markers (Taylor, Goubillon, Broad, & Robinson, 2007). The potential problem with this approach is the need for RNA amplification that adds additional

background noise and if the degree of linear amplification is equal across all transcripts and samples (Balla, 2005). However, rigorous data analysis coupled to extensive downstream verification procedures such as qRT-PCR, in situ hybridization and electrophysiology approaches as we have employed in the present studies, show that relevant physiological data can be obtained from large-scale microarray experiments. Another issue from our data is that we may have missed some regulated genes since the number of transcripts on the chip we used was small in comparison to Affymetrix chips, for example. The key is that we pre-selected transcripts known to change with estrogen so the data obtained was more focused. In addition, we started with the hypothesis that certain types of molecules would be regulated based on previous data from our lab and published literature on the negative feedback effects of estrogen in the guinea pig model. Furthermore, careful analysis of the microarray data steered us in directions that seem fruitful. For example, the microarray data revealed that many regulated transcripts were in some way related to PI3K signaling. These included not only the p55γ subunit but also many genes encoding proteins with pleckstrin homology (PH) domains, domains known to bind phosphoinositides generated from PI3K catalytic activity. Those data argue that a network of genes is regulated and therein resides the power of high-throughput assays. This extends to proteomics, lipidomics and transcriptosomal approaches as well (Bock et al., 2003; Cornier et al., 2006; Musatov et al., 2007; Naaby-Hansen, Waterfield, & Cramer, 2001; Pawson & Nash, 2003). Based on the number of PI3K-related molecules seemingly regulated by estrogen and the literature that show this pathway is clearly important for many events in the hypothalamus, we further pursued studies on this pathway.

PI3K signaling as a mediator of estrogen action in the hypothalamus

PI3K signaling in the nervous system has been implicated in development, survival, differentiation, neuroprotection, synaptic plasticity and other cellular activities. It is not surprising that blocking this pathway in the hypothalamus affects multiple physiological events. Hypothalamic PI3K signaling is important in mediating the anorectic effects of leptin and insulin and pharmacological inhibition of this pathway reverses the effects of leptin and insulin (Niswender et al., 2003). Pharmacological inhibition of PI3K also attenuates the estrogen-induced lordosis behavior in rats (Etgen et al., 2003). Synergistic activation by IGF1, which activates MAPK and PI3K, and estrogen is proposed to coordinate the timing of ovulation with the expression of sexual receptivity to maximize reproductive success (Etgen et al., 2003).

Further evidence of the importance of PI3K signaling involves its properties in promoting neuritic growth (Cepoi et al., 2004), which is also evidenced by estrogen treatment. For example, remodeling of axosomatic inhibitory synapses prior to the gonadotropin surge has been described (Calizo et al., 2000; Calizo et al., 2002) that is dependent on Akt activation. In addition, an interaction between the PI3K p85α subunit and ERα takes places within 1-3 h of systemic estrogen administration, and subsequently leads to the the activation of Akt (Cardona-Gómez et al., 2002; Mendez et al., 2003; Quesada et al., 2001), a downstream mediator of PI3K signaling. These data illustrate the complex interaction between the PI3K, growth factor and estrogen signaling pathways to affect synaptic remodeling and neuronal plasticity involved with the reproductive cycle. Unfortunately, there is no *in vivo* data on the function of the p55γ subunit and its role in PI3K signaling. However, based on data published within the past two months, I would speculate that an increase in p55γ subunit expression increases

PI3K signaling. My speculation is based in part from the findings described below on p85α association with PTEN.

Another layer of complexity of PI3K signaling involves the possible negative regulation of PTEN (phosphatase and tensin homologue deleted on chromosome 10) by estrogen to modulate PI3K signaling. PTEN is first described and its relationship to estrogen subsequently presented. PTEN was identified as a candidate tumor suppressor gene in 1997 by three independent groups, and hence is also named MMAC1 (mutated in multiple advanced cancers) and TEP1 (TGF-β-regulated and epithelial cell-enriched phosphatase) (Surmeier & Foehring, 2004; Jover-Mengual, Zukin, & Etgen, 2007; Dey, Furlanetto, & Nissley, 1998). The approach taken by the Parsons group highlights the advances made in hunting down disease-related genes through molecular biology. The Parsons group (Jover-Mengual et al., 2007) used representational difference analysis (RDA) to compare DNA from normal and breast tumor cells, and identified a dozen possibilities for such cancer-causing gene changes, including a deletion on chromosome 10. They were keen to follow up on this deleted region since many other cancers also showed a partially or completely deleted chromosome 10. By using a technique called exon trapping, they found two exons as candidates and screened expressed sequence tags (ESTs) in the GenBank database to subsequently piece together the whole gene.

PTEN is a 403-amino acid protein that functions as a $PI(3,4,5)P_3$ lipid phosphatase and antagonizes PI3K signaling. The crystal structure of PTEN reveals it is composed of two major structural domains, an N-terminal phosphatase domain and a C2-membrane binding domain (Suh, Horowitz, Hirdes, Mackie, & Hille, 2004). The N-terminus also has a stretch of basic and hydrophobic residues that form a $PI(4,5)P_2$-binding domain. The C-terminal tail of PTEN also has PEST (proline, glutamic acid,

serine) domains that regulate its protein stability and includes a PDZ (pSD-95/Dlg and ZO1) domain, which is necessary for protein-protein interactions and subcellular localization (Inukai et al., 2001; Pfaffl, 2001).

A recent flurry of publications implicates PTEN in much more than its well-known role in cancer. For example, its involvement in development of neuronal polarity (Aponte, Lien, Reisinger, & Jonas, 2006; Suh, Inoue, Meyer, & Hille, 2006), neuronal hypertrophy and plasticity (Chaban & Micevych, 2007; Slutsky, Sadeghpour, Li, & Liu, 2004; Kobayashi, Hori, Matsumura, Hosokawa, & Boulant, 2007), and behaviors such as autism (Tankersley, Nicholas, Deaver, Mikita, & Kenney, 1992; Slutsky et al., 2004), schizophrenia (Day et al., 2005) and fear-conditioning in rats (Rateau & Ropert, 2006) underscores the importance of the PI3K pathway in a neuronal physiology. Moreover, PTEN germline mutations are associated with a number of diseases in which some patients also show macrocephaly, mental retardation, ataxia and seizures (Berthoud, 2007).

More interestingly, recent data from the Ashford group suggests that while both insulin and leptin increase cellular PI3K activity, they do so by different mechanisms that involve PTEN (Christian et al., 2007). Most studies of hypothalamic signaling show STAT3 and PI3K activation with both leptin and insulin, however receptor deficits of insulin and leptin produce different obese phenotypes (Balla, 2006), suggesting their signal transductions mechanisms differ at some point. The Ashford group shows that although leptin and insulin are capable of increasing $PI(3,4,5)P_3$ in cells, differential outputs are possible by inhibiting (as is the case for leptin) or not inhibiting (in the case for insulin) PTEN activity (Christian et al., 2007). As an example, they cite that leptin depolarizes while insulin hyperpolarizes POMC neurons (Cowley et al., 2001; Kauffman, Bojkowska, Wills, & Rissman, 2006). In contrast, insulin depolarizes RIPCre neurons (mice lacking IRS-2 in beta cells and a population of hypothalamic neurons) whereas

leptin has no effect even though they express leptin receptors (Kauffman et al., 2006). They elegantly hypothesize that the generation of dual signals and coincidence detection (i.e., $PI(3,4,5)P_3$ production and whether PTEN is targeted to the membrane where it subsequently can be phosphorylated) are required to produce the differential outputs.

Even more exciting are the recent data of PTEN regulation by the p85α subunit of PI3K, and by estrogen (Bonsi et al., 2007; Ziegler & Gallagher, 2005; Kaplitt, Kleopoulos, Pfaff, & Mobbs, 1993; Coppola et al., 2007). Even though only a small fraction of PTEN is found in the plasma membrane, it is constituitively active to keep $PI(3,4,5)P_3$ levels low; this is demonstrated by the increased basal level of 3'phosphorylated polyphosphoinositides in PTEN-deficient cells (Titolo, Cal, & Belsham, 2006). This suggests that any regulatory mechanisms of PTEN would most likely act to limit its activity such that a transient increase in $PI(3,4,5)P_3$ is achieved after cell stimulation. A recent study describes a role for the p85α subunit in addition to its role as a regulatory subunit for the p110α catalytic subunit in insulin responsive cells (Emery et al., 1984). For example, it is proposed that p85α would bind and recruit p110α to the membrane after insulin stimulation, but then would interact with PTEN (by an as yet undetermined mechanism) to activate its phosphatase activity that would curb PI3K activity (essentially a feedback loop). This is interesting because it provides an explanation for why p85α knockout mice are more insulin-sensitive (insulin responsive cells more efficiently take in glucose), not hyperglycemic and do not develop type 2 diabetes as was originally predicted (Kawahara et al., 2007). In this model, p85α deletion removes the feedback signal that would normally occur after insulin stimulation (by anchoring and activating PTEN activity) and so $PI(3,4,5)P_3$ levels remain elevated.

PTEN may also be regulated by estrogen. It was studied in this context because PTEN mutations appear at a higher frequency in endometrial cancer than other cancers

(Kwon, Hofmann, & Montell, 2007). Mutter et al originally reported changes in endometrial PTEN expression throughout the menstrual cycle (Kaplitt et al., 1993). In vitro data from Guzeloglu-Kayisli et al show that estrogen rapidly decreases PTEN activity (5-15 min) by its phosphorylation and subsequent progesterone treatment significantly increases PTEN protein levels after 24 h (Ziegler et al., 2005). It is intriguing to consider that some of the rapid neuronal effects seen with estrogen to increase pAkt levels are perhaps due to phosphorylation of PTEN to then increase PI3K activity. Moreover, the increased p55γ expression may also increase PI3K signaling since p55γ may not even recruit PTEN to the membrane, unlike p85α, which does recruit PTEN to the membrane to limit PI3K activity (Emery et al., 1984).

Lastly, there has been an impediment to easily and accurately assess PI3K signaling. Most often, PI3K function is assessed by activation of known effectors of PI3K such as PKB (Akt) or the associations (or lack thereof) of PI3K subunits to other signaling protein complexes. Gene deletion studies have the disadvantage that many of the PI3K subunits also play roles as scaffolding or adaptor molecules and thus may lead to erroneous interpretations. Measuring the actual changes in membrane phospholipid content or the phosphoinositide products after a stimulus has been problematic mainly due to the nature of the technically demanding techniques. For example, the most accurate quantitative detection of PIs is to use the classical method of metabolically labeling cells with [^{32}P] phosphate or [^{3}H] myo-inositol, followed by lipid extraction, paper chromatography and autoradiograpy (Hu, Vervaeke, & Storm, 2007). A more modern version that is currently used involves separating the radiolabeled extracts via thin-layer chromatography (TLC) or reverse-phase high performance liquid chromatography (HPLC) (Singer-Lahat et al., 2007). These methods yield excellent quantitative results and are able to distinguish the various PIs; however, they are not practical for analyzing

tissue samples. But methods are currently being developed to overcome limitations, such as using mass spectrometry (Buijs et al., 2006). Again, the role of lipid dynamics at the plasma membrane in channel function is the subject of current investigations and may be another link to estrogen's effects in signaling at the membrane.

Estrogen and Inhibitory Neurotransmission in the Hypothalamus

For most of the cycle, estrogen plays an inhibitory role on the neuronal network regulating GnRH secretion (Sarkar et al., 1980; Caraty et al., 1989; Chongthammakun et al., 1993; Evans et al., 1994), which is considered the final output of a system that integrates numerous external and internal cues to regulate the release of LH and FSH from the anterior pituitary gland. Inhibition of GnRH secretion by estrogen is mediated by direct actions on GnRH neurons (Kelly et al., 1984; Lagrange et al., 1995; Abraham et al., 2003; Abraham et al., 2004) as well as indirect actions onto GABA and opioid neurons which synapse onto to GnRH neurons (Herbison et al., 1990; Herbison et al., 1991; Herbison, 1997; Wagner et al., 2001). The regulation of gec1 underscores the importance of $GABA_A$ and opioid receptor trafficking and insertion into the plasma membrane to modulate inhibitory neurotransmission, a key effector during negative and positive feedback. Finally, the regulation of other transcripts (as shown by our microarray data) synaptobrevin2, synaptogyrin, taxilin, rab11a, CAPS and neurobeachin supports the notion that an ensemble of genes critical for synaptic transmission are key regulators of mediating the sustained effects of estrogen in hypothalamic neurotransmission (Pfeffer, 2001) (Chen, Feng, Chen, & Wandinger-Ness, 1998; Krappa et al., 1999; Pfaff et al., 1979; Trimble, Gray, Elferink, Wilson, & Scheller, 1990; Jackson et al., 2000; Quilliam et al., 2002). Future studies will need to be carried out to more thoroughly examine their role in estrogen-mediated signaling to affect homeostasis.

Future Directions

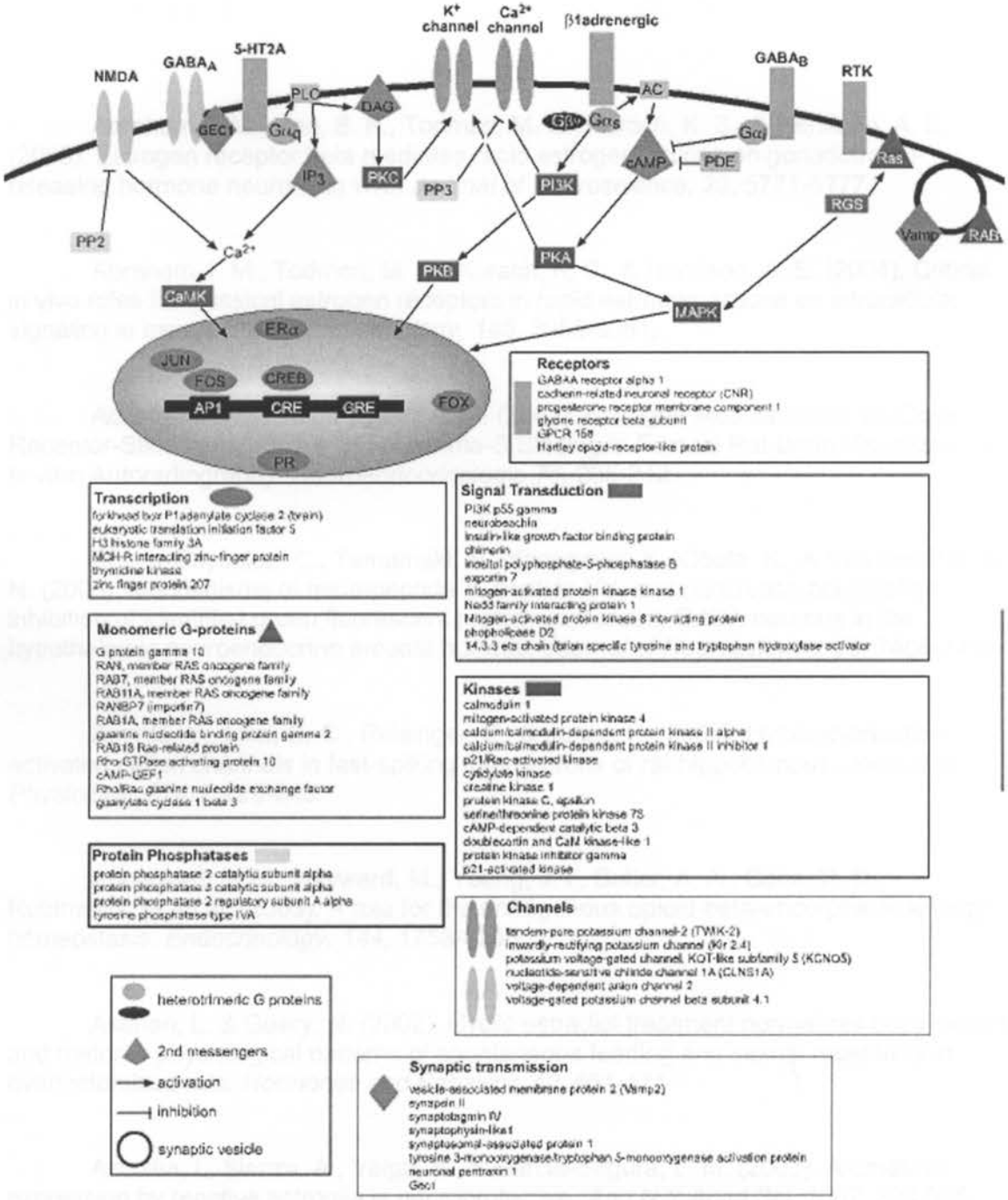
There are numerous directions in which the research from the work in this dissertation can be used to pursue studies to further examine the effects of estrogen in the hypothalamus to regulate homeostasis. A complete analysis of the list of regulated genes from our microarray studies using newer software that integrates multiple sources of information such as expression, pathway, distribution, etc. will be useful to define further gene networks that may be involved in estrogen signaling. In fact, a post-doctoral fellow in our laboratory has started this type of analysis and is currently following up with other techniques. Based on the microarray data, he is investigating the possible modulation by estrogen of a number of K^+ channels. In addition, single-cell RT-PCR experiments should be performed to fully characterize all of the PI3K catalytic and regulatory isoforms in arcuate or other hypothalamic nuclei. Considering that much of this work was arcuate-nucleus-centric, studies investigating the functional role of the gene expression changes in other nuclei would be interesting and may elucidate how events are coordinated between nuclei.

Another approach to address the synergistic effects of estrogen and PI3K signaling is the *in vitro* study Dr. Bryant and I are pursuing. This study involves comparing the pAkt levels in arc/vmh nuclei from 24 h estrogen- and vehicle-treated mice that have been incubated with leptin for 5-30 min. The details are still being teased out but the data would provide a functional output for the synergistic effects of estrogen and leptin. These studies may also provide clues to the gender difference seen where females are more sensitive to the effects of leptin.

By far, the most exciting experiments will be the functional characterization of estrogen's effects on membrane lipid dynamics. It would be interesting to develop hypothalamic extracts that can be transfected with a fluorescently coupled PH-domain

contain protein. This type of experiment would enable visualization and facilitate understanding the immediate effects estrogen at the plasma membrane. Using other agonists such as growth factors in addition to estrogen will reveal how these molecules are able to have synergizing effects. Finally, the most difficult area of investigation will be trying to understand the cross-talk mechanisms between pathways in different cells and how the individual pathways can be teased out for developing targeted pharmacological agents.

Figure 5-1 Cell model of estrogen-regulated genes in the hypothalamus



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