THE ROLE OF GPR30 IN MEDIATING ESTROGEN EFFECTS ON NEURONS AND COGNITIVE PERFORMANCE

by

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Basic and clinical research suggests the loss of estradiol following menopause may contribute to accelerated brain aging and the increased risk of age-related cognitive decline and dementia. Novel estrogenic compounds may confer positive cognitive effects without the added risk of side effects associated with current agents. G-1 is a recently developed agonist for the novel transmembrane estrogen receptor (ER) GPR30. Activation of the GPR30 pathway is independent of either the classical ER α or β pathways, raising the possibility of using G-1 as a novel estrogenic agent while avoiding the risks associated with other estrogenic compounds. Previous work in our laboratory has shown that estradiol enhances cognitive performance via effects on basal forebrain cholinergic neurons. We hypothesize the effects of estradiol on cholinergic function and cognitive performance are mediated by GPR30. If correct, then selective GPR30 agonists may provide useful alternatives to current estrogenic therapies. To test this hypothesis the first goal was to characterize GPR30 expression in the rat forebrain, focusing on co-expression by cholinergic neurons. A RT-PCR assay was developed to quantify GPR30 mRNA in specific brain regions. GPR30 protein and mRNA expression were found in regions of the brain important for spatial learning and memory. Moreover, GPR30 protein appears to be expressed by the vast majority of cholinergic neurons. The second goal was to examine the effects of GPR30 activation on cholinergic neurons. In vivo microdialysis and HPLC analysis of acetylcholine levels in dialysates were used to compare effects on acetylcholine release. Administration of a GPR30 agonist or estradiol (E2) to ovariectomized (OVX) rats produced a 3-

fold increase in potassium-stimulated acetylcholine release in the hippocampus relative to vehicle-treated controls. The third goal was to test the ability of GPR30 agonists and antagonists to enhance or impair cognitive performance in rats. GPR30 agonism enhanced the rate of acquisition on a delayed matching-to-position (DMP) T-maze task in OVX rats similar to estradiol while GPR30 antagonism dose-dependently impaired performance in gonadally intact and OVX+E2 rodents. Hence, GPR30 appears to play an important role in mediating direct effects of estradiol on basal forebrain cholinergic neurons, with corresponding effects on cognitive performance.

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I. INTRODUCTION

IA. Estrogen Effects on Cognition

Over the past few decades, we have seen vast improvements in medical technology and research that have resulted in better health care and increasing lifespan. In fact, in 1900 the life expectancy of a female was 54 years, whereas today the average is 83 years in industrialized countries (Singh, 1996). Since the age to menopause has not changed, and women are spending a longer portion of their lives in the post-menopausal years, increased efforts have been made to discover new ways to prevent and treat neurodegenerative diseases. Much of this work has focused on estrogens and the potential benefits it may have on cognitive function.

Animal studies show that estrogens have many beneficial effects on the brain, such as reducing neuronal loss following cardiac arrest and stroke (Suzuki et al., 2006), increasing neuronal connectivity (Spencer et al., 2008), improving cognitive performance (Daniel, 2006), and preventing or slowing age-related cognitive decline (Frick, 2009; Gibbs and Gabor, 2003). Young adult ovariectomized (OVX) rodents that are treated with estradiol (E2) perform better on learning and memory tasks such as T-maze and radial arm maze tasks (Daniel et al., 1997; Gibbs, 1999), place and object recognition tasks (Fernandez et al., 2008; Frye et al., 2007; Gresack and Frick, 2006a), and contextual or cued fear conditioning (Jasnow et al., 2006), than OVX controls. These task-effects are selective and can vary depending on the type of memory tested (Davis et al., 2005; Fader et al., 1999; Galea et al., 2001; Korol and Kolo, 2002), level of hormone (Wide et al., 2004), and prior stress (Wood and Shors, 1998). Human studies likewise show beneficial effects of estrogens on specific cognitive tasks in younger surgically menopausal

or perimenopausal women, particularly in the realm of verbal memory and executive functioning (Sherwin and Henry, 2008). Several studies have reported estrogen-mediated increases in cerebral blood flow (Greene, 2000; Resnick and Maki, 2001), and reductions in hippocampal and cortical atrophy associated with aging and Alzheimer's disease (AD) (Erickson et al., 2007; Hu et al., 2006), as well as significant reductions in memory decline (Resnick et al., 1997) and in the risk of developing Alzheimer's disease (AD)-related dementia (Figure 1) (Henderson, 2009; Zandi et al., 2002). A recent study also reported that women who experience an early menopause are at significantly greater risk for age-related cognitive decline and dementia, and that this risk is mitigated by early estrogen therapy (Shuster et al., 2010).

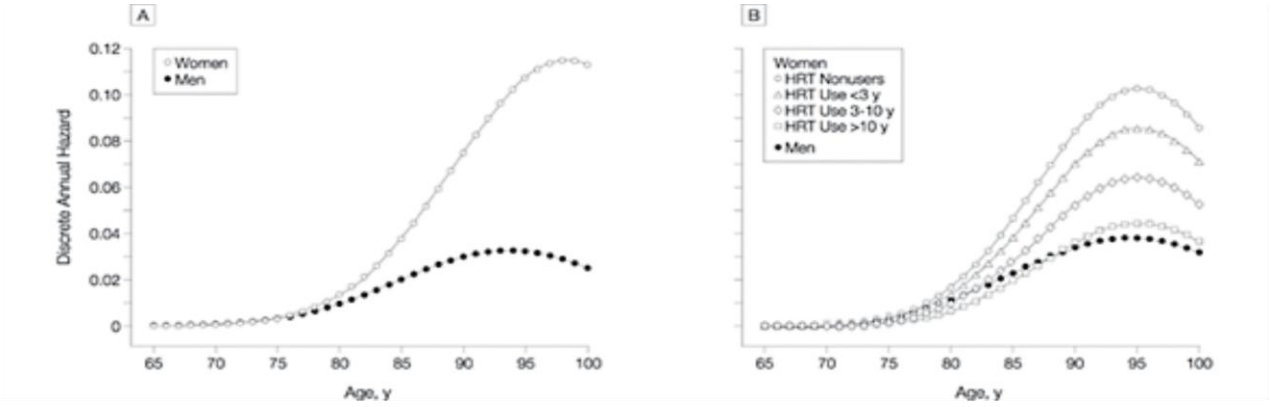

Figure 1. Risk of developing Alzheimer's Disease in men and women over time (A) and in women who have been placed hormone replacement therapy (HRT) for varying amounts of time. Graphs from(Henderson, 2009; Zandi et al., 2002).

Despite these encouraging findings, recent randomized trials suggest far fewer benefits when estrogen therapy is initiated in older women. In fact, several large trials have reported either no beneficial effect or increased harm for women receiving hormone therapy (HT) in old age (Resnick et al., 2006; Shumaker et al., 2003; Shumaker et al., 2004). The Women's Health Initiative Memory Study (WHIMS), one of the largest prospective randomized trials conducted to date, found that older women (mean age 69 years at enrollment) treated daily with a

combination of conjugated equine estrogens and medroxyprogesterone acetate were at significantly greater risk for stroke and dementia than placebo-treated controls (Shumaker et al., 2004). Likewise, attempts to use estrogen therapy to reduce AD-related cognitive impairment in women have been largely unsuccessful (Henderson et al., 2000; Mulnard et al., 2000; Mulnard et al., 2004; Wang et al., 2000). Taken together, these studies have led to the 'Window of Opportunity' or 'Critical Period' hypothesis, which posits that estrogen therapy must be initiated at or near the time of menopause in order to positively affect brain aging and cognition. This is supported by recent animal studies which show that the beneficial effects of estrogens on cognitive performance in rats and mice diminish with age and time following loss of ovarian function when treatment is delayed (Daniel et al., 2006; Gibbs et al., 2009; Markowska and Savonenko, 2002; Talboom et al., 2008).

IB. The Cholinergic Hypothesis

The reasons for the loss of estrogen effect with age and time following menopause have yet to be explained. Cholinergic inputs to the hippocampus and cortex are known to play an important role in mediating effects of estrogens on cognitive performance (reviewed in (Gibbs, 2010)). Therefore, it is possible that deficits in cholinergic function that occur with age and time following menopause are responsible, at least in part, for the loss of estrogen effects.

Cholinergic neurons in the medial septum (MS), diagonal band of Broca (DBB), and nucleus basalis magnocellularis (NBM) are the primary source of cholinergic inputs to the hippocampus and cerebral cortex (Figure 1) (Gritti et al., 1997; Mesulam, 1996; Woolf, 1991). These cholinergic projections are important for learning, memory, and attention (Baxter and Chiba, 1999; Everitt and Robbins, 1997). Early human studies demonstrate that anticholinergic

drugs produce cognitive impairments in young healthy adults similar to impairments seen in patients with dementia (Deutsch, 1971; Drachman and Leavitt, 1974), while cholinergic enhancing drugs improve performance in older patients (Drachman, 1977). Cholinergic lesions in the MS or NBM as well as use of selective muscarinic antagonists impair learning and memory (Everitt and Robbins, 1997). Moreover, cholinergic neurons in the basal forebrain (BFCNs) undergo degenerative changes during aging that correlate with progressive memory deficits (Bartus et al., 1982; Baxter and Chiba, 1999). Loss of BFCNs is a hallmark of Alzheimer's disease and has been shown to play a role in the cognitive deficits associated with the dementia (Lanari et al., 2006; Linstow and Platt, 1999; Schliebs and Arendt, 2006; Smith et al., 1999).

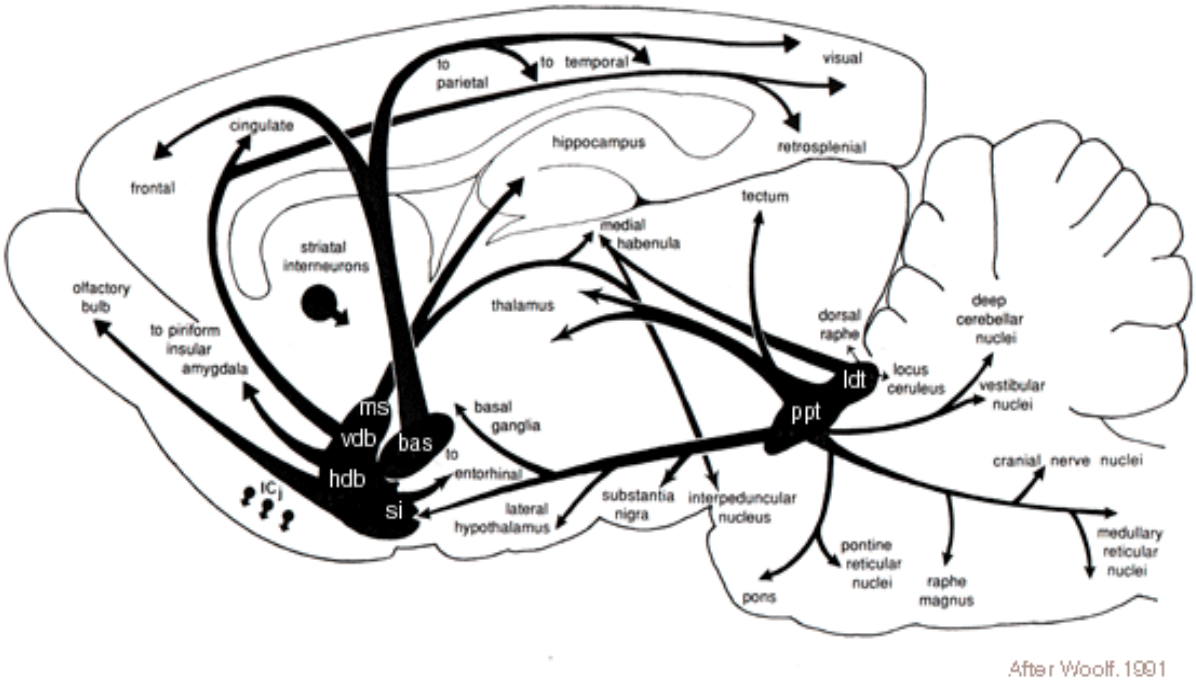

Figure 2. Illustration of the cholinergic pathways in the rat brain. Regions containing cholinergic neurons include the medial septum (ms), vertical and horizontal limbs of the diagonal band of

Broca (vdb and hdb), nucleus basalis (bas), substantia innominata (si), laterodorsal tegmental nuclei (ldt), and peduncolopontine tegmental nuclei (ppt) (Image from Woolf, 1991).

The ability of E2 to influence the functionality of the cholinergic system may contribute to the timing of the Window of Opportunity hypothesis. Following ovariectomy, E2 treatment can significantly enhance basal forebrain cholinergic function in females. E2 treatment increases choline acetyltransferase (ChAT) activity (Luine, 1985), ChAT mRNA and protein (Gibbs, 1997; Gibbs, 2000; Singh et al., 1994), high-affinity choline uptake (O'Malley et al., 1987; Tinkler et al., 2004), and potassium-stimulated acetylcholine release in the hippocampus (Gabor et al., 2003; Gibbs et al., 1997). E2 treatment also can increase the density of cholinergic fibers innervating specific regions of frontal cortex (Gibbs et al., 1997; Tinkler et al., 2004). These effects are relevant to effects on cognitive performance. For example, E2 treatment can attenuate the amnestic effects of scopolamine, a muscarinic acetylcholine receptor blocker in rats (Fader et al., 1998; Fader et al., 1999; Gibbs, 1999). A similar effect has been reported in humans (Dumas et al., 2006; Dumas et al., 2008). The effects of E2 on NMDA receptor binding and dendritic spine density in region CA1 of the hippocampus also appear to be mediated via cholinergic inputs (Daniel and Dohanich, 2001; Lam and Leranth, 2003). In addition, selectively destroying cholinergic projections to the hippocampus (Gibbs, 2002; Gibbs, 2007) or blocking M2 muscarinic receptors in the hippocampus (Daniel et al., 2005) prevents E2 effects on spatial learning. This demonstrates that the cholinergic inputs are essential for mediating beneficial effects of E2 on certain tasks. Notably, recent studies suggest that the effects of E2 on ChAT expression and cholinergic function are altered by age and time following ovariectomy (Bohacek et al., 2008; Gibbs, 1998; Gibbs, 2003; Gibbs et al., 2009; McMillan et al., 1996), which may account for the loss of beneficial effects of estrogen therapy on cognition in older women and in

women with AD. We propose that a better understanding of the mechanisms that underlie the effects of estrogens on BFCNs will lead to the development of more effective therapies for preventing and treating cognitive decline associated with aging and with AD.

IC. Mechanisms of Estrogen Effects on Cholinergic Function

The mechanism(s) by which E2 increases the functionality of BFCNs is unclear. Two nuclear estrogen receptors (ERs) have been identified, ERα and ERβ (Toran-Allerand, 2004). These nuclear receptors regulate gene transcription but are also capable of activating second messenger signaling pathways such as mitogen-activated protein kinases (MAPK), calcium/calmodulin-dependent protein kinases (CamKII), and cAMP response element-binding proteins (CREB) (Manavathi and Kumar, 2006; McEwen, 2002). Approximately 30% of cholinergic neurons in the MS express ERα, while less than 5% express ERβ (Shughrue et al., 2000). E2 can stimulate CREB activation in BFCNs in adult mice, and this effect was blocked by inhibition of MEK1/2 and by treatment with ICI182780 (an ERα and β antagonist) (Szego et al., 2006). These effects were observed in ERβ knockout mice but not in ERα knockout mice, suggesting that E2 may act through ERα to activate MAPK signaling and CREB phosphorylation in a subset of the cholinergic neurons. The different pathways of E2 signaling are shown in Figure 2.

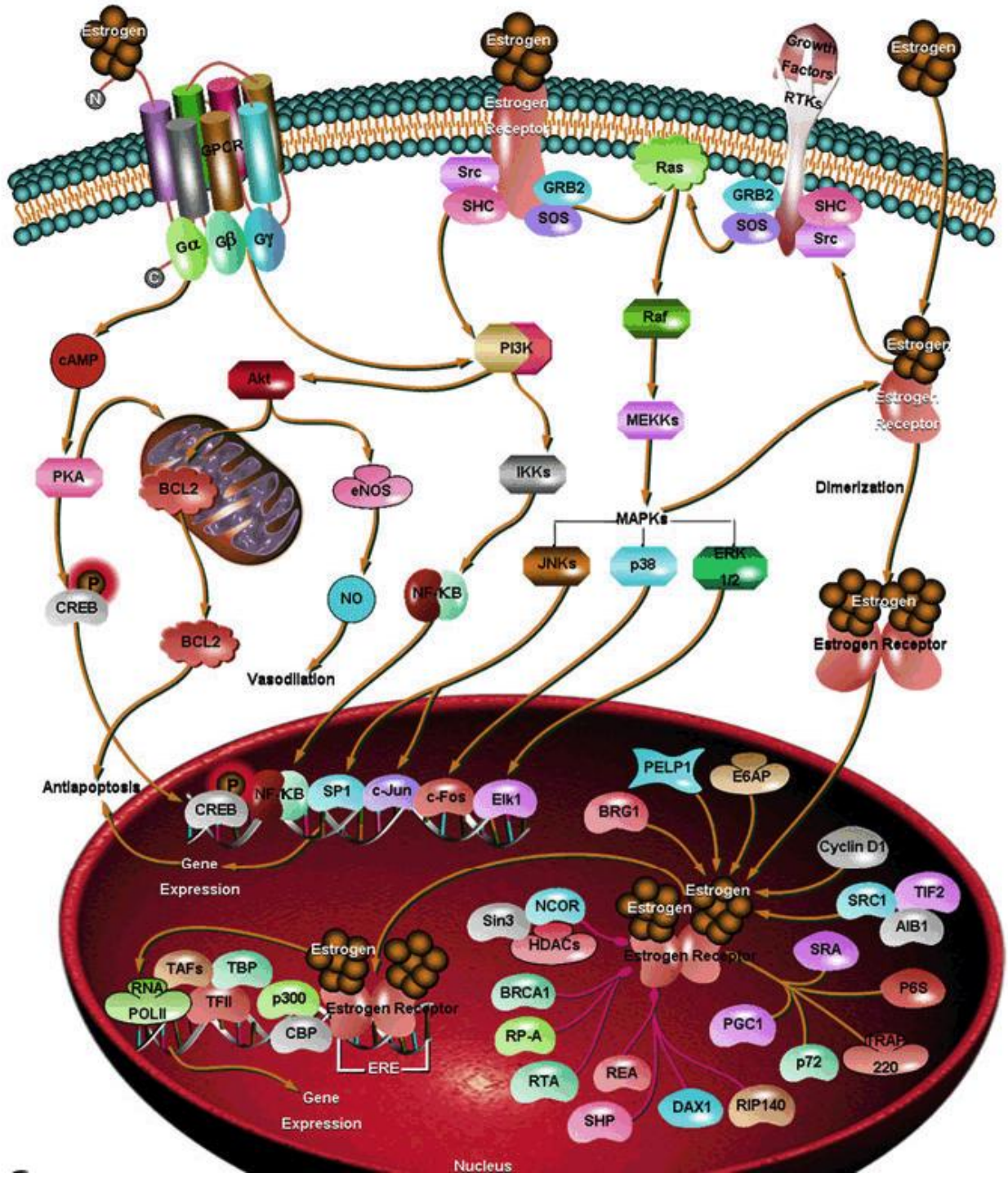

Figure 3. Illustration of E2-signaling pathways (image by SABiosciences). Note that E2 can bind to membrane-bound receptors (left and middle pathways) to activate signaling cascades,

or directly enter the nucleus (far right) and bind to classical receptors to activate transcription factors.

Recently, the novel membrane-associated estrogen receptor GPR30 was identified (Moriarty et al., 2006; Prossnitz et al., 2007). GPR30 is a seven-transmembrane-spanning G-protein coupled receptor that is localized to both intracellular and plasma membranes (Funakoshi et al., 2006) and promotes rapid estrogen signaling in a variety of cell types including breast cancer cells, uterine epithelial cells, keratinocytes, thymus, and select cell lines (Albanito et al., 2007; Filardo and Thomas, 2005; Thomas et al., 2005; Vivacqua et al., 2006). Radioligand assays have found that GPR30 shows specific, high affinity binding to E2 and related estrogens. In SKBR3 cells, GPR30 confers high affinity (Kd: 2.7 nM), saturable, low capacity (Bmax: 114 pM) estrogen binding (Thomas et al., 2005). In Cos-7 cells, transfection with GPR30 confers E2 binding with a Ki of 6.6 nM, and a Kd similar to that calculated for E2 binding in SKBR3 cells (Revankar, 2005). Heterologous expression studies and RNAi or antisense knock-down experiments confirm that binding is associated specifically with GPR30, and does not require classical estrogen receptors. Competitive binding assays show that steroid binding to GPR30 is specific for E2 and for certain phytoestrogens, whereas other steroid hormones (e.g., 17α-estradiol, progesterone, cortisol, and testosterone) have little binding at concentrations up to 10 μM. In contrast, the antiestrogens ICI182,780 and tamoxifen compete effectively, with relative binding affinities approximately 10% that of E2 (Thomas et al., 2005). Functional studies using breast cancer cells lacking classical estrogen receptors found that GPR30 mediates up-regulation of the c-fos proto-oncogene by E2, as well as by other estrogen-like compounds such as the phytoestrogens genistein and quercetin (Maggiolini et al., 2004). Using Ishikawa cells (which express ERα) and human endometrial cancer cells (HEC1A cells; which do not express

functional ERα), Vivacqua et al. (Vivacqua et al., 2006) demonstrated that E2 and the major antiestrogenic metabolite of tamoxifen (4-hydroxytamoxifen) up-regulate c-fos expression specifically through interaction with GPR30, and that this effect involves activation of epidermal growth factor receptors.

Both Northern blot and Western blot studies have confirmed that GPR30 is expressed in a variety of tissues, including the central nervous system. In the brain, GPR30 immunoreactivity has been detected in many regions including the hippocampus, hypothalamus, and midbrain (Brailoiu et al., 2007; Hammond et al., 2010). GPR30 also is expressed in spinal cord and in dorsal root ganglion neurons, and G-1, a selective GPR30 agonist (Bologa et al., 2006), can depolarize spinal cord neurons as well as stimulate protein kinase Cξ-dependent hyperalgesia in rats (Dun et al., 2009; Kuhn et al., 2008). The role that GPR30 plays in mediating estrogen effects in brain is still largely unclear. A GPR30-selective agonist referred to as G-1 and the selective antagonist G-15 have been developed to better understand the role of GPR30 (Figure 3 shows the structures of G-1 and G-15). One recent study found that G-1 reduces 5-HT1A receptor signaling in the paraventricular nucleus of the hypothalamus, analogous to E2 (Xu et al., 2009). Other studies suggest that GPR30 is involved in estrogen-mediated regulation of GnRH neurons in non-human primates (Noel et al., 2009; Terasawa et al., 2009). Another study reports that G-1 significantly reduced freezing behavior in a mouse model of depression (analogous to E2) and that this effect was blocked by G-15 (a selective GPR30 antagonist) (Dennis et al., 2009).

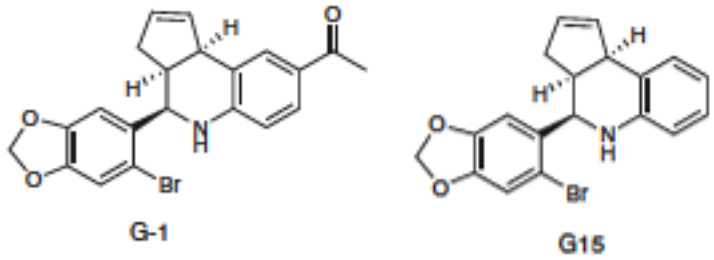

Figure 4. Structures of G-1 (GPR30-selective agonist) and G-15 (GPR30-selective antagonist).

Despite the evidence shown above, there is still controversy over whether GPR30 directly mediates estrogen effects. Otto et al. examined binding of E2 to GPR30 in different cellular systems and found no evidence for specific, saturable binding (Otto et al., 2008). Pedram et al. used SKBR3 cells (ERα negative, GPR30 positive) and found no significant binding of E2 and found non stimulation or ERK, PI3K, or cAMP signaling (Pedram et al., 2006). Moreover, a third group showed that the GPR30-selective agonist G-1 did not alter E2-induced gene expression in MCF-7 cells nor stimulate the expression of E2-responsive genes (Madak-Erdogan et al., 2008). Thus, these experiments do not support the hypothesis that GPR30 is a membrane-bound estrogen receptor.

Recently, four GPR30-knockout mice lines were generated to better delineate the role of GPR30 (Isensee et al., 2009; Martensson et al., 2009; Otto et al., 2009b; Wang et al., 2008). However, the observed phenotypes from these lines were diverse with only partial overlap. The varying phenotypes included increased body weight and visceral adiposity, impaired glucose tolerance, elevated blood pressure, or no phenotype at all. Different targeting strategies, genetic backgrounds, and experimental conditions may explain the conflicting observations (reviewed in (Langer et al., 2010)).

ID. Overview of Thesis:

We have investigated the role of GPR30 in mediating estrogen effects on cholinergic neurons in the basal forebrain in rats, and have generated evidence that GPR30 is an important regulator of BFCN function with corresponding effects on cognitive performance. The major themes are as follows: (1) GPR30 mRNA and protein expression were observed throughout the rat forebrain and in regions important for learning and memory; (2) GPR30 agonism enhanced basal forebrain cholinergic function in a similar manner as E2; (3) GPR30 agonism enhanced spatial learning to that of gonadally intact rats, whereas antagonism impaired performance in a dose-dependent fashion. These themes will be described in greater detail in the following chapters.

II. GPR30 EXPRESSION IN THE FOREBRAIN

A. GPR30 Protein Expression

i. Overview

Since estrogens have beneficial effects on learning and memory, and we hypothesize that GPR30 may directly mediate these effects, we first sought to determine whether GPR30 protein is found in regions of the brain that are implicated in learning and memory. At the start of this project no one had explored GPR30 expression in the rat brain. Therefore, GPR30 protein expression was first characterized in the rat forebrain using immunohistochemical methods.

ii. Methods

Eight 3-4 month old OVX rats were anesthetized with a mixture of ketamine and xylazine and perfused with 0.9% saline followed by 4% paraformaldehyde/0.05 M sodium acetate (pH 6.5) and then with 4% paraformaldehyde/0.05 M Tris (pH 9.0). Brains were removed, post-fixed for 2 hours in 4% paraformaldehyde/50 mM Sorenson's phosphate buffer, and then stored in 20% sucrose/50 mM PBS. Adjacent series of 40 μm coronal sections were cut through the forebrain. Series were first incubated with a rabbit anti-GPR30 antiserum directed against the human C-terminus of GPR30 (0.1 μg/mL in 50 mM PBS containing 0.05% Triton X-100 and 5% normal horse serum). The GPR30 antibody was a gift from Edward Filardo (Brown University). Sections were placed in primary antibody solution for 3 days at 4°C. The sections were rinsed with 50 mM PBS and then incubated with biotinylated goat-anti-rabbit IgG (Vector Laboratories, 1:200 dilution) for 1 hour at room temperature. Sections were rinsed again with PBS and incubated with an avidin-HRP complex (Vectastain Elite kit; Vector Laboratories) for 1 hour at room temperature. Sections were rinsed with PBS, placed in 50 mM Tris-HCl (pH 7.6) containing 3,3'-diaminobenzidine (DAB; 0.5 mg/mL), then reacted with 50 mM Tris containing DAB (0.5 mg/mL), H_2O_2 (0.01%) and $NiCl_2$ (0.032%) for 10 min. Sections were then rinsed with PBS, mounted onto Superfrost Plus slides (Fisher Scientific, Inc.), dehydrated, and coverslipped. Specificity was tested by blocking staining with purified C-terminal peptide (Novus Biologicals, Inc.) at a concentration of 10 μg/mL. Additional negative control sections were incubated with the secondary antibody but no primary antibody.

iii. Results

GPR30 immunoreactivity was detected in many rat forebrain regions, including the hippocampus, medial septum, frontal cortex, striatum, and nucleus basalis (Figure 1). Strong staining was seen in the cytoplasm and in some processes. Some cells were more strongly

labeled than others. Staining was particularly strong in presumptive cholinergic cell groups (Figure 1B and 1C). Staining was greatly reduced in sections incubated with the GPR30 antibody plus 10 μg/mL C-terminal blocking peptide (Figure 1F). Staining was not observed in sections that received no primary antibody.

iv. Discussion

The data show that GPR30 is expressed in many regions of the forebrain that are important for learning and memory including the hippocampus, frontal cortex, and striatum. These findings are consistent with and augment previous studies showing significant GPR30 expression in hippocampus, and in specific regions of the hypothalamus and spinal cord (Brailoiu et al., 2007; Dun et al., 2009). In the hippocampus, E2 has many effects on connectivity, synaptic plasticity, and function (Spencer et al., 2008; Woolley, 2007). Among these effects are a significant increase in the number of dendritic spines and NMDA receptors on the apical dendrites of CA1 pyramidal cells, which are thought to contribute to estrogen effects on memory (Sandstrom and Williams, 2001; Sandstrom and Williams, 2004). Notably, these effects also are dependent on cholinergic afferents (Daniel and Dohanich, 2001; Lam and Leranth, 2003). In the frontal cortex, OVX decreases and E2 increases dendritic spine density in specific regions of the cortex in rats and nonhuman primates (Hajszan et al., 2007; Hao et al., 2006; Tang et al., 2004), which likewise has been associated with effects on memory (Li et al., 2004; Wallace et al., 2006; Wallace et al., 2007). The extent to which GPR30 plays a role in mediating these effects is currently unknown and needs to be investigated.

Currently there are conflicting results regarding the cellular localization (cell surface versus endoplasmic reticulum) of GPR30 (Prossnitz et al., 2007). Previous studies using confocal fluorescence microscopy reported localization of GPR30 to the endoplasmic reticulum and Golgi

(Filardo et al., 2006; Revankar, 2005). Others have reported GPR30 localizes to the cell surface (Funakoshi et al., 2006; Thomas et al., 2005), as it co-localizes with concanavalin A, a plasma membrane marker (Filardo et al., 2007). In this study, GPR30 appeared to be located both on the cell surface and in the cytoplasm, which is consistent with the localization on the plasma membrane and endoplasmic reticulum (Figure 1). It should be emphasized, however, that this analysis was not designed to isolate expression within any given cellular compartment. There may be differences in the intracellular distribution of GPR30 based on cell type. For example, GPR30-IR in endothelial cells lining the third ventricle appear to localize differently within the cells compared with GPR30-IR in neurons (not shown). Further work is needed to better identify the subcellular localization of GPR30-IR within specific cells in the brain.

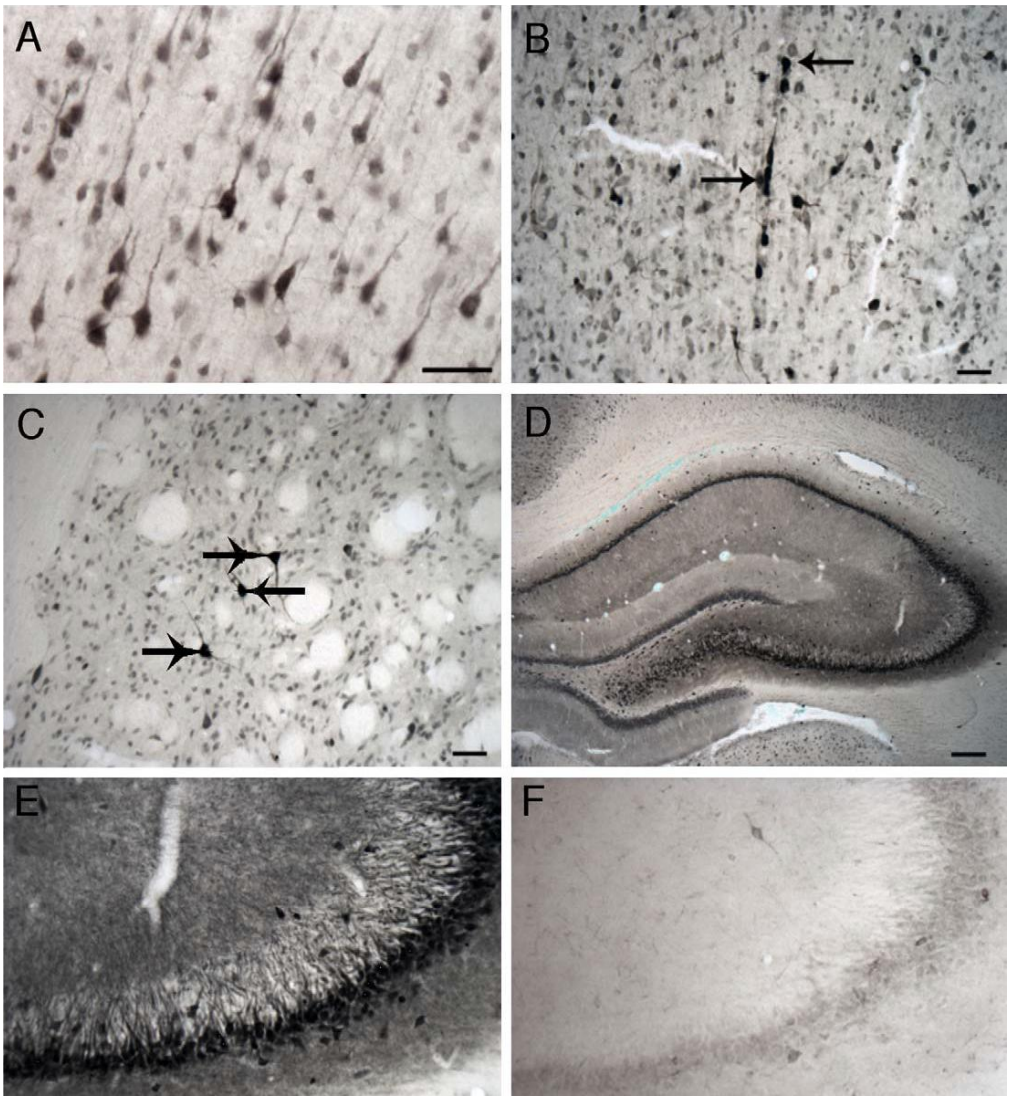

Figure 5. Photomicrographs illustrating GPR30-like immunoreactivity in different forebrain regions. (A) Frontal cortex, (B) medial septum, (C) striatum, (D) hippocampus, (E) CA3 region of hippocampus, and (F) CA3 region that was incubated with GPR30 antibody plus C-terminal peptide (Hammond et al., 2010). Scale bar = 50 μm in panels A-C, E, and F. Scale bar = 200 μm in panel D. Arrows point to several intensely labeled cells in regions containing cholinergic neurons.

B. GPR30 Co-localization with Selected Neuronal Types

i. Overview

Cholinergic neurons in the medial septum, diagonal band of Broca, and nucleus basalis of the rat forebrain are part of the basal forebrain cholinergic system and play roles in learning, memory, and attention (Baxter and Chiba, 1999; Everitt and Robbins, 1997). We hypothesized that GPR30 may be expressed by these neurons, thereby directly mediating estrogen effects on the cholinergic system and learning. Immunohistochemistry was used to evaluate GPR30 expression in cholinergic and GABAergic neurons.

ii. Methods

For co-localization studies, tissues were prepared from five 3-4 month old OVX rats and sections were incubated with the GPR30 antibody as described above. Sections were next incubated with a Cy3-labeled anti-rabbit IgG raised in donkey at a dilution (diluted 1:400; Jackson ImmunoResearch). Sections were then rinsed with PBS and incubated either with an antibody against ChAT (goat anti-ChAT, 1:1200 dilution; Chemicon International, Inc.) or with an antibody against parvalbumin (mouse anti-Parv, 1:16,000 dilution; Sigma-Aldrich, Inc.). Parvalbumin is one type of calcium- binding protein that is expressed by GABAergic neurons in the basal forebrain (Freund, 1989; Kiss et al., 1990). These antibodies were detected using Alexa488-labeled secondary antibodies raised in donkey (diluted 1:400; Invitrogen). Negative controls underwent all processing steps, but omitted one or both primary antibodies. This controlled for non-specific staining and ruled out cross-reactivity between secondary antibodies. The order in which primary antibodies were added also was varied to control for order effects.

Labeled cells were imaged using an Olympus Fluoview 1000 confocal microscope. Regions containing populations of cholinergic neurons were analyzed. These included the medial

septum, vertical (VDB) and horizontal (HDB) limbs of the diagonal band of Broca, nucleus basalis magnocellularis (NBM), and striatum (STR). Areas were defined by plate numbers in Paxinos and Watson (Paxinos et al., 1980). Sections through each region were selected from each rat and matched according to plate number (Table I). Within each section, the total number of ChAT- or Parv-immunoreactive cells in the region of interest and the percentage of these cells containing GPR30-IR were counted and calculated. Specific brain regions in which cells were counted are illustrated in Figure 1. Cells were included in the counting if they contained ChAT- or Parv-IR, had a defined cell body and a detectable nucleus. Defined ChAT or Parv-immunoreactive cells were first located and then examined for GPR30 labeling. Data are presented as average percentage of ChAT- and Parv-IR cells containing GPR30-IR per section.

iii. Results

Double-staining for GPR30 with ChAT and Parv is shown in Figure 2. No staining was seen in control sections incubated with secondary but not primary antibodies. Many ChAT-GPR30 double-labeled cells were detected in regions containing basal forebrain cholinergic nuclei. In comparison, few Parv-GPR30 double-labeled cells were detected. In the MS and STR, nearly all of the ChAT-IR cells detected also contained GPR30-IR (97% and 99%, respectively). In the VDB, HDB, and STR, 63%, 64%, and 80%, of ChAT-IR cells also contained GPR30-IR. In contrast, only 17%, 35%, 27%, and 0.4% of Parv-IR cells in the MS, VDB, HDB, and STR contained GPR30-IR. These data suggest that GPR30 is preferentially expressed by cholinergic neurons and that estrogens may bind to GPR30 to directly affect these neurons.

iv. Discussion

While the focus of this work was on cholinergic neurons, it should be noted that most of the cells containing GPR30-IR in the forebrain were not cholinergic. Many of these cells have

not been phenotypically identified; however, we did find that a subset of the parvalbumin-IR neurons in specific regions of the forebrain also contain GPR30-IR. In the medial septum, these neurons are GABAergic and synapse onto the dendrites of cholinergic neurons in the septum/diagonal band as well as project to the hippocampal formation (Leranth and Frotscher, 1989). In addition, these neurons are excited by muscarinic agonists (Wu et al., 2000), and it has been shown that cholinergic neurons in the medial septum provide an excitatory drive to the GABAergic projections (Alreja et al., 2000). Like the cholinergic neurons, these GABAergic projections have been shown to play a role in specific cognitive processes (Zarrindast et al., 2002). Hence, it is possible that activation of GPR30 on GABAergic neurons in the medial septum may contribute to the effects of E2 on cognitive performance.

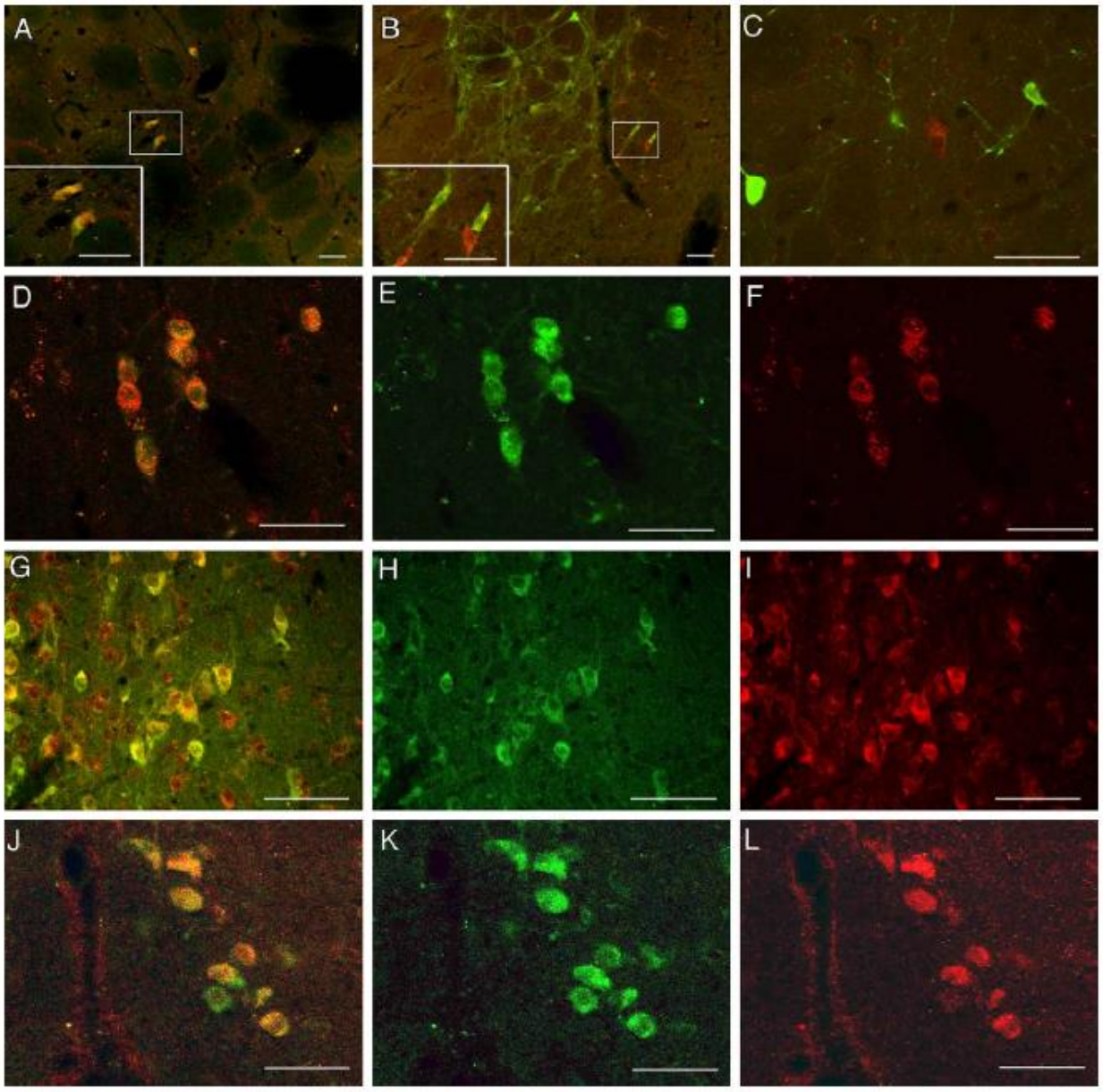

Figure 6. Confocal images illustrating co-localization of GPR30-IR with ChAT (A, D, G, J), as well as examples of Parv-labeled cells lacking GPR30-IR (B and C) (Hammond et al., 2010). Panels A and B show sections through the striatum, C-F show sections through the medial septum, G-I show sections through the nucleus basalis, and J-L show sections through the vertical limb of the diagonal band of Broca. The insets in panels A and B show indicated neurons at double the magnification. Panels E and F are unmerged images of D. H and I are unmerged

images of G. K and L are unmerged images of J. ChAT- or Parv- staining is green. GPR30-IR staining is red. Double-labeled cells appear orange/yellow. Scale bar = 50 μm in A-F and J-L and 80 μm in G-I.

C. Expression and Co-localization in the Monkey Forebrain

i. Overview

We hypothesize that GPR30 protein and co-localization with cholinergic neurons is not limited to the rat brain and can be seen in other species. Immunohistochemistry was used to evaluate GPR30 expression in the rhesus monkey forebrain.

ii. Methods

Perfused rhesus brain tissues were a gift from Dr. Tony Plant (University of Pittsburgh). Blocks of tissue containing the septum from untreated 4 one year old gonadally intact adult male rhesus monkeys (Macaca mulatta) were sectioned at 30 μm and processed for GPR30 and ChAT immunoreactivity as is described in the preceding section.

iii. Results/Discussion

Double-staining for GPR30 with ChAT is shown in Figure 3. No staining was seen in control sections incubated with secondary but not primary antibodies. Many ChAT-GPR30 double-labeled cells were detected in regions containing basal forebrain cholinergic neurons. These data are consistent with the hypothesis that GPR30 is preferentially expressed by cholinergic neurons and that estrogens may bind GPR30 to directly affect these neurons. Moreover, they show that GPR30 is expressed in the forebrains of other animal models.

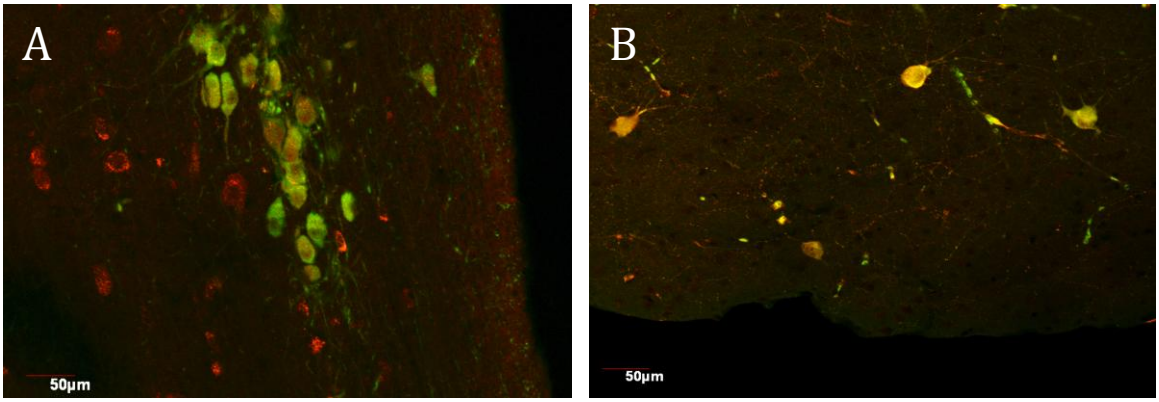

Figure 7. Confocal images illustrating co-localization of GPR30-IR with ChAT in the medial septum (A) and vertical limb of the diagonal band of Broca (B) in the rhesus brain. ChAT staining is green and GPR30 staining is red. Double-labeled cells appear orange/yellow. Scale bar = 50 µm

D. GPR30 mRNA Expression

i. Overview

One limitation of immunohistochemical studies is that antibodies can bind to proteins other than the target protein that have similar structures. To address this limitation a RT-PCR assay was developed to verify the presence of GPR30 mRNA as well as compare relative levels of GPR30 mRNA in different regions of interest, such as the hippocampus, cortex, medial septum, and striatum. We hypothesized that GPR30 mRNA is present in the regions in which we saw GPR30-like immunoreactivity, and that mRNA levels would also be highest in regions with the largest numbers of immunoreactive neurons.

ii. Methods

Three adult OVX rats were anesthetized, decapitated, and brains were removed and dissected. Using a brain matrix, the anterior portion of the brain (+4.70 mm from Bregma) was discarded. The next 3 mm (4.70 - 1.70 mm from Bregma) containing frontal and prefrontal

cortex were collected. Prefrontal cortex included regions Cg1, Cg2, and Fr2. Frontal cortex included regions Fr1 and Fr3. Next, a 3 mm slab (1.70 to -1.30 mm from Bregma) was collected containing the septum/VDB and striatum. The septum/VDB was dissected by collecting tissue between the lateral ventricles and anterior commissure. The hippocamapus was dissected from the remaining tissues. An example of a rat brain in the brain matrix is shown in Figure 4.

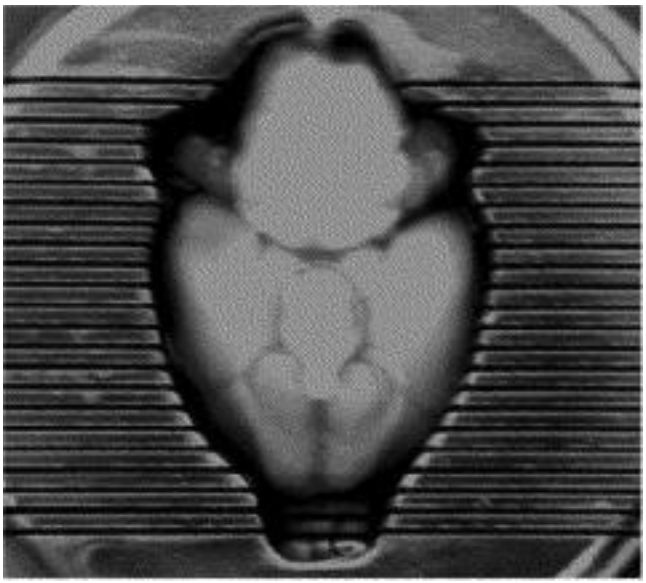

Figure 8. Rat brain in a matrix. Horizontal lines delineate where the brain is cut. Spacing between lines is 1 mm. Image from (Liu and Smith, 2003).

All tissues were frozen at -80°C. TRIzol (Invitrogen, Inc.) was added to each sample and total RNA was isolated as per manufacturer's instructions. mRNA was then reverse transcribed using the SuperScript III kit (Invitrogen, Inc.). Real-time PCR was performed using SYBR green and an ABI 7300 Sequence Detection System (ABI). All samples were run in duplicate, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to control for amount of mRNA added to the reaction. Negative controls for RT and PCR reactions included no template and no enzyme and also were performed in duplicate. The following primer sequences were used (listed in 5'-3' direction):

GPR30 sense: AGGAGGCCTGCTTCTGCTTT

GPR30 antisense: ATAGCACAGGCCGATGATGG

GAPDH sense: TGCCACTCAGAAGACTGTGG

GAPDH antisense: GGATGCAGGGATGATGTTCT

These primers were created using Invitrogen's Oligoperfect Designer. The coding sequence near the C-terminus was amplified, and these primers were generated to be 18-20 bases long, to have a primer Tm of 60-63°C, a GC content of 50%-55%, and to generate a product size of 80-130 base pairs. Data were analyzed using Sequence Detection System (SDS) software (ABI, Inc.), and results were obtained as C_t (threshold cycle) values. The average C_t for each set of duplicates was calculated. Relative expression of GPR30 was normalized to GAPDH by subtracting the average C_t for GAPDH from the average C_t for GPR30 (ΔC_t) for each region per rat. The average ΔC_t for each region was then calculated. Levels of GPR30 mRNA relative to the hippocampus were than calculated by subtracting the average ΔC_t for each region from the average ΔC_t for the hippocampus ($\Delta\Delta C_t$). Approximate GPR30 mRNA levels for each region were then calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). This entire process was performed three times, and ANOVA was used to determine significant differences in expression across brain regions.

Studies were first done to validate this assay and specifically to verify that the designed primers worked and that increasing amounts of starting material resulted in a logarithmic increase in detection of mRNA levels. cDNA was created from the hippocampus of four vehicle treated rats. Results of this validation are shown in the Figure 5.

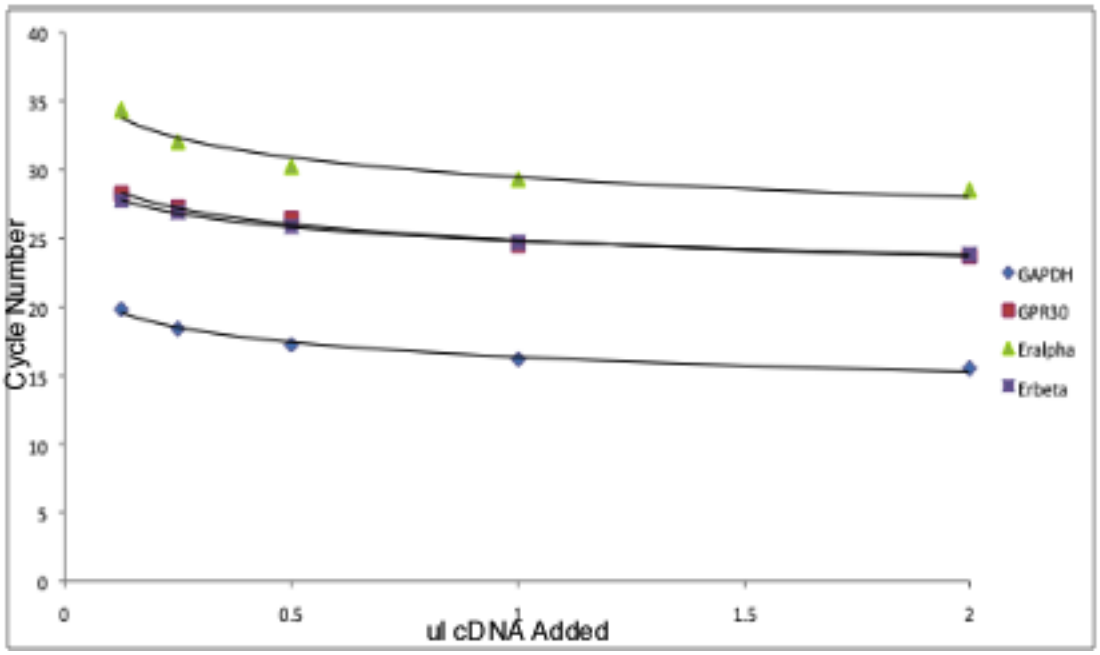

Figure 9. Graph showing that increasing amounts of starting material (cDNA) resulted in decreasing cycle number to detection threshold. n=4 per primer.

iii. Results/Discussion

Results confirmed that GPR30 mRNA could be detected in each region analyzed. Relative levels differed depending on the region of interest. The highest levels were detected in the hippocampus, whereas lowest levels were detected in the septum and striatum (Figure 4). Post-hoc analysis confirmed that levels of GPR30 mRNA in the septum were significantly lower than in the hippocampus ($p<0.05$). This data confirms immunohistochemical data showing GPR30 immunoreactivity in the regions analyzed and supports the hypothesis that GPR30 may play a role in mediating estrogen effects in these regions.

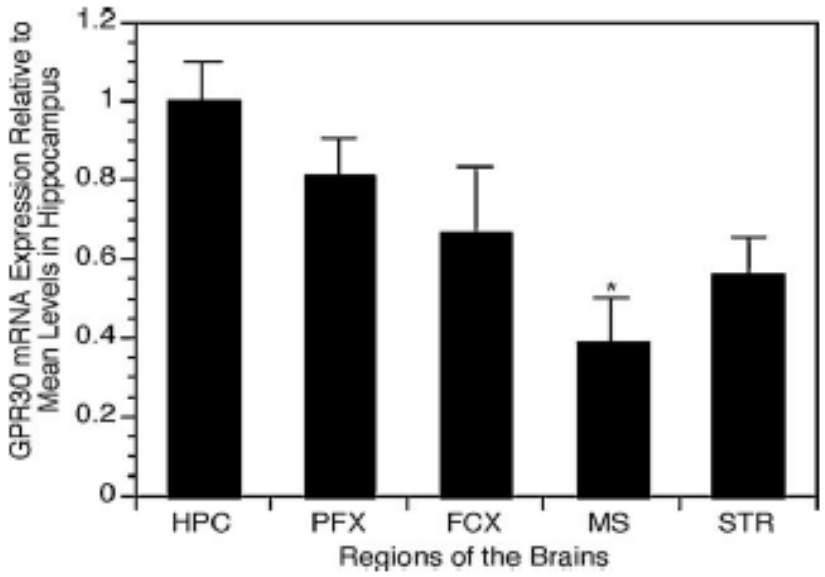

Figure 10. Bar graph comparing relative GPR30 mRNA levels in different brain regions (Hammond et al., 2010). Bars represent mean levels of GPR30 mRNA ± SEM relative to mean levels in the hippocampus. HPC: hippocampus; PFX: prefrontal cortex; FCX: frontal cortex; MS: medial septum; STR: striatum. N = 3, *p < 0.05 relative to mean levels in the hippocampus.

III. GPR30 AND BASAL FOREBRAIN CHOLINERGIC FUNCTION

A. Acetylcholine Release

i. Overview

Since we hypothesize that GPR30 mediates the ability of E2 to enhance the basal forebrain cholinergic system, i*n vivo* microdialysis and HPLC were used to compare the effects of G-1 with E2 on potassium-stimulated ACh release in the hippocampus, one measure of basal forebrain cholinergic function.

ii. Methods

Twelve OVX rats were administered E2, G-1, or vehicle using an Alzet osmotic minipump (model 2002; Durect Corp., Inc.) implanted subcutaneously in the dorsal neck region. Rats were anesthetized with a combination of ketamine (50 mg/ml) and xylazine (10 mg/ml) (0.25 cc/250 g.b.w. i.p.) For the pumps a small in incision in the skin overlying the dorsal neck region was made, the capsule was implanted, and the incision was closed with a 9 mm wound clip. Drugs were administered continuously at a dose of 5 μg/day for one week. This dose was selected based on our previous study which showed that 5 μg/day of either G-1 or E2 enhanced acquisition of a DMP T-maze task (Hammond et al., 2009). E2 was diluted in a vehicle of 13.9% DMSO + 20% hydroxypropyl-ß-cyclodextrin (HPβCD). G-1 was administered in a vehicle 25% DMSO+ 13.3% HPβCD. Half of the control group was treated with each of the vehicle solutions. At the time of pump implantation rats also received a microdialysis guide cannula (CMA 12 Elite Guides, CMA Microdialysis, Inc.) lowered into the right hippocampus (-3.4 mm bregma, 1.18 mm lateral, -3.4 mm ventral) and fixed to the skull using stainless steel screws and craneoplastic cement (Plastics One, Inc.). Dummy cannulae were inserted into the guide cannulae to retain patency. Microdialysis was performed 7 days later. Following implantation and cannulation rats were placed on a warm heating pad during recovery. Ketofen (1 mg/kg s.c.) was administered twice per day for three days to reduce discomfort.

Concentric, 3 mm microdialysis probes were used (CMA 12 Elite Probes, CMA Microdialysis, Inc.). Probes were perfused at a rate of 1 μl/min with artificial cerebrospinal fluid (ACSF; 144.3 mM NaCl, 4.0 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.7 mM Na_2HPO_4, 0.5 mM NaH_2PO_4) containing 0.8 μM neostigmine bromide. On each day of microdialysis, probes were first dialyzed for 30 minutes against a solution of ACSF containing 0.1 pmol ACh/20 μL. This sample was used to calculate and correct for probe efficiency (efficiencies averaged around

30%). The probe was then inserted through the cannula into the hippocampus. The rat was placed into a large plastic container for the duration of the experiment. Dialysates were collected continuously. Samples were collected and frozen every 30 min. After waiting 90 minutes for basal ACh release to stabilize, two additional samples were collected over one hour and were averaged to represent basal ACh release. Next probes were perfused with identical ACSF containing 60 mM potassium and samples were collected after 30 and 60 min. Probes were then perfused again with the ACSF containing 4.0 mM potassium and samples were collected every 30 minutes for an additional 60 minutes.

Dialysates were analyzed using HPLC, enzymatic conversion, and electrochemical detection as previously described (Rhodes et al., 1996). An ESA HPLC system was used. Flow rate was 0.35 μL/min. Mobile phase consisted of 100 mM di-sodium hydrogen phosphate anhydrous (Fluka, Inc.), 1-Octanesulfonic acid (Sigma-Aldrich, Inc.), Reagent MB (ESA, Inc), and the pH was adjusted to 8.0 using phosphoric acid. Samples were passed through a Shiseido Capcell Pak C18 MGII column, 100A, 3micron, 1.5 x 75mm, and then through a solid-phase reactor (ESA, Inc.) containing acetylcholinesterase and choline oxidase at 35 ˚C. The resulting H_2O_2 was detected with a model M5040 electrochemical cell attached to a Coulochem model 5300 detector (ESA, Inc.). Chromatograms were analyzed using EasyChrome software. ACh standards were prepared in ACSF. Twenty microliters of each sample was injected. All standards were run in duplicate. Quantity of ACh was determined by measuring area under the ACh peak. This assay was able to detect as little as 30 fmol of ACh per 20 μL sample. Values from *in vivo* dialysates were then corrected for probe recovery and expressed as picomoles per 20 μl sample. The two samples collected prior to the switch to high potassium were averaged as an estimate of basal ACh release. Subsequent samples were used to calculate percent change in

ACh release relative to baseline for each rat. In this way, each rat served as its own control for estimating potassium-stimulated release. Percent change that was calculated from the two samples collected over 60 minutes after switching to high potassium were averaged as a measure of percent change from basal release during the 60 minute period. Remaining samples were evaluated to verify that release declined after switching back to low potassium. This was done for each rat. Average basal and potassium-stimulated release was then calculated for each treatment group. Effects of treatment on basal and potassium-stimulated release were analyzed using one-way ANOVA followed by a Tukey test.

iii. Results

Treatment with either G-1 or E2 had no significant effect on basal extracellular levels of ACh in the hippocampus. Basal levels were 0.38±0.02, 0.34±0.03, and 0.46±0.03 pmol/20μL for controls, G-1 and E2-treated rats respectively. In contrast, treatment with G-1 or E2 produced substantial increases in potassium-stimulated ACh release (Figure 5). The average percent change relative to baseline was 135.4±6.8% for controls, versus 290.7±21.2% and 289.8±28.9% for G-1 and E2-treated groups. ANOVA revealed a significant effect of Treatment ($F[2,9]=4.50$, $p < 0.04$). Post-hoc analysis revealed that E2 and G-1 treatments differed significantly from OVX controls but not from each other. Following return to low potassium, levels of extracellular acetylcholine declined (not shown).

iv. Discussion

G-1 was able to enhance potassium-stimulated ACh release to a similar extent as E2. This suggests that activation of GPR30 alone may be sufficient to mediate this effect. Cell culture studies show that G-1 does not bind to ERα or ERβ, produces effects in cells expressing GPR30 but lacking ERα or ERβ, and does not produce effects in cells lacking GPR30 (Bologa et al.,

2006). This suggests that the effects of G-1 on ACh release are not due to G-1-mediated activation of ERα or ERβ. In the SKBR3 cell line, which expresses GPR30 but not ERα or ERβ, knockdown of GPR30 decreases E2 binding by 80% and blocks E2 signaling (Thomas et al., 2005), suggesting that GPR30 alone can mediate E2 effects. These results are somewhat controversial. Results showing a lack of GPR30 involvement in E2 signaling in mouse endothelial cells as well as SKBR3 cells also have been reported (Pedram et al., 2006), although there is evidence for a direct role of GPR30 signaling in keratinocytes (Kanda and Watanabe, 2004). It remains to be determined whether GPR30 alone is sufficient to account for effects of E2 on ACh release.

Even if GPR30 plays a significant role (as suspected) in mediating effects of E2 on ACh release, this does not mean that all effects of E2 on BFCNs are mediated by GPR30. Szego et al. showed that E2 administered either *in vivo* or *in vitro* induces pCREB in approximately 30% of the cholinergic neurons in the medial septum and substantia innominata of OVX mice, that this involved activation of the MAP kinase pathway, and that this occurred in ERβ knock-out, but not ERα knock-out mice (Szego et al., 2006). These data suggest that E2 can directly affect a subpopulation of cholinergic neurons in medial septum and substantia innominata via ERα. Whether E2 activation of MAPK and CREB involve activation of GPR30 is not yet known. The fact that effects were not observed in ERβ knock-out mice, however, suggests that activation of GPR30 alone cannot be sufficient. One possibility is that activation of GPR30 does not play a role in mediating these effects. Another possibility is that signaling via ERα cooperates with signaling via GPR30 to mediate effects on cholinergic function, perhaps via different signaling pathways. For example, the MCF-7 cell line expresses both GPR30 and ERα, and some studies show that E2 signaling via either receptor can induce c-fos expression in these cells (Maggiolini

et al., 2004). Further, it has been shown that E2-bound ERα and ERβ can regulate the expression of target genes either directly or indirectly via protein-protein interactions with other transcription factors (reviewed in (Truss and Beato, 1993). Further studies are needed to identify the extent to which GPR30 is responsible for mediating the effects of E2 on basal forebrain cholinergic function.

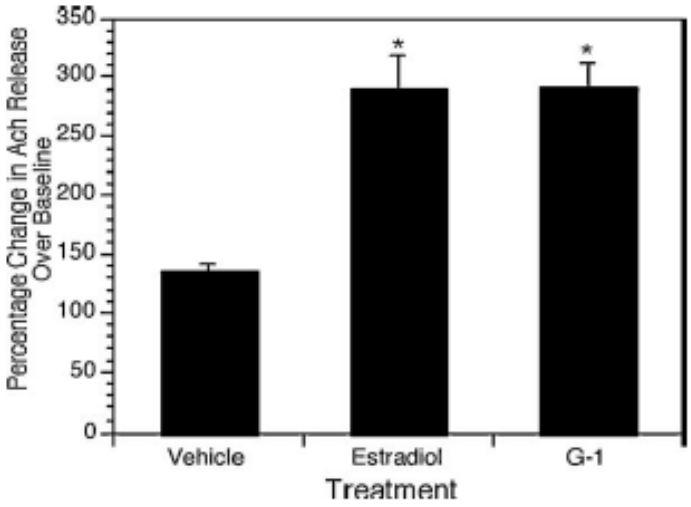

Figure 11. Bar graph showing percent change in ACh release in response to elevated potassium (Hammond et al., 2010). Bars represent average percentage change from baseline ± SEM. *$p < 0.05$ relative to vehicle-treated animals. $n = 4$ per group.

B. Induction of pCREB and pERK in cholinergic neurons

i. Overview

To further test the hypothesis that GPR30 mediates the ability of E2 to enhance the basal forebrain cholinergic system, we next sought to examine E2 effects on cholinergic neurons at the cellular level. Immunohistochemical methods were used to evaluate the rapid induction of pCREB and pERK within ChAT-positive neurons in response to intracerebroventricular (ICV) infusions of E2 or vehicle.

ii. Methods

8 adult Ovx Sprague-Dawley rats were used. Rats were anesthetized with a mixture of ketamine (50 mg/ml) and xylazine (10 mg/ml) and placed into a standard stereotaxic apparatus. The skull was exposed and a hole drilled 1.2 mm lateral from midline and +0.2 mm from Bregma. A cannula was lowered 3.3 mm into the lateral cerebral ventricle. Rats received 1 μl infusions of 1, 3, or 10 μM E2 or vehicle for a total of five minutes to allow for diffusion. Rats were then euthanized after five minutes and the brains processed as described above. Sections were pretreated with 1 % $NaBH_4$ in 50 mM PBS for 30 minutes followed by treatment with 0.3% H_2O_2 in 50 mM PBS for 10 minutes. They were then incubated with an antibody against pCREB (1:500 dilution) or pERK (1:5000) for three days at 4°C. Sections were then incubated for one hour at room temperature with a biotinylated donkey anti-rabbit secondary antibody (1:200 dilution) and then processed using avidin-biotin-HRP methods. Next, sections were incubated with a goat anti-ChAT primary antibody (1:1200 dilution) for three days at 4°C and then incubated with a biotinylated horse anti-goat secondary antibody (1:200) for one hour at room temperature. Sections were then processed using a Vector Red alkaline phosphatase kit. The reaction product was assessed using standard light microscopy. We quantified the number of ChAT labeled neurons that also stained positive for pCREB and pERK in the medial septum. We used approximately 5 sections per region per animal between plate numbers 14-18. Sections were selected based on plate number and similar plate numbers were compared across treatment groups.

iii. Results/Discussion

No differences were seen when the number of ChAT and pCREB double-labeled cells were compared across the different E2 treatments. In the plates analyzed pCREB staining could

be seen throughout the septum, but this staining was seen in less than 2% the nuclei of cholinergic neurons in all treatment groups (Figure 4). Also, no differences were seen between the different estradiol treatments and the number of ChAT+pERK double-labeled cells. The majority of pERK staining was seen in the lateral areas of the septum and in the cortex.

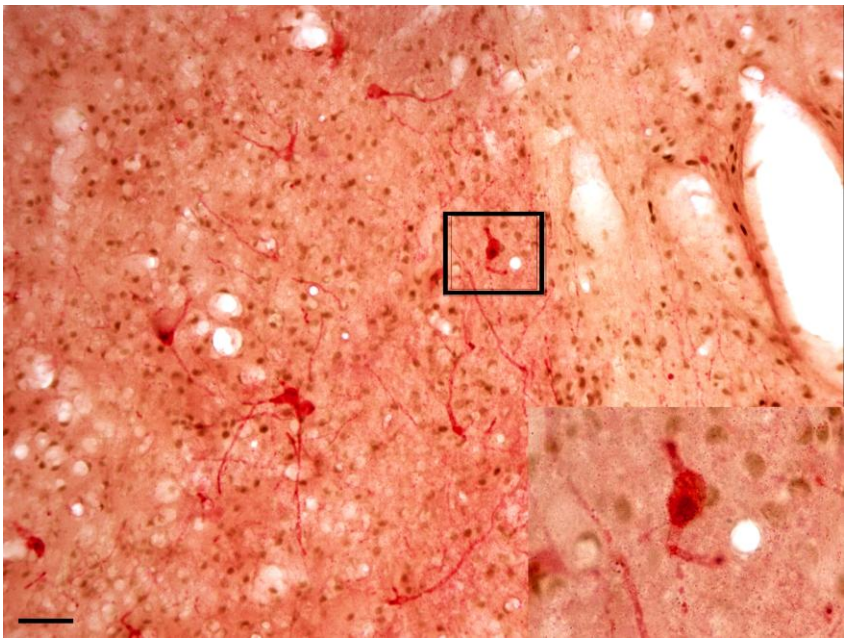

Figure 12. Image showing one of the few examples of ChAT and pCREB co-localization in the medial septum. The neuron in the rectangle is shown at the bottom right at a higher magnification. ChAT neurons are labeled in red and pCREB neurons are labeled in brown-black. Scale bar equals 50 μm.

However, dose-dependent changes in pERK and pCREB expression in the cortex and hippocampus were seen, as evidenced by increased staining in the hippocampus (Figure 6), but these were not quantified.

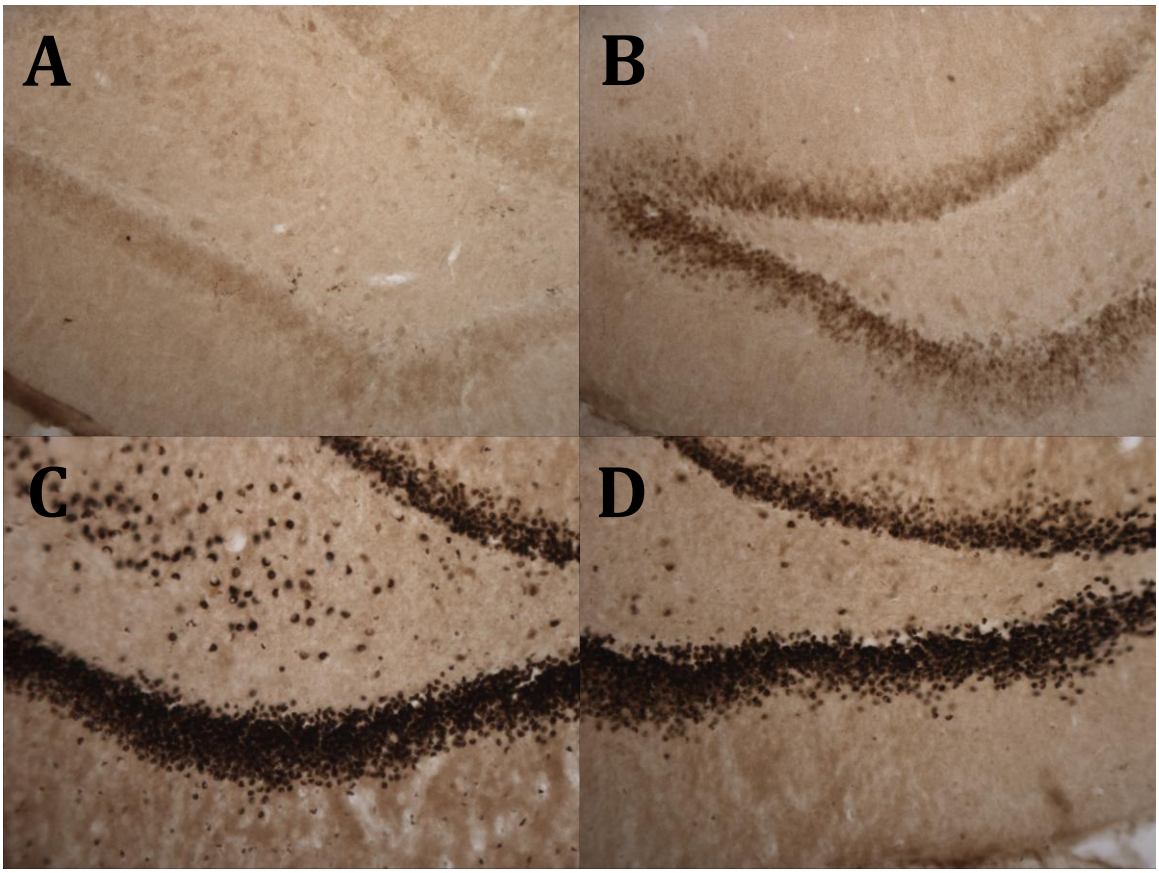

Figure 13. Images comparing pCREB expression in the dentate gyrus and CA3 regions of the hippocampus in response to E2 infusions. Panel A shows minimal pERK expression in response to vehicle infusion. Panel B shows moderate expression of pERK following 1 μM E2 treatment. Panels C and D show intense pCREB labeling following 3 μM and 10 μM E2, respectively.

We planned to examine the ability of G-1 to induce activation of pERK or pCREB in cholinergic neurons similar to E2. Since we were unable to find any acute E2-induced changes in pCREB or pERK expression in cholinergic neurons, we did not examine G-1 effects. The fact that we saw concentration-dependent changes in pCREB expression in the hippocampus suggests that the methodology used was valid. Thus, no change in pCREB or pERK expression in cholinergic neurons across varying E2 doses could be due to the time of sacrificing. Future work

could examine the effects of varying doses at longer time points after the initial E2 infusion. Currently we are replicating the finding of E2-induced pCREB expression in the hippocampus and will determine whether G-1 induces a similar effect.

C. Choline Acetyltransferase Activity

i. Overview

Choline Acetyltransferase (ChAT) is the enzyme that couples acetyl-CoA to choline, resulting in the formation of acetylcholine, and its activity is a commonly used measure of basal forebrain cholinergic function. E2 has been shown to increase ChAT activity in regions such as the frontal cortex and hippocampus (Kaufman et al., 1988; Luine, 1985), so a ChAT assay was used to compare the effects of G-1 with E2 on ChAT activity. This experiment further tests the hypothesis that GPR30 mediates estrogen effects on cholinergic function, and we predicted that G-1 would increase ChAT activity similar to E2.

ii. Methods

ChAT activity was measured in the prefrontal cortex, frontal cortex, medial septum/diagonal band, and hippocampus of 17 OVX rats treated with vehicle, E2 (5 μg/day for 1 week), or G-1 (5 μg/day for 1 week). Frozen tissues were thawed and dissociated by sonication in a medium containing EDTA (10 mM) and Triton X-100 (0.5%) (10 mg tissue/ml). An aliquot of each sample was used for the determination of total protein. To measure ChAT activity, three 5-μl aliquots of each sample were incubated for 30 min at 37°C in a medium containing [^{3}H]acetyl-CoA (50,000-60,000 d.p.m./tube, final concentration 0.25 mM acetyl CoA, Sigma, St. Louis, MO), choline chloride (10.0 mM), physostigmine sulfate (0.2 mM), NaCl (300 mM), sodium phosphate buffer (pH 7.4, 50 mM), and EDTA (10 mM). The reaction was terminated

with 4 ml sodium phosphate buffer (10 mM) followed by the addition of 1.6 ml of acetonitrile containing 5 mg/ml tetraphenylboron. The amount of [^{3}H]acetylcholine produced was determined by adding 8 ml of EconoFluor scintillation cocktail (Packard Instruments, Meriden, CT) and counting total c.p.m.s in the organic phase using an LKB beta-counter. Background was determined using identical tubes to which no sample was added. For each sample, the three reaction tubes containing sample were averaged and the difference between total c.p.m.s and background c.p.m.s was used to estimate the total amount of acetylcholine produced per sample. ChAT activity was then calculated for each sample as pmol acetylcholine produced/hr/μg protein, and these values were analyzed for each treatment group by one-way ANOVA.

iii. Results/Discussion

No significant effects were observed between either the E2 or G-1 group compared with vehicle controls, though there were trends for higher ChAT activity in the prefrontal cortex ($p=0.1$) in rats treated with E2 or G-1 versus controls. One explanation is that our sample size was not large enough to capture a significant difference, especially since there was considerable variability in some regions. Another explanation is that E2 and G-1 do not have a significant effect on ChAT activity at this specific dose and/or time point. Previous work from our lab shows that E2 does enhance cholinergic activity, but that this enhancement varies with the manner, regimen, and duration of hormone treatment (Gibbs, 2000). Specifically, it was found that short-term treatment (i.e. 2 weeks) with E2 enhanced cholinergic function within specific targets, whereas long-term repeated or continuous administration (greater than 4 weeks) produced no significant changes.

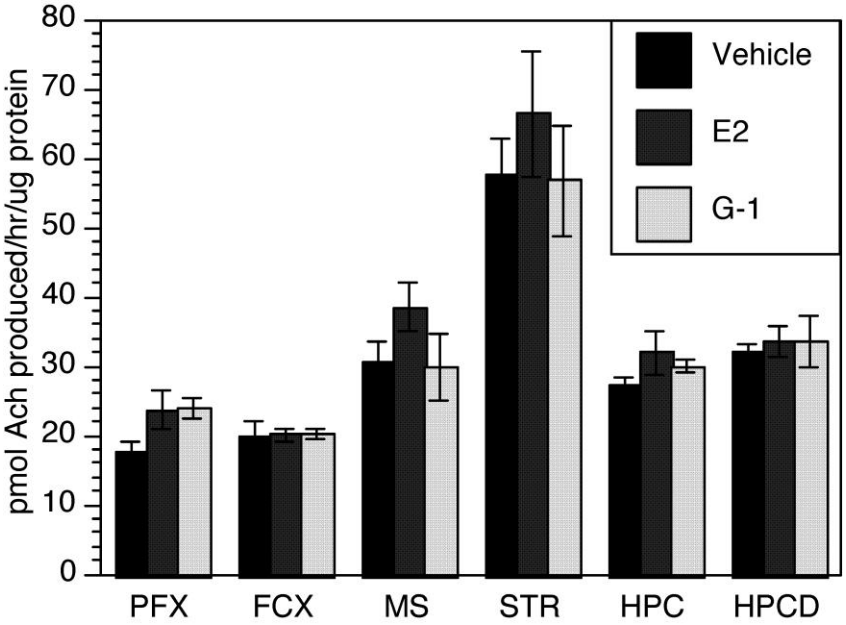

Figure 14. Bar graph comparing acetylcholine production in different regions following treatment with vehicle, E2, or G-1. Bars represent pmol acetylcholine produced/hr/μg protein ± SEM. PFX: prefrontal cortex; FCX: frontal cortex; MS: medial septum; STR: striatum; HPC: hippocampus; HPCD: hippocampus from side containing cannula. n=5-6 per group.

D. GPR30 mRNA Expression and Cholinergic Lesions

i. Overview

Cholinergic lesions in the medial septum have been shown to impair the ability of estradiol to enhance spatial learning (Gibbs, 2002). We hypothesized that this may be due to downregulation of estrogen receptor expression in the hippocampus, a key brain region that receives basal forebrain cholinergic input and that is required for memory consolidation. RT-PCR was used to examine the effects of cholinergic lesions on estrogen receptor mRNA expression in the hippocampus.

ii. Methods

Six young adult female rats were used. Rats received intraseptal injections of 192IgG-saporin (SAP) or vehicle. 192IgG-SAP is a conjugate of a mouse monoclonal antibody to rat p75NTR and the ribosome-inactivating protein saporin. Since only cholinergic neurons express the p75NTR antigen, they are selectively destroyed by the immunotoxin. One week after treatment rats were sacrificed and the brains dissected. Tissues from the septal region were immersion-fixed and later sectioned. Immunohistochemical methods were used to examine ChAT labeling and verify successful lesions. The hippocampus was dissected and frozen at -80°C. TRIzol (Invitrogen, Inc.) was added to each sample and total RNA was isolated as per manufacturer's instructions. mRNA was then reverse transcribed using the SuperScript III kit (Invitrogen, Inc.). The following primer sequences were used (listed in 5'-3' direction):

GPR30 sense: AGGAGGCCTGCTTCTGCTTT

GPR30 antisense: ATAGCACAGGCCGATGATGG

ERα sense: TCCGGCACATGAGTAACAAA

ERα antisense: TGAAGACGATGAGCATCCAG

ERβ sense: AAAGTAGCCGGAAGCTGACA

ERβ antisense: ACTGCTGCTGGGAGGAGATA

GAPDH sense: TGCCACTCAGAAGACTGTGG

GAPDH antisense: GGATGCAGGGATGATGTTCT

Real-time PCR was performed using SYBR green and an ABI 7300 Sequence Detection System (ABI). All samples were run in duplicate, a standard curve was generated, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to control for amount of mRNA added to the reaction.

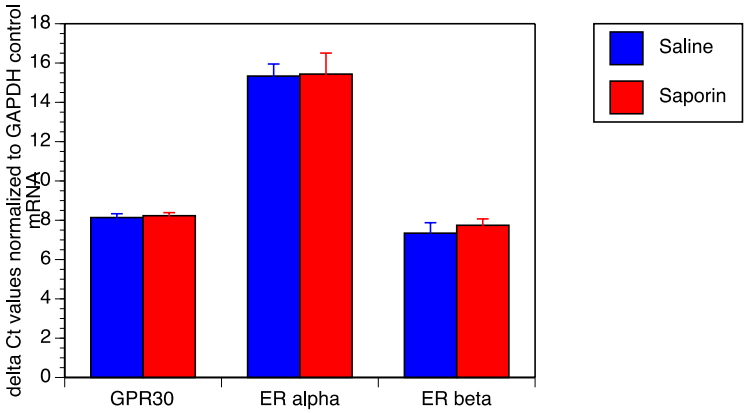

Figure 15. Bar graph showing relative ER expression in response to saline or saporin infusions. Bars represent delta Ct values of ER levels that are normalized to GAPDH control mRNA ± SEM. n=3 per group.

iii. Results/Discussion

No significant differences were found between SAP-treated and control rats for any of the ERs examined. Though SAP selectively destroyed the cholinergic neurons in the septum that project to the hippocampus (immunostaining was used to verify that cholinergic neurons in the MS were destroyed), the immunotoxin had no noticeable effect on ER expression in the hippocampus. This suggests that SAP does not impair E2 performance on spatial learning via disruption of estrogen receptors in the hippocampus. It is possible that disruption of the direct effects of E2 on cholinergic neurons in the medial septum is responsible for the impaired learning seen with cholinergic lesions.

IV. GPR30 AND COGNITIVE PERFORMANCE

A. T-Maze Acquisition

1. Comparison of G-1 and selective estrogen receptor agonists

i. Overview

We hypothesize that GPR30 mediates estrogen effects on spatial learning, so we tested the ability of a GPR30 selective agonist to enhance learning and compared this with other estrogen receptor selective agonists. Rats were chronically administered G-1 (GPR30 selective agonist), PPT (ERα selective agonist), DPN (ERβ selective agonist), or E2 and tested on a delayed matching-to-position (DMP) T-maze, a task that tests spatial learning and working memory.

ii. Methods

Animals:

A total of sixty-eight 3-4 month old female Sprague-Dawley rats (300-350 g) were purchased from Hilltop Laboratories. Rats were ovariectomized (with the exception of the gonadally intact group) prior to delivery and individually housed on a 12-hour day/night cycle with food and water available ad libitum. All procedures were carried out in accordance with PHS policies on the use of animals in research, and with the approval of the University of Pittsburgh's Institutional Animal Care and Use Committee.

Treatments:

Two weeks prior to behavioral training, animals were handled daily, food restricted, and maintained at 85% of normal body weight during acquisition and testing. Rats were administered estradiol (Sigma Chemicals; St Louis, MO), G-1 (Calbiochem; La Jolla, CA), DPN (Tocris Cookson; Ellisville, MO), PPT (Tocris Cookson; Ellisville, MO), or vehicle by Alzet model 2006 mini-osmotic pumps implanted s.c. in the dorsal neck region. Gonadally intact rats received sham surgeries. The pumps delivered a volume of 0.15 μl per hour over 42 days. G-1 has been shown to be an agonist specific to GPR30 – competitive binding studies showed that G-1 did not

compete with estradiol binding in COS7 cells transfected with either ERα or ERβ, whereas as it did compete with binding in COS7 cells transfected with GPR30 (Bologa et al., 2006). The ERα agonist used was propylpyrazole triol (PPT), a triarylpyrazole found to be approximately 410-fold more selective for ERα than ERβ (Kraichely et al., 2000; Stauffer et al., 2000). The ERβ agonist used was diarylpropiolnitrile (DPN), a compound with 70-fold higher relative binding affinity and 170-fold higher relative potency for ERβ over ERα (Meyers et al., 2001).

E2, DPN, and PPT treatments were administered at a rate of 5 μg/day at concentrations of 1.39 mg/ml in vehicles consisting of 13.9% DMSO + 20% hydroxypropyl-β-cyclodextrin (HPβCD). G-1 treatments were also administered at a rate of 5 μg/day at a concentration of 1.39 mg/ml in a vehicle 33.7% DMSO+ 13.3% HPβCD. Previous literature was used to select doses of E2, PPT, and DPN (Gibbs et al., 2004; Walf et al., 2004), but because no previous research has been published using G-1, the dose was chosen to be similar to the other agonists based on molecular weight. Vehicle controls contained either one of the vehicles listed above minus any drug treatment.

Behavior:

The DMP task is a spatial learning and memory T-maze task. The T-maze consists of an approach alley (4 in. wide x 14 in. long) and two goal arms (4 in. wide x 12 in. long). The walls of the maze are 5 in. high, and the doors are constructed of clear plexiglass, thus allowing animals to view the surrounding room. Sliding doors are positioned 8 in. down the approach alley and at the entrance to each goal arm.

Behavior training was performed as previously described (Gibbs, 1999). Testers were blinded to what rat belonged in each treatment group. Animals were first adapted to the maze by placing them in the maze with food (formula 5TUM 45 mg pellets from Test Diets, Inc.) for 5

days. Starting on day 6 through 9 animals were trained to run to the ends of the goal arms by using a series of forced choices and rewarding with four pellets. Right and left arms were alternated to avoid introducing a side bias. Next, animals began DMP testing, which was performed as 8 trial pairs per day. The first trial of each pair consisted of a forced choice in which one arm was blocked, forcing the animal to enter the unblocked arm to receive the food reward. The animal was then returned to the approach alley for the second trial in which both goal arms were open. A choice was defined as the animal placing both front legs and part of both rear legs into a goal arm. Returning to the same arm as the forced choice trial resulted in a food reward, while entering the incorrect arm resulted in no food reward and confinement for 10 seconds. Forced choices were randomized and balanced to avoid introducing a side bias. Animals were run in squads of 4 to 6. After each trial pair, the animal was returned to its home cage for 5-10 min while other animals were tested. Animals received 8 trial pairs per day until they reached the criterion of at least 15/16 correct choices over two days.

After reaching criterion, animals received a probe trial during which the T-maze was rotated 180° (relative to extramaze cues) between the forced and open trial. This was done to assess whether rats were using a place strategy (relying on extramaze cues) or a response strategy (independent of extramaze cues) to perform the task. Rats using a place strategy would be impaired by maze rotation, whereas rats using a response strategy would be unaffected. For analysis purposes, rats were given a score of 1 if they entered the same goal arm during the open choice and a score of 0 if they entered the opposite arm. After the probe trial, animals received 8 trial pairs per day for 4 days with increased intertrial delays (10, 30, 60, 90 seconds on each of the 4 consecutive days).

Following training, animals were given an overdose of ketamine (40 mg/kg) and xylazine (28 mg/kg) injected i.p. and euthanized by decapitation. Trunk blood was collected for the determination of serum E2 and luteinizing hormone (LH) levels. For estradiol analysis, samples were extracted with ether and resuspended in buffer. Samples were then analyzed in duplicate by RIA using a kit from Diagnostic Systems Laboratories, Inc. (Webster, TX). All samples were analyzed in one assay. The estradiol assay was performed by the assay core of the Center for Reproductive Physiology at the University of Pittsburgh. The minimum detectable dose was 1.2 pg/ml, and the intra-assay CV was 5.8%. Samples also were analyzed for circulating levels of LH. Samples were assayed in singlet using a sensitive sandwich immunoradiometric assay by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. This assay had a reportable range of 0.07 – 37.4 ng/ml with a sensitivity of 0.07 ng/ml. The inter- and intra-assay CVs were 10% and 5%, respectively.

Data Analysis

Rate of acquisition was defined as the number of days to reach criterion (DTC). DTC was analyzed for each treatment group by one-way ANOVA. Learning curves were constructed by plotting the mean performance (percent correct) for each group across Blocks of training. Each Block represented average performance across three consecutive days of training. Upon reaching criterion, a value of 93.8% correct, reflecting the criterion of 15/16 correct choices over 2 days, was used in calculating group performance on subsequent days. The learning curves were compared using a two factor (Treatment x Block) ANOVA with repeated measures on Block. Performance on the probe trial was analyzed by contingency table and Chi-square. Performance during the increased intertrial delays was analyzed by ANOVA with repeated measures on

Delay. Post-hoc comparisons were made using a Tukey test. Significance was set at $p \leq 0.05$. All statistical analysis was performed using JMP software for Macintosh.

iii. Results

Serum estradiol levels:

The mean levels of E2 in the E2 treatment group were 116.0 ± 39.6 pg/ml, with a range of 65.0-166.1 pg/ml. Mean E2 levels in the gonadally intact vehicle group were 15.0 ± 7.0 pg/ml, with a range of 4.7-23.1 pg/ml. Levels of E2 in non-E2 OVX treatment groups were undetectable.

Serum LH levels:

The mean serum LH levels are summarized in Table 1. ANOVA revealed a significant effect of Treatment ($F(5,61)=14.9$, $p < 0.01$), and post-hoc analysis revealed vehicle, G-1, DPN, and PPT treatment groups differed significantly from intact and estradiol groups.

Table 1. Effects of Treatment on LH levels.

Treatment	Mean LH Levels ± SEM
Intact	0.96 ± 0.85
OVX+E2	0.25 ± 1.02
OVX	8.45 ± 0.88
OVX+G-1	10.16 ± 0.99
OVX+DPN	9.08 ± 1.04
OVX+PPT	10.22 ± 0.95

DMP acquisition:

Results show that ovariectomized (OVX) animals took more days to reach criterion than gonadally intact animals, and that E2 and all three selective estrogen receptor agonists were able to restore the rate of acquisition to that of gonadally intact controls (Fig. 1). On average, OVX controls took 13±0.59 days to learn the task. All other groups took an average of approximately 9 to 10 days to reach criterion on the task. ANOVA revealed a significant effect of Treatment ($F(5,62)=6.60$, $p < 0.01$). Post-hoc analysis revealed that E2 treatment and all selective estrogen

receptor agonist treatments differed significantly from OVX controls. E2 and selective estrogen receptor agonist treatments did not significantly differ from gonadally intact controls. There was no significant correlation between DTC and circulating E2 levels in the E2-treated or intact groups. Likewise there was no significant correlation between DTC and serum LH levels in any of the treatment groups.

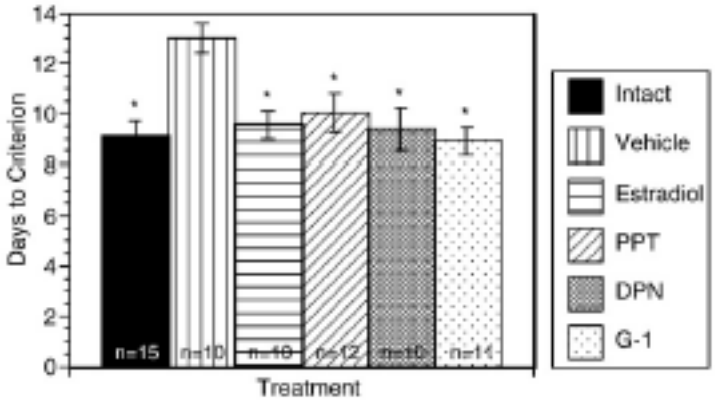

Figure 16. Bar graph summarizing Days to Criterion (DTC) for each treatment group (Hammond et al., 2009). Bars indicate mean number of days ± SEM. *p < 0.05 relative to OVX vehicle.

Examination of the learning curves revealed that all groups performed similarly below chance at the start of training (no effect of treatment on Block 1), and that rats receiving E2 or agonist treatments acquired the task at a faster rate than the vehicle group (Fig. 2). ANOVA revealed a significant effect of Treatment ($F(5,62)= 5.74$, $p < 0.01$), a significant effect of Block ($F(3,60)= 94.4$, $p < 0.01$), and a significant Treatment x Block interaction ($F15,166)=1.88$, $p<0.028$). Post-hoc analysis of the main effects of treatments collapsed across Blocks 2-5 revealed significant differences between OVX controls and intact ($p<0.01$), E2 ($p<0.0041$), G-1 ($p<0.01$), PPT ($p<0.0025$), and DPN ($p<0.01$) treatments. Significance was adjusted to $p \leq 0.01$ to account for multiple comparisons. One way ANOVA for each block followed by a post-hoc

Tukey test showed that the intact group was significantly different from OVX controls at Blocks 2-3, and that the G-1 treatment group was significantly different from OVX controls at Blocks 2-4.

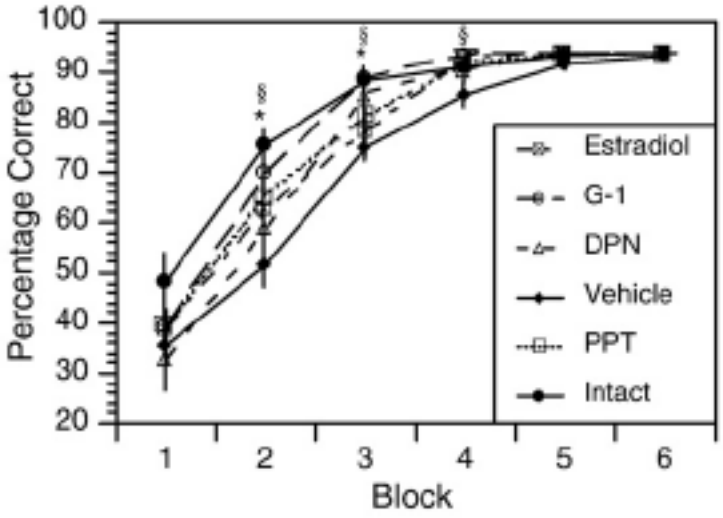

Figure 17. Learning curves showing acquisition of the DMP task over time (Hammond et al., 2009). Values represent the mean percent correct ± SEM within a 3-day block of training for each treatment group. *$p < 0.05$ for intact group relative to OVX vehicle. $^{\delta}p < 0.05$ for G-1 treatment relative to OVX vehicle.

Previous studies show that some rats will adopt a persistent turn early on during testing and that this can affect DTC (Gibbs, 2007; Gibbs and Johnson, 2007). To quantify this, we examined whether rats adopted a persistent turn, defined as entering the same arm at least 15/16 times during the choice trial over two days. Results show that a minority (26%) of rats adopted a persistent turn (consistently entering either arm of the maze). Treatments had no affect on the percentage of rats that adopted a persistent turn (X^2 (5)=2.9, p=0.71). Among rats that did adopt a persistent turn, treatments did not affect the number of days that this pattern persisted (F(5,17)=1.3, p=0.32).

Post-criterion Testing:

After reaching criterion, rotating the maze 180° between the forced and open choices significantly disrupted performance within most groups. Performance dropped from 93.8% (criterion level) as a result of rotating the maze 180˚ between the forced and open trials (Table 2). Analysis showed that none of these values differed significantly from chance with the exception of the PPT-treated group, which scored significantly above chance (X^2=9.7, p<0.02). Nevertheless, no significant overall effect of Treatment on performance during the probe trial was detected (X^2=5.9, p > 0.4).

Table 2. Effect of Treatment on Performance of 180°-Rotated Maze

Treatment	Performance on Rotated Maze
Intact	60%
OVX+E2	44.4%
OVX	53.3%
OVX+G-1	72.7%
OVX+DPN	72.7%
OVX+PPT	83.3%

Once rats had reached criterion, increasing the intertrial delay impaired performance for all treatment groups (Fig. 3). ANOVA revealed a significant effect of Delay (F(3,60)=8.9, p < 0.01), no significant effect of Treatment (F(5,62)=0.7, p > 0.6), and no significant interaction between Treatment and Delay (F(15,166)=1.15, p > 0.3).

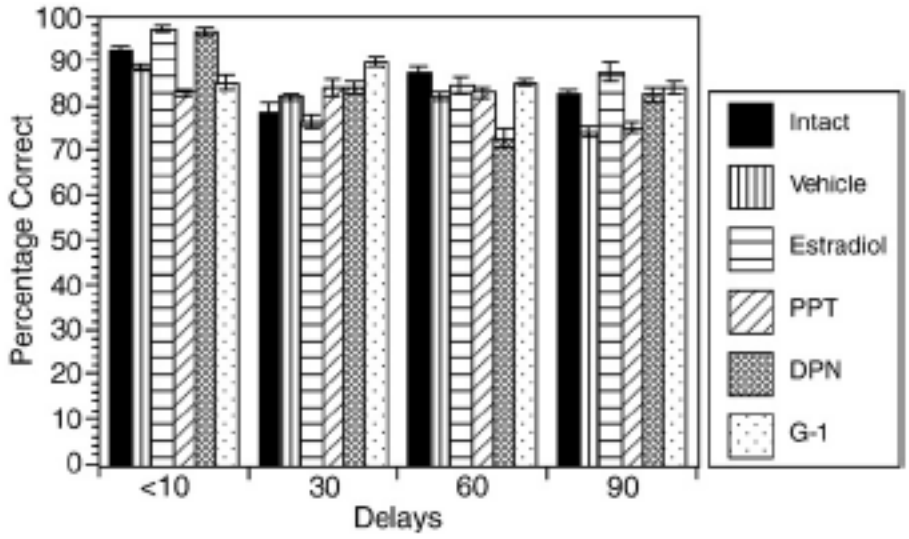

Figure 18. Effects of increasing the delay between the forced and open trials to 30, 60, and 90 seconds on DMP performance (Hammond et al., 2009). Bars represent the percentage of correct choices ± SEM for each group.

iv. Discussion

Effects of E2 and ER agonists on DMP acquisition

Our results confirm that OVX slowed the rate of DMP acquisition in young adults rats, and that PPT, DPN, G-1, and E2 each were able to restore the rate of DMP acquisition to that of gonadally intact controls. This is consistent with previous studies showing that E2 enhances DMP acquisition in young rats (Gibbs, 1999; Gibbs, 2007), and suggests that activity at any of the three established ERs might underlie this effect. Moreover, similar performance was observed in intact cycling rats and OVX rats with sustained E2 replacement. Although we did not monitor cycles, we assume that the intact rats were undergoing estrous cycles. The fact that E2 levels were low in some of these rats suggests that they were killed on diestrus, whereas others were killed at other phases of the cycle. This suggests that sustained elevated E2 levels are not required to enhance acquisition of the DMP task. Controversy exists over whether cyclical or sustained E2 treatment is more effective in enhancing cognitive performance (Gresack and Frick,

2006b; Iivonen et al., 2006; Rapp et al., 2003). We also note that the effect of E2 on the learning curve, though significant, was not as robust as in some previous reports, possibly due to the relatively high levels of E2 achieved in the E2 treatment group. Some studies have reported that high doses of E2 can impair spatial working memory in rats(Wide et al., 2004).

Recent studies suggest that elevated levels of LH that occur in response to ovariectomy may contribute to the cognitive impairment associated with ovariectomy, aging, and neurodegenerative diseases (Casadesus et al., 2007; Webber et al., 2006). It has been shown that intraperitoneal or intracerebroventricular administration of human chorionic gonadotropin produces deficits on T-maze tasks in rats (Lukacs et al., 1995), and that increased LH levels in mice are associated with declines in cognitive performance on a Y-maze task (Casadesus et al., 2007). In the present study, we detected no correlation between LH levels and the number of days required to reach criterion on the task in any of the treatment groups. In addition, G-1, DPN, and PPT all significantly enhanced the rate of DMP acquisition despite the fact that LH levels were very high relative to intact and E-treated groups. This suggests that the effects of ovariectomy and estrogen treatments on the rate of DMP acquisition are not due to effects of LH.

Treatment Effects on Learning Strategy and Working Memory

Rats can use various strategies to solve T-maze tasks, such as place strategies, which use extramaze visual cues, and response strategies, which use body positioning and kinetic cues (Dudchenko, 2001b; Korol et al., 2004). Rats also have been shown to change strategy with repeated testing or in response to estradiol treatment (Korol and Kolo, 2002; Packard and McGaugh, 1996). Evidence suggests place learning is mediated by hippocampal circuits (Zurkovsky et al., 2006) while response learning is mediated by extrapyramidal circuits, such as through the caudate (Packard and McGaugh, 1996; Squire, 1998). Moreover, place learning is

acquired faster than response learning (Chang and Gold, 2003; Packard and McGaugh, 1996). Results of the probe trial suggest that upon reaching criterion most treatment groups used extramaze cues to a significant degree while performing the DMP task. PPT-treated rats were slightly less affected by rotating the maze than the other groups, which may indicate a slightly greater predisposition to use a response vs. a place-based strategy; however, this difference was not statistically significant. In addition, treatments had no effect on the predisposition to adopt a persistent turn. This suggests that the effects of the selective ER agonists on DMP acquisition were not due to differential effects on strategy. The hippocampus also plays an important role in spatial working memory. Results of the delay trials indicate that the effects of the selective ER agonists on DMP acquisition cannot be accounted for by effects on spatial working memory. These findings are consistent with a recent analysis of the effects of E2 on DMP acquisition in young rats (Gibbs, 2007).

Comparison with Previous Studies

This study showed that chronic E2 treatment throughout training and testing enhanced acquisition of the DMP task. Others have used a post-training regimen and have shown that E2 can be administered in the period immediately after training to enhance performance. For example, OVX rats that were trained in a hidden platform water maze and given E2 injections less than two hours post training performed better than vehicle controls when returned to the maze 24 hours later for a retention test. This enhancement in retention did not occur when E2 was given later than two hours post training (Packard, 1998). In another case, OVX rats were trained in an inhibitory avoidance task and tested following a 24 hour delay, and it was shown that E2 administration immediately, but not 1, 2, or 3 hours post training, increased crossover

latencies compared to vehicle (Rhodes and Frye, 2004). Taken together, these suggest that E2 may affect both acquisition and memory consolidation.

In addition to spatial learning and memory, others have examined the effects of DPN and PPT on stress and anxiety. Daily treatment with DPN has been shown to decrease anxiety-related behaviors on tasks such as the elevated plus maze or open field, suggesting that ERß mediates select anxiolytic effects of E2 (Lund et al., 2005; Walf and Frye, 2005). In contrast, daily injections of PPT have been reported to increase anxiogenic behaviors such as the number of fecal boli and time spent grooming (Lund et al., 2005). Thus, it is important when analyzing effects of PPT and DPN on learning tasks to consider the degree to which stress and anxiety play a role in each task. Though we did not measure corticosterone levels, to our knowledge stress and anxiety are not major components of the DMP task, analogous to other food-motivated land-based navigational tasks. While rats do experience the stress of food deprivation, the task does not include an acute stressor such as foot shock or swimming, which are major components of standard inhibitory avoidance and water maze tasks.

The parallel effect of the three agonists in facilitating acquisition raises the possibility that E2 and the three agonists may affect non-mnemonic processes such as sensory-motor functions, motivational factors, or attentional mechanisms. Previous work from our lab has shown that OVX and E2 treatments that affect DMP acquisition have no effect on a configural association operant conditioning task (Gibbs and Gabor, 2003). This task requires rats to distinguish between visual and auditory stimuli and is motivated by the same food reward associated with the DMP task. The fact that OVX and E2 treatments have no effect on this task reduces the likelihood that effects on motivation and sensory perception underlie the effects on DMP acquisition.

What circuits underlie the effects of the selective estrogen receptor agonists on DMP acquisition is unknown. As mentioned earlier, ERα immunoreactivity and mRNA have been found in the hippocampus, and ERβ immunoreactivity and mRNA have been found in the hippocampus and neocortex (Mitra et al., 2003; Osterlund et al., 1998). This raises the possibility that DPN and PPT may act directly at one of these two regions to affect DMP acquisition.

This is the first study to show an effect of G-1 administration on a cognitive task. Results show that G-1 had as much of a beneficial effect on DMP acquisition as any of the other agonists, suggesting that activation of GPR30 is a viable strategy for enhancing performance within specific cognitive domains.

2. Effects of G-15, a GPR30 Antagonist

i. Overview

The previous section showed that administration of G-1 to OVX rats enhances acquisition of a delayed matching-to-position (DMP) T-maze task similar to E2. The next goal was to evaluate the effects of a selective GPR30 antagonist (G-15) administered to gonadally intact rats, and to OVX rats treated with or without E2. We hypothesized that use of a GPR30 antagonist would impair the ability of E2 to enhance spatial learning. Rats were chronically administered G-15 at 5, 10, or 40 μg/day and tested on DMP T-maze as already described.

ii. Methods

A total of 75 3-4 month old female Sprague-Dawley rats were used Two weeks prior to treatments and testing, rats were handled daily for 5 minutes, food restricted, and maintained at 85% of normal body weight during acquisition and testing on both tasks. 2-3 weeks following ovariectomy rats were given drug treatments. Rats were administered G-15 (a gift from Eric Prossnitz, University of New Mexico, also purchased from Calbiochem, Inc.; La Jolla, CA) or

vehicle by Alzet model 2006 mini-osmotic pumps implanted s.c. in the dorsal neck region. The pumps delivered a volume of 0.15 μl per hour over 42 days. G-15 was administered at a rate of 5, 10, or 40 μg/day in a vehicle of 33.7% DMSO + 20% hydroxypropyl-β-cyclodextrin (HPβCD). Doses were selected based on our previous work showing that 5 μg/day of the selective GPR30 agonist G-1 enhanced learning on the DMP task similar to estradiol (Hammond et al., 2009). Rats receiving E2 received 3-mm silastic capsules (length 5 mm; inner diameter 1.47 mm) implanted s.c. in the dorsal neck region. The E2 capsules produce levels of E2 in the physiological range for up to 2 months post-implantation. These levels have been shown to enhance DMP acquisition in young adult OVX female rats (Gibbs, 1999; Gibbs, 2002; Gibbs et al., 2004). One week following implantation of pumps and/or capsules rats began T-maze training.

Following training, rats were given an overdose of ketamine (40 mg/kg) and xylazine (28 mg/kg) injected i.p. and euthanized by decapitation. Trunk blood was collected for the determination of serum estradiol levels, which were determined using a sensitive LC-MS/MS assay recently developed by the Small Molecules Biomolecular Core Facility in our Department of Pharmaceutical Sciences. Samples were spiked with internal standard (2,4,16,16,17-d3-17ß-estradiol) and then extracted with n-butyl chloride. After centrifugation and evaporation, the residue was derivatized in 0.1 mL buffered dansyl chloride solution (pH 10.5). E2 was eluted from a Waters Acquity UPLC BEH C18, 1.7 um, 2.1 × 150 mm reversed-phase column, with an acetonitrile:water (0.1% formic acid) gradient. Detection and quantitation were achieved in the positive mode with a Thermo Fisher TSQ Quantum Ultra mass spectrometer interfaced via an electrospray ionization (ESI) probe with the Waters UPLC Acquity solvent delivery system. Transitions used for analysis were 506 → 171 for E2, and 511 → 171 for the deuterated internal

standard. Area under the peak was quantified and used to determine absolute levels of E2/mL of sample by comparison with a series of standards. The limit of detectability for this assay is 2.5 pg/mL. Intra-assay statistics show errors below 8.1% and relative standard deviations below 10.4%. Inter-assay statistics show errors below 5.0% with relative standard deviations below 7.4%.

Rats were tested in the T-maze as described in the previous chapter.

Partway through the study we realized that E2 levels in many of the gonadally intact rats were relatively low. This was likely due to the fact that rats were euthanized randomly at different points along the estrous cycle. Nevertheless, we thought it best to test any potential effect of G-15 on the estrous cycle and on levels of E2. A separate cohort of gonadally intact rats were treated with G-15 (40 μg/day) or vehicle for a period of four weeks. Daily vaginal smears were collected beginning several days following the initiation of treatment. Smears were stained with cresyl violet and analyzed to determine cycle stage. After four weeks of treatment, rats were euthanized and plasma was collected and analyzed to determine serum levels of E2 as described above.

iii. Results

Serum estradiol levels:

Mean levels of E2 in the gonadally intact rats are summarized in Table 3. Levels in many rats were below detectability and were assigned values of 0 for the purpose of calculating averages. However, these low values are not unexpected given that rats were euthanized randomly with respect to the estrous cycle. Group differences were not statistically significant. E2 levels for all OVX rats were undetectable. Levels in the OVX+E2 group were 50.7 ± 1.52 pg/ml, with a range of 32.32 – 75.28 pg/ml. Levels in the OVX+E2+G-15 group were 49.56 ± 1.47 pg/ml, with a

range of 34.52 – 77.31 pg/ml. These levels are consistent with the reported peak levels obtained on the afternoon of proestrus (McGinnis et al., 1981). In the separate cohort of rats whose cycles were monitored, mean levels of E2 were 5.05 ± 4.40 pg/ml and 9.60 ± 5.17 pg/ml for vehicle- and G-15-treated rats. These differences were not statistically significant. Again, levels were undetectable in some rats, consistent with the fact that some rats were sacrificed during diestrus or metestrus.

Table 3. Effects of Treatment on Serum E2 Levels

Treatment	Serum E2 Levels ± SEM
Intact	4.6 ± 0.65 pg/ml
OVX+G-15 (5 µg/day)	1.9 ± 0.14 pg/ml
OVX+G-15 (10 µg/day)	2.32 ± 0.34 pg/ml

Analysis of the vaginal smears indicated that all rats in both the vehicle and the G-15-treated groups experienced estrous cycles of 4-5 days in length. Cycle stage was determined by classifying the epithelial cells into parabasal, intermediate, or superficial cells. Parabasal cells are the smallest cells seen in a smear and are prevalent during diestrus, anestrus, and early proestrus, but absent during estrus. Intermediate cells are typically 2-3 times the size of parabasal cells and are seen during all stages except estrus. Superficial cells are the largest cells with polygonal shapes and pyknotic or absent nuclei. They are the defining cell type during estrus. Examples of these cell types are shown in Figure 13.

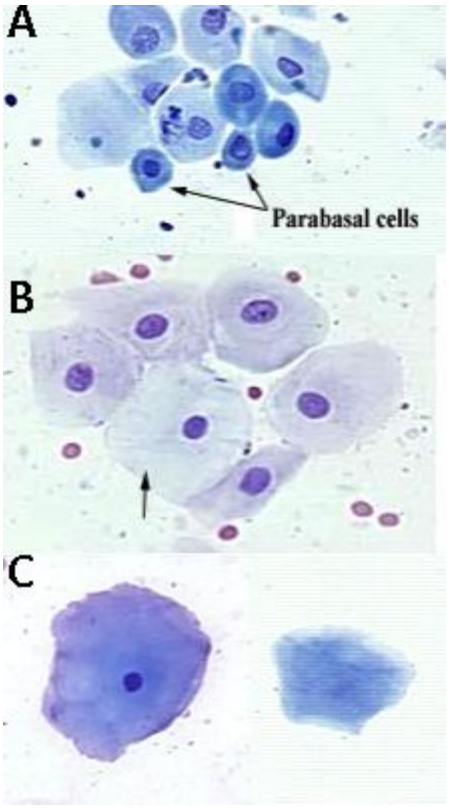

Figure 19. Examples of typical epithelial cell types seen in vaginal smears (Bowen, 1998). A. Arrows point to parabasal cells. B. Arrow points to a large intermediate cell. C. Examples of two superficial cells. The left cell has a pyknotic nuclei and the right has none (these cells are sometimes referred to as fully cornified).

DMP T-maze acquisition:

G-15 dose-dependently impaired performance in gonadally intact rats (Figure 1). Gonadally intact rats treated with 10 μg/day of G-15 required significantly more days on average to reach criterion (13.3 ±0.9 days for rats treated with 10 μg/day G-15) than vehicle treated intact controls (10.0 ± 0.3 days) and performed similarly to OVX controls. ANOVA revealed a significant effect of Treatment ($F(2,23)=7.66$, $p < 0.01$). Post-hoc analysis revealed that in intact rats treated with G-15 at a dose of 10 μg/day differed significantly from gonadally intact controls.

G-15 dose-dependently impaired performance in OVX+E2-treated rats (Figure 1). OVX rats treated with E2 + 40 μg/day G-15 required significantly more days to reach criterion (14.0 ± 0.9) than OVX rats treated with E2 alone (9.2 ± 0.6). ANOVA revealed a significant effect of Treatment ($F(4,44)=5.64$, $p < 0.01$). Among OVX rats, those treated with E2 learned significantly faster than OVX controls, and OVX+E2+G-15 (40 μg/day) rats took significantly longer than OVX+E2 rats ($p<0.05$ in each case). Rats treated with G-15 alone did not differ significantly from OVX controls but differed significantly from OVX+E2-treated rats.

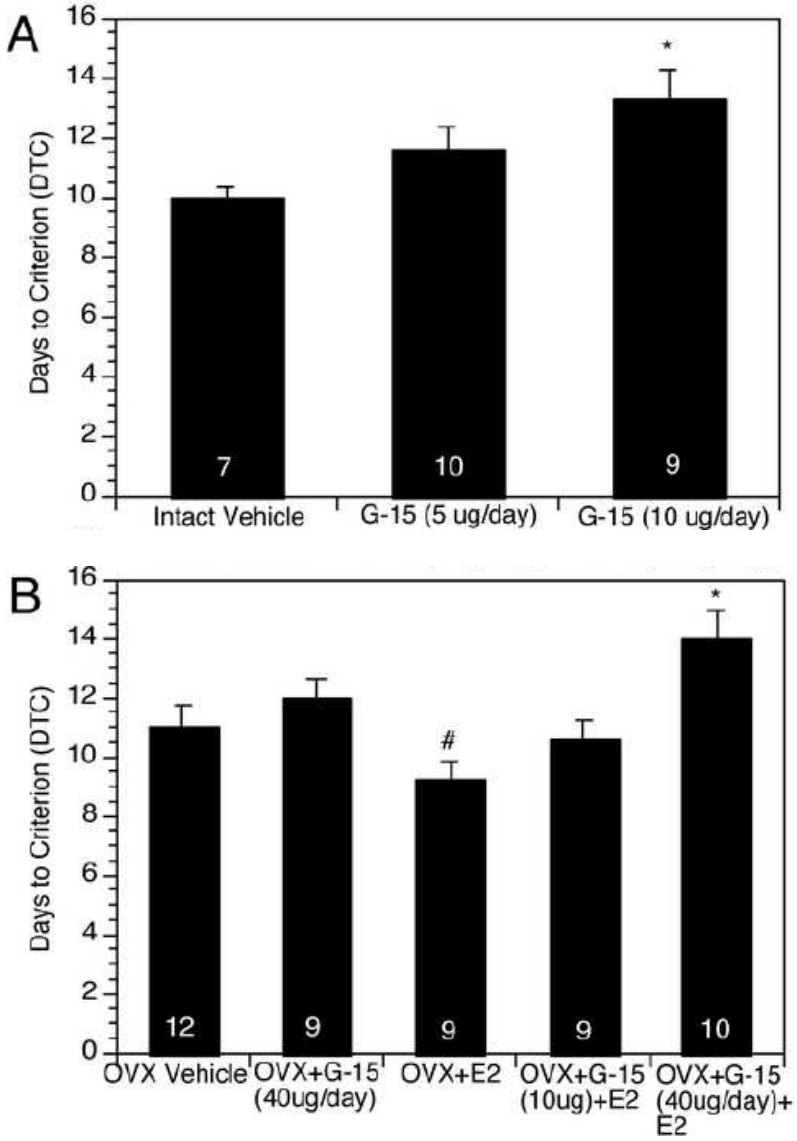

Figure 20. Effects of G-15 on DTC in gonadally intact rats and in OVX rats. 1A shows G-15 dose-dependently increased DTC in gonadally intact rats, similar to the effects of OVX. 1B shows E2 treatment significantly decreased DTC in OVX rats and this was dose-dependently reversed by G-15. G-15 alone had no significant effect on DTC in OVX rats. Bars indicate mean number of days to react criterion ± s.e.m. *$p < 0.05$ relative to gonadally intact controls or OVX+E2-treated rats. # $p < 0.05$ relative to OVX vehicle controls.

Learning curves show that all groups performed similarly below chance at the start of training and then improved at different rates. Among the gonadally intact rats, controls acquired the task at a significantly faster rate than those that recevied G-15 at 10 μg/day (Figure 2A). In analyzing Blocks 3-5, ANOVA revealed a significant effect of Treatment ($F(2,23)=3.43$, $p < 0.05$) and a significant effect of Block ($F(2,22)= 18.5$, $p < 0.01$). Post-hoc analysis revealed significant differences between the controls and the G-15 (10 μg/day) group ($p < 0.01$) on Block 4.

Among OVX rats, those treated with E2 acquired the task at a significantly faster rate than all other OVX groups (Figure 2B). OVX controls did not differ significantly from any of the G-15-treated groups. In analyzing Blocks 3-5 ANOVA revealed a significant effect of Treatment ($F(4,44)= 4.83$, $p < 0.01$) a significant effect of Block ($F(2,43)= 44.2$, $p < 0.01$), and a significant Treatment x Block interaction ($F(8,86)=2.1$, $p < 0.05$). Post-hoc analysis revealed significant differences between the OVX+E2 group and the OVX+G-15 (40 μg/day) group and OVX+E2+G-15 (40 μg/day) group on Blocks 3 and 4.

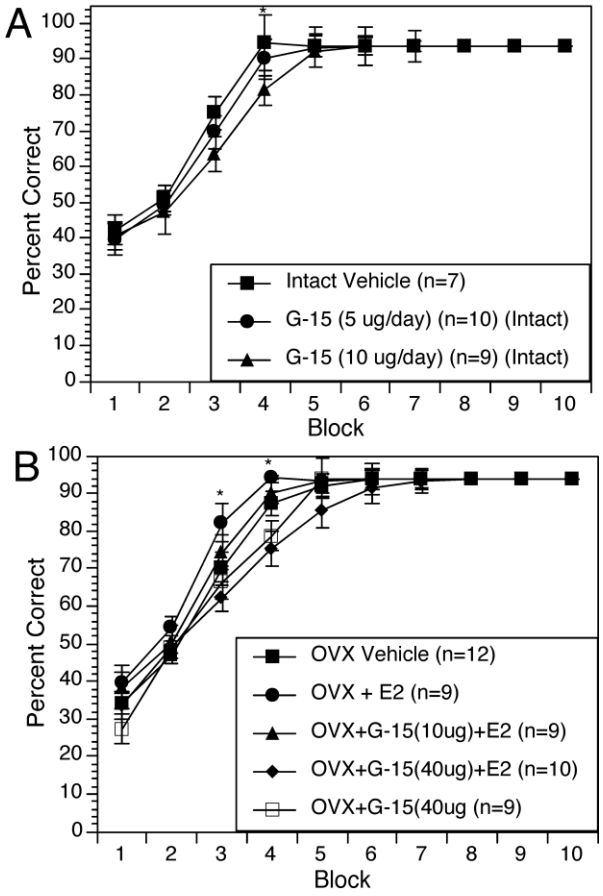

Figure 21. Learning curves showing acquisition of the DMP task over time. Values represent the mean percent correct ± s.e.m. within a 3-day block of training for each treatment group. Panel 2A shows learning curves for all gonadally intact rats. *p < 0.05 for G-15 (10 μg/day) relative to intact vehicles. Panel 2B shows learning curves for all OVX rats. *p < 0.05 for OVX+G-15 (40 μg/day) and OVX+E2+G-15(40 μg/day) relative to OVX+E2-treated rats.

Previous studies show that some rats adopt a persistent turn early on during DMP training and that this can affect DTC (Gibbs, 2007; Gibbs and Johnson, 2007). To quantify this, we examined whether rats adopted a persistent turn, defined as entering the same arm at least 15/16 times during the choice trial over a two day period. Results show that ovariectomy significantly increased the percentage of rats that adopted a persistent turn (28.6% of gonadally intact controls

vs. 83.3% of OVX controls). Treatments also affected the percentage of rats that adopted a persistent turn. Among gonadally intact rats, G-15 dose-dependently increased the percentage of rats that adopted a persistent turn (X^2=6.9, $p < 0.05$) as well as the average numbers of days that rats engaged in a persistent turn (Table 1). Among OVX rats, E2 significantly decreased the percentage of rats that adopted a persistent turn and decreased the average number of days that rats engaged in a persistent turn. However, G-15 at the highest dose blocked this effect. G-15 alone had no effect on persistent turn on OVX rats (X^2=23.1, $p < 0.01$, Table 1).

Table 1: Effects of Treatments on Adoption of a Persistent Turn During DMP Training

Group	**Percentage that Adopted a Persistent Turn**	**Average Length of Persistent Turn (Days ± s.e.m.)**
Intact Vehicle Controls	28.6	0.6 ± 0.4†
Intact+G15 (5ug/day)	50.0	2.0 ± 0.8
Intact+G15 (10ug/day)	88.9*	3.6 ± 0.7*
OVX Vehicle Controls	83.3*	4.1 ± 0.6*
OVX+G15 (40ug/day)	88.9*	3.4 ± 0.6
OVX+E2	33.3†	2.0 ± 0.3†
OVX+E2+G15 (10ug/day)	11.1†	2.0 ± 0.2†
OVX+E2+G15 (40ug/day)	90.0*	5.4 ± 1.2*

*p<0.05 relative to Intact Vehicle controls; †p<0.05 relative to OVX Vehicle Controls

Post-criterion Testing:

After reaching criterion, rotating the maze 180° between the forced and open choices significantly disrupted performance within most groups. Performance dropped from 93.8% (criterion level) to 42% for intact groups and to 49% for all OVX groups as a result of rotating the maze 180˚ between the forced and open trials. Analysis showed no significant effects of Treatment on performance during the probe trial (X^2=0.95, $p > 0.6$ for intact groups; X^2=2.1, $p > 0.7$ for OVX groups), suggesting that treatments did not differentially affect the degree to which rats relied upon extramaze cues to solve the task.

During post-criterion testing, increasing the intertrial delay impaired performance for all treatment groups (Figure 3). Treatments did not differentially alter this effect. For gonadally intact rats, ANOVA revealed a significant effect of Delay ($F(3,21)=5.7$, $p < 0.01$), no significant effect of Treatment ($F(2,23)=0.11$, $p > 0.3$) and no significant interaction between Treatment and Delay ($F(6,42)=0.71$, $p > 0.3$). For OVX groups, ANOVA revealed a significant effect of Delay ($F(3,42)=13.4$, $p < 0.01$), no significant effect of Treatment ($F(4,44)=0.46$, $p > 0.8$), and no significant interaction between Treatment and Delay ($F(12,111)=0.77$, $p > 0.5$).

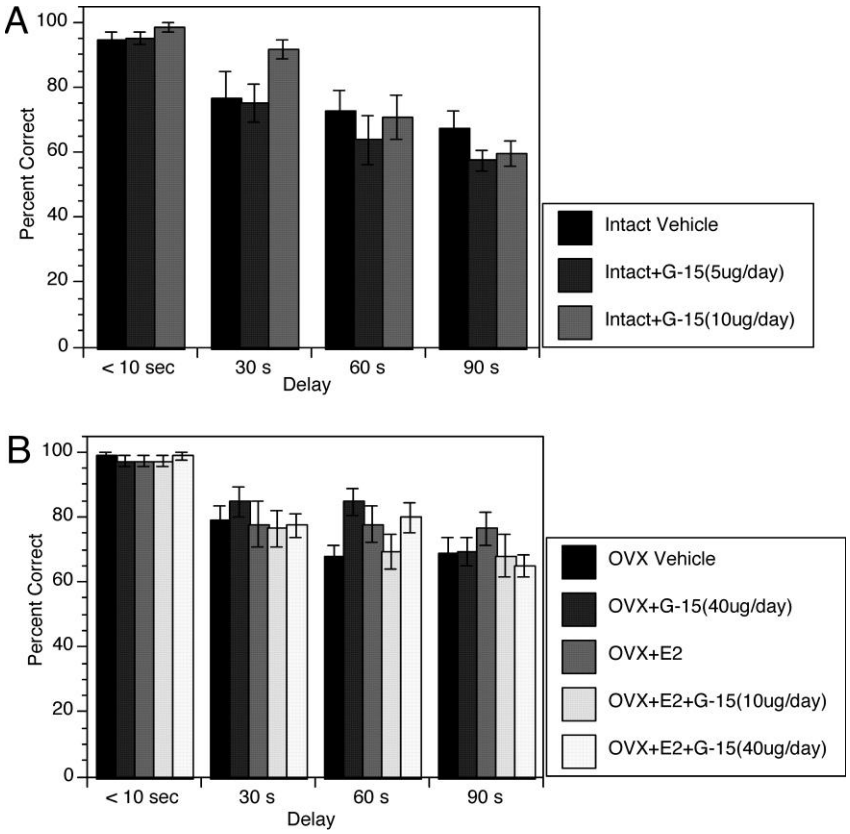

Figure 22. Bar graphs showing effects of increasing the delay between the forced and open trials to 30, 60, and 90 seconds on performance. Bars represent the percentage of correct choices ± s.e.m. for each group. Panel A shows results for gonadally intact rats. Panel B shows results for OVX rats.

iv. Discussion

Effects of E2 and G-15 on DMP acquisition

Our findings corroborate previous work showing that ovariectomy slows the rate of DMP acquisition in young adult rats, and that E2 can restore the rate of DMP acquisition to that of gonadally intact controls (Gibbs, 1999; Gibbs, 2007). The present study showed that G-15 dose-dependently impaired acquisition in gonadally intact rats and blocked the effect of E2 on DMP acquisition in OVX rats. Rats treated with the higher doses of G-15 performed comparable to OVX controls. Notably, the dose required to block the effect of E2 in OVX rats was higher than that needed to affect performance in gonadally intact controls, possibly because OVX+E2-treated rats had sustained elevated levels of E2 in the high physiological range.

G-15 at the dose used had no apparent effect on DMP acquisition in OVX rats, suggesting that results were not due to a non-specific effect of G-15 on performance. Recent work from Smejkalova et al. suggests that E2 synthesized in the brain may be important for neural plasticity in the hippocampus (Smejkalova and Woolley, 2010). The finding that G15 fails to influence learning or working memory in OVX rats also suggests that local release of E2 and subsequent signaling through non-nuclear means does not play a substantial role in the observed effects of E2 treatment on this task. Moreover, there were no treatment effects on delay-dependent performance once rats had reached criterion. This is consistent with previously published work from our lab showing that E2 enhances the rate of acquisition but not performance during increasing intertrial delays (Gibbs et al., 2004; Gibbs, 2007). These finding are consistent with the hypothesis that the effects of E2 on DMP acquisition are task-specific and may be mediated, at least in part, via GPR30.

Effects of treatments on learning strategy

Rats use various strategies to solve T-maze tasks such as place strategies (use of extramaze visual cues) and response strategies (use body positioning and kinetic cues) (Dudchenko, 2001a; Korol et al., 2004). Strategy choice is hormone-sensitive, as Korol et al. showed changes in learning strategies from place to response across the estrous cycle (Korol et al., 2004), and others have shown that acute and chronic E2 treatment biases rats towards place strategies (Daniel and Lee, 2004; Davis et al., 2005; Korol and Kolo, 2002). Results from the probe trial suggest that while rats in each treatment group used both strategies to solve the task, there was a trend for intact and E2-treated rats to use place strategies and OVX or G-15 treated rats to use response strategies.

Effects of G-15 were not due to effects on estrous cycles or ovarian function

E2 levels in the gonadally intact rats were low. This can be explained by the fact that rats were killed randomly with respect to the estrous cycle. Nevertheless, we considered the possibility that G-15 might affect performance by interfering with ovarian function. In a separate cohort of rats, we observed no effect of sustained G-15 delivery on estrous cycles or on serum levels of E2. This is consistent with the fact that in mice, knock-out of the GPR30 gene does not impair normal reproduction (Otto et al., 2009a). This suggests that the effects of G-15 on DMP acquisition in gonadally intact rats were not due to an effect on ovarian function or on circulating levels of E2.

Effects on persistent turn and relevance to basal forebrain cholinergic neurons

Results also show that gonadally intact rats treated with G-15, and OVX+E2-treated rats that received G-15, were more likely to adopt a persistent turn during DMP acquisition than rats that did not receive G-15. In fact, in cycling rats 8 out of 9 rats receiving 10 μg/day of G-15 adopted a persistent turn, in comparison with only 2 out of 7 vehicle treated controls. G-15 also

increased the average number of days that rats engaged in a persistent turn. The same trend was observed in the OVX+E2 rats treated with the highest dose of G-15. This suggests that G-15 impairs performance on this task by increasing the predisposition to adopt and maintain a persistent turn. Notably, these same effects are produced by basal forebrain cholinergic lesions (Gibbs and Johnson, 2007), suggesting that both G-15 and lesions affect response pattern, strategy selection, and the ability to alter strategy (i.e., cognitive flexibility) in a similar way.

As mentioned above, cholinergic neurons in the medial septum and nucleus basalis magnocellularis are the primary source of cholinergic inputs to the hippocampus and cortex and play an important role in learning, memory, and attentional processes (Baxter and Chiba, 1999; Everitt and Robbins, 1997; Gritti et al., 1997; Mesulam, 1996). Studies show that selectively lesioning these cells produces an increase in the predisposition to adopt a persistent turn during DMP acquisition (Gibbs, 2002; Gibbs, 2007). A similar effect is observed in aged rats and can be reversed, in part, by treating with selective cholinesterase inhibitors (Bohacek et al., 2008; Gibbs et al., 2009) (Gibbs et al., 2011a; Gibbs et al., 2011b). This suggests that cholinergic inputs to the hippocampus and cortex have a strong effect on perseveration and the adoption of a persistent turn during DMP training. Prior studies show that E2 has a number of effects on basal forebrain cholinergic neurons including increasing choline acetyltransferase mRNA and protein (Gibbs et al., 1997; Gibbs, 2000; Singh et al., 1994), increasing high affinity choline uptake in the hippocampus and frontal cortex (O'Malley et al., 1987; Tinkler et al., 2004), and increasing potassium-stimulated acetylcholine release (Gabor et al., 2003; Gibbs, 1997). In addition, we have shown that septal cholinergic neurons are essential for E2-mediated enhancement of DMP acquisition (Gibbs, 2002). Recently we showed that cholinergic neurons in the medial septum and nucleus basalis magnocellularis also contain GPR30, and that G-1, a selective GPR30

agonist, enhances potassium-stimulated acetylcholine release in the hippocampus similar to E2 (Hammond et al., 2010). Collectively, these data suggest that E2 can enhance basal forebrain cholinergic function via GPR30, and support the hypothesis that the effects of G-15 on DMP acquisition are due, at least in part, to a blockade of E2 effects on basal forebrain cholinergic neurons. Future studies will focus on testing this hypothesis. Specifically, studies looking at G-15 effects on the ability of E2 to enhance potassium-stimulated acetylcholine release or to enhance choline acetyl transferase activity will help further test this hypothesis.

B. OPERANT CONDITIONING

i. Overview

As was discussed in the introduction, E2 enhances learning in a task-specific manner. Previous work from our lab has shown that E2 does not enhance learning in operant conditioning tasks (Gibbs et al., 2011a). We examined the effects of G-15 on a non-spatial learning task. A subset of rats that were chronically administered G-15 at 5 or 10 μg/day and completed the DMP T-maze task were next tested in a successive conditional discrimination (SCD) task. We hypothesized that, like E2, G-1 would only enhance learning on the spatial (T-maze) task.

ii. Methods

Upon completion of the DMP T-maze task rats were trained on the SCD task. Training was performed in operant chambers (Med. Associates, Inc., Georgia, VT) connected to a computer running Med-PC software. Each operant chamber contained a dim red house light, a ventilation fan, a 6 W stimulus panel light, a speaker calibrated to present a 1500 Hz tone, a pellet dispenser, and a recessed food cup located immediately below the panel light. Entry into the food cup was monitored by a photosensor. Rats were adapted to the chamber by receiving

one 60 min session during which they received a total of 16 food pellets delivered at intervals ranging from 2 to 6 min. Testing began the following day. Each rat received one training session per day for a total of 14 days. During the session, rats received 30 presentations of a tone stimulus and 30 presentations of a light stimulus. Rats were randomly divided into 2 groups and trained to respond to only one of the stimuli (either tone or light), which will be referred to as the reinforced stimuli. If the rat entered the food cup within 10 sec of the presentation of the reinforced stimuli, the stimulus discontinued and the animal received a food reward (one 45 mg pellet). If an animal entered the food cup during the non-reinforced stimulus, the stimulus was discontinued, the house light turned off for 60 sec, and no food was delivered. This is referred to as a time out. The stimuli presentations were randomly distributed throughout the session and occurred at one of 30 randomly selected intertrial intervals ranging from 12 to 70 sec. After 6 days of testing, testing was reversed and rats had to learn to respond to the initially non-reinforced stimulus in order to receive a food reward. Reversal learning was tested for 8 days.

Following training, animals were given an overdose of ketamine (40 mg/kg) and xylazine (28 mg/kg) injected i.p. and euthanized by decapitation. Trunk blood was collected for the determination of serum estradiol levels. For estradiol analysis, samples were extracted with ether and resuspended in buffer. Samples were then analyzed in duplicate by RIA using a kit from Diagnostic Systems Laboratories, Inc. (Webster, TX). The estradiol assay was performed by the assay core of the Center for Reproductive Physiology at the University of Pittsburgh.

iii. Results

In contrast to effects on DMP acquisition, neither OVX nor E2 treatment had any significant effect on OD/RL performance. Likewise, G-15 treatment had no effect on OD/RL acquisition or reversal learning in gonadally intact rats (Figure 16). ANOVA revealed no

significant effect of Treatment on number of correct choices ($F(3,29)= 0.95$, $p > 0.4$) (Figure 4A) or incorrect choices ($F(3,29)= 0.51$, $p > 0.7$) (Figure 4B) during the acquisition or reversal learning phases.

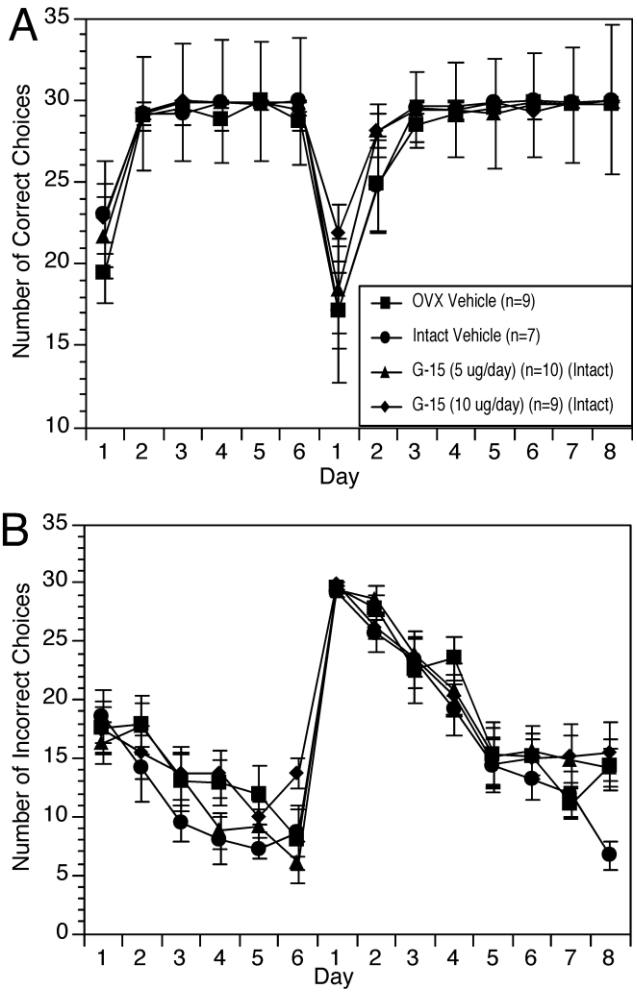

Figure 23. Learning curves showing number of correct choices (A) and number of incorrect choices (B) made by rats during successive choice discrimination. Rats were trained to respond to either light or sound for the first six days and then trained to respond to the opposite stimulus for the next eight days. The arrow indicates the day on which the stimulus contingency was reversed.

iv. Discussion

In contrast to effects on the DMP task, no effects of ovariectomy or E2 replacement were observed on the OD/RL task. Likewise, G-15 had no effect on OD/RL learning in gonadally intact rats. The OD/RL task is a non-spatial operant learning and memory task. Learning requires that rats be able to distinguish visual and auditory cues and to associate individual cues with a food reward. The fact that neither OVX, E2 nor G-15 had any affect on this task suggests that treatments did not significantly impact these processes. In addition, as both the DMP task and the OD/RL task utilize the same food reward, this suggests that effects on the DMP task are not due to an effect on the motivation for food. Many studies have shown that effects of E2 on cognitive performance are task selective. For example, E2 has been shown to improve performance on spatial learning (Daniel et al., 1997; Gibbs, 1999), object placement and recognition (Fernandez et al., 2008; Gresack and Frick, 2006a), and fear condition tasks (Jasnow et al., 2006), but not on specific reference or working memory tasks (Fader et al., 1999; Galea et al., 2001), nor on a configural association operant conditioning task (Gibbs and Gabor, 2003; Gibbs et al., 2009). Thus, our results agree with previous work showing that the effects of E2 on learning and memory tasks are task-specific, and suggest that the effects of G-15 are due to interference with the effects of E2, rather than to non-specific effects on learning and memory processes.

V. SUMMARY AND CONCLUSIONS

SUMMARY

In conclusion, the data shown support the hypothesis that GPR30 mediates E2 effects on the basal forebrain cholinergic system with corresponding beneficial effects on cognitive performance. We have shown that GPR30 mRNA and protein are expressed in brain regions important for learning and memory. In fact, GPR30 is expressed by the majority of cholinergic neurons in the basal forebrain. Using microdialysis, we showed that treatment with a GPR30 agonist can enhance potassium-stimulated acetylcholine release similar to E2. This suggests that GPR30 plays a significant role in mediating effects of E2 on acetylcholine release. In our behavioral studies we found that GPR30 agonism restores performance of OVX rats on a spatial learning task to that of gonadally intact controls, while antagonism impairs performance in a dose-dependent fashion. While we showed that other estrogen receptor agonists can also enhance performance on this task, the fact that a GPR30 antagonist could block E2 effects suggests that GPR30 is necessary for E2-mediated enhancement of learning. Future endeavors will further define the role of GPR30 in mediating E2 effects on cholinergic neurons as well as elsewhere in the brain, and on the mechanisms by which GPR30 activation elicits these effects.

LIMITATIONS OF STUDIES PRESENTED

Future endeavors will also aim to overcome some of the limitations inherent to the work presented here. A limitation of any chemical agonist/antagonist study is the possibility that the agonist/antagonist is affecting a different protein than the receptor of interest. Future work could use tools such as siRNA to more definitively prove that E2 effects on cholinergic function and DMP acquisition are GPR30 mediated. Moreover, we only examined the effects of a couple doses of G-1 and G-15. More details about these compounds, such as how easily they get into the brain, what levels are required to illicit an agonistic or antagonistic effect, or what is the selectivity window for each compound, would prove useful when choosing dosing regimens. A limitation of the microdialysis and behavior studies is that while we did measure circulating levels of E2, we did not measure levels of G-1 or G-15. Assays are currently being developed to be able to measure agonist and antagonist levels in serum samples. While these assays will answer questions regarding circulating levels, the question still remains of what circulating levels are in the brain. Being able to reliably measure minute quantities in the brain is not a trivial matter, so future technologies that permit that level of detection should be investigated. A limitation of the immunostaining studies is that the antibody used was a polyclonal antibody, which raises the possibility that it is binding to a similar protein as GPR30. As was mentioned earlier, there still exists controversy in the field over GPR30 localization (reviewed in (Langer et al., 2010). Future work using monoclonal antibodies or doing in situ hybridization would more definitively prove GPR30 localization in different regions.

The fact that we and others have observed GPR30 expression in many non-cholinergic neurons and in other regions that do not contain cholinergic projections suggests that GPR30 may mediate E2 effects on other systems besides the basal forebrain cholinergic system. For example, estrogens are known to regulate the release the hypothalamic hormones necessary in

reproduction. Brailoiu et al. showed that G-1 increases intracellular calcium levels in cultured rat hypothalamic neurons (Brailoiu et al., 2007). G-1 can inhibit serotonin 5HT1A receptor-mediated attenuation of oxytocin release (Xu et al., 2009). Noel et al. used GPR30 siRNA to show that the rapid excitatory affect of E2 on primate LHRH neurons in vitro is partially dependent on GPR30 (Noel et al., 2009). Our work has focused on how GPR30 may mediate E2 effects on the cholinergic system to enhance learning, but the fact that GPR30 is highly expressed in the regions such as the hippocampus and cortex suggests the possibility that E2 can directly act on these regions to enhance learning and memory, thereby bypassing the cholinergic system. Thus, more work needs to be done to clearly define the role of GPR30 in mediating E2 effects on these different systems.

HOW GPR30 FITS IN WITH ESTROGEN THERAPY

As was mentioned in the Introduction, the beneficial effects of estrogen therapy on cognitive performance diminish with age and time following loss of ovarian function, and this finding has led to the Window of Opportunity hypothesis. Effects of estrogen therapy on cognition are due, in part, to the effects on cholinergic afferents innervating the hippocampus and cortex, and it has been suggested that the loss of estrogen effects with age and time following menopause is due to a substantial reduction in the functionality of these projections. GPR30 is expressed in brain regions important for learning memory and attention, is expressed by the majority of cholinergic neurons in the basal forebrain, and appears to be an important regulator of basal forebrain cholinergic function. Therefore, enhancing cholinergic activity, whether indirectly via a GPR30 agonist or acetylcholine esterase inhibitors, or directly via selective acetylcholine receptor agonists, could be an effect means of re-opening the window of

opportunity and restoring beneficial effects of estrogen therapy on cognition. This is supported by a recent study from our lab showing that donepezil, a cholinesterase inhibitor used in treating Alzheimer's disease, was effective at restoring effects of E2 on DMP acquisition in aged rats (Gibbs et al., 2009). Similar effects have been observed with donepezil + E2 treatment in young rats with cholinergic lesions, as well as with galanthamine, another cholinesterase inhibitor, and its ability to enhance E2 effects on DMP acquisition in aged rats (Gibbs et al., 2011a). These findings are all consistent with clinical studies showing that postmenopausal women with Alzheimer's disease who received estrogen therapy responded better to tacrine (a cholinesterase inhibitor formerly used to treat Alzheimer's disease), than women not on estrogen therapy (Schneider et al., 1996). Therefore, combining estrogen therapy with cholinergic enhancers may be an effective strategy for re-opening the window of opportunity. Sustained estrogen therapy, however, does carry significant risks and side effects, especially in older women (Grady et al., 1995; Grady et al., 2000; Ross et al., 2000). Selective GPR30 agonists such as G-1 may offer added benefits by avoiding these adverse side effects. Whether G-1 is effective at enhancing cholinergic function and cognition in aged rats and is similar affected by the co-administration of cholinesterase inhibitors needs to be evaluated. Thus, GPR30 may be an important target for developing new therapies that can enhance or restore estrogen effects on cognition in older women.

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