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Effects of glyceollin on mRNA expression in the female mouse brain.

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EFFECTS OF GLYCEOLLIN ON MRNA EXPRESSION IN THE FEMALE MOUSE BRAIN

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DEDICATION

I dedicate this dissertation to my mother, Nergish Bamji. You have been my role model and my guide through the years, always supporting my dreams and ambitions, encouraging me to achieve my professional goals through hard work and dedication, but most importantly, reminding me that the greatest achievement in life is to be the best human being you can be. I also dedicate this dissertation to my father, Firdaus Bamji and my sister, Farah Bamji. You both have been a big part of my life and support system. I could not have accomplished any of this without you all. I love you all very much and I hope to make you all proud of me one day!

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ABSTRACT

EFFECTS OF GLYCEOLLIN ON MRNA EXPRESSION IN THE FEMALE MOUSE BRAIN

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Glyceollins (Glys), produced by soy plants in response to stress, have anti-estrogenic activity in breast and ovarian cancer cell lines *in vitro* and *in vivo*. In addition to known anti-estrogenic effects, Glys exhibit mechanisms of action not involving estrogen receptor (ER) signaling. To date, effects of Glys on brain physiology and function are unknown. The purpose of the experiments summarized in this dissertation was to gain an understanding of the effects of Gly on brain-related functions in the female mouse brain through the observation of changes in gene expression. For our initial studies, we treated ovariectomized Swiss Webster (CFW) mice with 17-β estradiol (E2) or placebo pellets, followed by 11 days of exposure to Glys or vehicle i.p. injections. We then performed microarray (Chapter 2) and RNA-sequencing analyses (Chapter 3) on total RNA extracted from whole brain hemispheres and identified differentially expressed genes (DEGs) between our treatment groups. Our results suggested that Glys, when in combination with E2 (E2+Gly), can oppose the E2 effects on gene expression and vice versa, can regulate genes similarly to E2, and can also have E2-independent effects on gene expression in the female brain. However, the whole brain experiments did not take into account the heterogeneity of the brain. Different brain regions perform unique and

distinct functions and can differ markedly in terms of gene expression, so we wanted to determine if Glys had any brain region-specific effects on gene expression. Additionally, as the whole brain studies only included a single time point of exposure to Glys, we evaluated the effects of a single acute dose of Glys (2, 24 and 48 hr) as well as chronic exposure to Glys (multiple doses of Glys for 7 consecutive days) on gene expression in distinct brain regions. Therefore, in Chapter 4, we evaluated the effects of acute vs. chronic doses of Glys alone and in combination with E2 on gene expression in the hypothalamus, hippocampus, and cortex of the female mouse brain. Our results suggest that Glys can rapidly upregulate the expression of genes like growth hormone (*Gh*) in the hypothalamus, hippocampus and cortex and prolactin (*Prl*) in the hypothalamus and cortex, 2h or 24h after administration of a single acute dose. Thus Glys may potentially affect neuronal processes like food intake, stress and cognition through its effects on *Gh* and *Prl* gene expression in the female mouse brain. As all of the above chapters involve a peripheral administration of Glys (intraperitoneal injections), it was unclear if Glys affect gene expression through direct action at the neuron or through some indirect peripheral effect. To address this issue, in Chapter 5 we screened five immortalized neural cell lines derived from the adult female mouse hypothalamus (mHypoA-50, 51, 55, 59 and 63) for the presence of our genes of interest and E2 responsiveness. Based on consistency of mRNA transcript detection and E2 responsivity, we selected two cell lines (mHypoA-55 and 63) that may be suitable for future experiments to determine the direct effect of Glys on gene expression at the neuron. Together this work provides novel information on the effects of Glys in the brain, which is important in order to develop its use as a dietary supplement and/or therapeutic agent.

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CHAPTER 1

GLYCEOLLINS: SOYBEAN PHYTOALEXINS THAT EXHIBIT A WIDE RANGE OF HEALTH-PROMOTING EFFECTS

Introduction

In the last few decades, there has been a remarkable increase in research on compounds with established health benefits. Among these foods, soybeans (*Glycine max*) have been especially well studied, mainly due to their ability to produce proteins and isoflavones, such as genistein and daidzein, with well-known health-promoting effects (Dixon and Sumner 2003; Nwachukwu et al. 2013; Sacks et al. 2006). When soybean tissues are challenged by a microbial or physical stimulus, a special class of low molecular weight plant defense compounds (phytoalexins) called glyceollins (Glys) are synthesized *de novo* and accumulate in the plant's tissues (Burden and Bailey 1975). Glys have received much attention due to their known antibacterial, antioxidant, antifungal, antitumor and insulinotropic actions both *in vitro* and *in vivo* (Kim et al. 2011a; Kim et al. 2010b; Ng et al. 2011; Park et al. 2010; Salvo et al. 2006). The focus of this chapter is to review the known health-promoting effects of Glys and to discuss potential central nervous system effects that have not been previously described.

Biosynthesis of Glys in soybeans

The biosynthesis of Glys includes enzymes that are generally dedicated to the synthesis of isoflavonoids and phytoalexins (Ebel 1986). Phenylalanine is first converted

to *trans*-cinnamic acid through the action of enzyme phenylalanine ammonia-lyase, followed by a hydroxylation reaction catalyzed by cinnamic acid 4-hydroxylase that converts the *trans*-cinnamic acid to p-coumaric acid (Dixon and Paiva 1995). A series of subsequent reactions involving enzymes 4-coumarate:coenzyme A ligase, chalcone-synthase, chalcone reductase, chalcone isomerase and isoflavone synthase, lead to the formation of the isoflavonoid daidzein (Dixon and Paiva 1995). Daidzein then undergoes cyclization and hydroxylation reactions to give rise to glycinol, which is the non-prenylated precursor for Glys (Dixon and Paiva 1995). Two separate prenyl transferases, viz. glycinol 4-dimethylallyl transferase (G4-DT) and glycinol 2-dimethylallyl transferase (G2-DT), then bring about the prenylation of glycinol to glyceollidins I and II, respectively (Akashi et al. 2009; Yazaki et al. 2009). The final step of Gly biosynthesis is the cyclization of glyceollidins by glyceollin synthases to convert glyceollidin I to Gly I and glyceollidin II to Glys II and III (Welle and Grisebach 1988) (Fig.1.1). Of importance is that most studies on Glys use a mixture of Glys I, II and III instead of isolated Gly isomers. Although these are the three main isomers of Glys, recent studies have also reported the existence of Glys IV, V and VI along with glyceofuran (Simons et al. 2011) (Not shown in Fig.1.1).

Glys antitumor effects

Glys have been extensively studied for their antitumor effects through estrogen receptor (ER)-dependent (mainly ERα and ERβ) and ER-independent mechanisms. Initial research on Glys focused on their anti-estrogenic effects and ability to inhibit estrogen-responsive tumors. Glys (applied as a mixture of Glys I, II, and III) suppressed the 17-β estradiol (E2)-induced proliferation of ER-positive (ER^+) human breast cancer cells

(MCF-7) and ER-negative (ER^-) human embryonic kidney cells (HEK 293) transiently transfected with either ERα or ERβ *in vitro* (Burow et al. 2001). Through competition binding assays, the study also provided evidence that Glys had a 3-fold higher affinity for ERα than for ERβ (Burow et al. 2001; Nikov et al. 2000). Similar to the *in vitro* study, Glys suppressed the E2-induced *in vivo* proliferation of both breast (MCF-7) and ovarian (BG-1) tumor cells implanted in ovariectomized athymic nude mice (Salvo et al. 2006). In addition to breast and ovarian tumors, Glys also inhibited the growth of human androgen-responsive prostate cancer cells (LNCaP) through the induction of cyclin-dependent kinase inhibitors (CDKN1A and CDKN1B) involved in cell cycle arrest (Payton-Stewart et al. 2009). Importantly, Glys suppressed the E2-induced growth of LNCaP cells and not dihydrotestosterone (DHT)-induced growth, which indicated an ER-mediated anti-estrogenic effect of Glys on prostate tumor cells (Payton-Stewart et al. 2009). A subsequent study found that of all the Gly isomers (*i.e.*, Glys I, II and III), Gly I had the highest affinity for ERα, exhibited the strongest anti-estrogenic activity compared to Glys II and III and also downregulated E2-responsive stromal-derived factor (SDF-1) and progesterone receptor (PgR) expression in both MCF-7 and BG-1 cells (Zimmermann et al. 2010). This finding led to further interest in determining the effectiveness of the Gly I isomer on tumor proliferation and overall anti-estrogenic activity at ERs. A comparison of naturally occurring (-) Gly I and its chemically synthesized enantiomer (+) Gly I showed that both had similar binding affinity to both ERα and ERβ (Payton-Stewart et al. 2010). The difference in their activity was that (-) Gly decreased the ER-induced transcriptional activation of estrogen-response element (ERE) transcriptional activity, whereas (+) Gly increased ERE activity in both MCF-7

and ERα-transfected HEK 293 cells (Payton-Stewart et al. 2010). The natural (-) Gly I isomer also showed the highest growth inhibition activity against ER^+ breast and ovarian tumors as well as androgen receptor-positive (AR^+) prostate tumors *in vitro*, compared to a Gly racemic mixture and unnatural (+) Gly I isomer (Khupse et al. 2011). In contrast to the aforementioned anti-estrogenic effects of Glys, one study reported estrogenic effects of Glys, increasing the proliferation of ER^+ MCF-7 cells, mainly through activity at ERβ (Kim et al. 2010a). The study showed Gly acting anti-estrogenically via ERα, while exhibiting estrogenic effects via action at ERβ (Kim et al. 2010a). These results suggest that Gly may not be a pure anti-estrogen but may potentially be a selective estrogen-receptor modulator (SERM). This SERM-like activity has recently been attributed to differences in prenylation patterns, backbone structure and the lengths of the different Gly isomers, with the effects being SERM-like at ERα (Gly I) and agonistic at ERβ (Gly I, II and III) (van de Schans et al. 2016).

Although all of the above studies provided evidence for ER-mediated effects of Glys, subsequent studies showed that Glys may act through non ER-mediated mechanisms as well. The very first study of this kind showed that the natural (-) Gly I isomer, but not the unnatural (+) Gly isomer, significantly inhibited the growth of ER^- breast and ovarian tumors as well as androgen receptor-negative (AR^-) prostate tumors *in vitro*, but to a lesser extent than its inhibition of ER^+ and AR^+ tumors (Khupse et al. 2011). Further evidence for a potential ER-independent mechanism came from experiments that showed the ability of Glys to suppress tumorigenesis in triple-negative breast carcinoma cells (MDA-MB-231 and MDA-MB-468) that lack ERs, PgR and human epidermal growth factor receptor 2 (Her2) (Rhodes et al. 2012). A recent report

indicated that in addition to ERα signaling, Glys can suppress proliferation of breast tumors (MCF-7 and T-47D) through suppression of the mechanistic target of the rapamycin (mTOR) - ribosomal protein S6 kinase, 70kDa (p70S6) pathway (Bratton et al. 2015), thus indicating a dual inhibitory mechanism of Gly action on breast cancer progression. Glys are also reported to affect breast, prostate, liver, lung and gastric carcinoma cells through the inhibition of the activity of hypoxia-inducible factor (HIF-1α), a molecule that plays a critical role in regulation of genes involved in tumor metabolism, angiogenesis and metastatic ability (Lee et al. 2014). The mechanism of Gly inhibition of HIF-1α was through the inhibition of the phosphatidylinositol-3 kinase/protein kinase B/mTOR (PI3K/AKT/mTOR) pathway that regulates translation of the HIF-1α protein and through interception of the interaction of HIF-1α-heat shock protein (Hsp90), which stabilizes the HIF-1α protein (Lee et al. 2014). Furthermore, a very recent study reported that Gly I can reverse the epithelial to mesenchymal transition (EMT) of letrozole-resistant breast cancer cells (LTLT-Ca) through the inhibition of zinc-finger E-box binding homeobox 1 (ZEB1) expression (Carriere et al. 2016). Letrozole is an aromatase inhibitor, which is approved for treatment of ER^+ breast cancer in postmenopausal women, and ZEB1 is a transcription factor that is known to play a role in EMT in breast cancer cells. Therefore, in addition to the mTOR/p70S6 or PI3K/AKT/mTOR pathway, Glys can also suppress tumor metastases through inhibition of ZEB1 gene and protein suppression. In addition to hormone-responsive tumors, Glys inhibited melanogenesis in B16 melanoma cells through the suppression of tyrosinase, tyrosinase-related protein 1 (TRP-1) and microphthalamia-associated transcription factor (MITF) expression (Lee et al. 2010), without any cytotoxic effects. Moreover, Glys

inhibited the induction of melanogenesis by alpha melanocyte-stimulating hormone (α-MSH) by suppressing cyclic adenosine monophosphate (cAMP) levels induced by α-MSH in melanoma cells (Lee et al. 2010). This suppression of melanin synthesis indicated a potential use for Glys as cosmetic skin whitening agents. A subsequent study also showed that Glys' skin whitening effect may be through the inhibition of stem cell factor-kit receptor (SCF/c-kit) signaling in addition to tyrosinase inhibition in B16F10 melanoma cells (Shin and Lee 2013). In addition to tumor inhibition, treatment with Glys also suppresses the migration and tube formation of endothelial progenitor cells (EPC), hematopoietic stem cells that play a role in early tumor vasculogenesis (Choi et al. 2013). This study also showed that Glys inhibited the expression of genes involved in EPC function such as SDF-1, C-X-C chemokine receptor-4 (CXCR4), angiopoietin-1 (Ang-1) and angiopoietin receptor (Tie-2) (Choi et al. 2013). Further, Glys also inhibited the activation of Akt, ERK1/2 and endothelial nitric oxide synthase (eNOS) induced by SDF-1 or vascular endothelial growth factor (VEGF) (Choi et al. 2013).

Thus, Glys significantly suppress several different types of tumors both *in vitro* in cancer cell lines and *in vivo* in xenograft mouse tumor models, directly through ER-mediated mechanisms or pathways involving PI3K/AKT/mTOR signaling as well as indirectly through the suppression of tumor vasculogenesis. These studies together provide good evidence in support of Glys as potential chemotherapeutic agents for effective treatment of various cancers.

Glys antimicrobial effects in soybeans

Recently, there has been a growing interest in exploring naturally occurring antimicrobial agents to replace synthetically produced bactericides or fungicides in the food industry (Nwachukwu et al. 2013). Glys exhibit potent antibacterial and antifungal activity against various bacterial and fungal strains. Glys inhibited the growth of bacterial strains such as *Pseudomonas syringae* and *Xanthomonas campestris* and was effectively bactericidal towards *Bacillus subtilis, Bacillus licheniformis, Staphylococcus aureus* and *Corynebacterium flaccumfaciens* in three different bacterial bioassays (Fett and Osman 1982). Subsequent studies showed that Glys potentially exhibit their antibacterial effects by inhibiting cellular processes and altering the structural integrity of bacterial membranes, thereby impeding bacterial growth (Weinstein and Albersheim 1983). Furthermore, Glys were not only more abundant but also the most inhibitory against the growth of *Fusarium solani* f. sp. *glycines* (soil-borne fungus and causative agent for sudden death syndrome in soybeans) in a genotype of *Glycine max* (PI 567374) that is partially resistant to fungal infections (Lozovaya et al. 2004). Glys also inhibited the growth of various fungal pathogens such as *D. phaseolorum* var. *meridionales, Macrophomina phaseolina, Phytphthora sojae, Sclerotinia sclerotiorum, Cercospora sojina, Phialophora gregata,* and *Rhizoctonia solani* in the hairy roots of non-transformed soybean genotypes Spencer and PI 567374 (Lygin et al. 2010). Importantly, this Gly inhibition was much lower in soybeans transformed with isoflavone synthase or chalcone synthase genes, which led to markedly low levels of total isoflavones, including Glys (Lygin et al. 2010). This led to a study on the effects of similar isoflavone synthesis suppression on the susceptibility of the whole soybean plant or the plant seeds to fungal infections (Lygin et al. 2013). Suppression of isoflavone synthesis led to significantly

lower levels of Gly production, which rendered both the whole plant and seeds susceptible to infection by *Phytophthora sojae* and *Macrophomina phaseolina* (Lygin et al. 2013). Another study showed that Glys produced by soybean seeds elicited with *Aspergillus sojae* had remarkable antifungal effects against *Fusarium oxysporum, Phytophthora capsici, Sclerotinia sclerotiorum*, and *Botrytis cinerea* (Kim et al. 2010c). Moreover, a very recent study showed that pre-treatment with calcium is beneficial for soybeans infected with *Sclerotinia sclerotiorum* and enhances the expression of defense-related genes, including chalcone synthases, isoflavone synthase and isoflavone reductase, which are enzymes involved in the production of Glys (Arfaoui et al. 2016) (Fig.1.1). Pre-treatment with calcium, through the upregulation of these enzymes, improved the accumulation of daidzein, genistein and acetyl glycitin and enhanced the *de novo* synthesis of Glys, especially in soybeans inoculated with the aggressive strain of *Sclerotinia sclerotiorum* (Arfaoui et al. 2016). In combination, these studies indicate that rapid Gly biosynthesis during infections forms an important aspect of innate disease resistance in soybean. Thus, Glys hold promise in the development of natural fungicides and bactericides in the agriculture and food industry.

Glys antioxidant properties

Oxidative damage, especially prolonged exposure to reactive oxygen species (ROS) can lead to severe damage to DNA, proteins and lipids. Antioxidant defenses to minimize ROS accumulation and fluctuations are critical to cell survival and can be of importance in the treatment of cancers and age-related deterioration of functional activity. Glys along with other phytoalexins exhibit antioxidant properties that protect the soybean from

oxidative damage caused by stressors. A study measuring the antioxidant activity of total isoflavones in soybean extracts elicited by germination, wounding or exposure to *Aspergillus sojae* using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and β-carotene cooxidation in a linoleate system, found that soybean extracts exposed to *Aspergillus sojae* had the highest accumulation of total isoflavones, including Glys, and also displayed the highest antioxidant activity in both DPPH and β-carotene assays (Boué et al. 2008). This work led to further research focusing specifically on the antioxidant activity of Glys. One study showed that a mixture of Gly I, II and III isomers exhibited strong antioxidant potential in mouse brain, liver and kidney extracts treated with hydrogen peroxide (H_2O_2), measured using radical ABTS and DPPH scavenging activity, reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), inhibition of singlet oxygen formation and thiobarbituric acid reactive substances (TBARS) (Kim et al. 2010b). Moreover, this Gly antioxidant activity was found to be greater than that of its parent compound daidzein. The same study also showed Glys inhibition of H_2O_2-induced ROS production in hepatoma cells (hepa1c1c7) (Kim et al. 2010b). Subsequent studies indicated that Glys antioxidant activity in hepatoma cells was through the upregulation of antioxidant enzymes (heme oxygenase 1, gamma-glutamylcysteine synthase, and glutathione reductase), by the induction of the nuclear translocation of nuclear factor eythroid-derived 2-like 2 (*Nrf2*), a transcription factor and regulator of cellular antioxidant enzymes (Kim et al. 2011b). Moreover, the study suggested that Gly caused the *Nrf2*-mediated enzyme induction through activation of thePI3K signaling pathway and by regulating *Nrf2* interaction with kelch-like ECH-associated protein 1 (*Keap1*), which is an inhibitor of *Nrf2* function (Kim et al. 2011b). More recently, a study indicated that

soybeans infected with *Aspergillus oryzae* had a high accumulation of Gly I and phytoalexin coumestrol, which significantly prevented H_2O_2-induced ROS production and lipid peroxidation in human HepG2 hepatoma cells (Jeon et al. 2012). Other antioxidant effects of Glys include the inhibition of proton-induced ROS generation and cell death in human umbilical vein endothelial cells *in vitro*, which has an important application in development of dietary supplements for astronauts who are exposed to proton radiation and high-energy particles (Jung et al. 2009). Furthermore, Glys also suppressed ROS production in platelet-derived growth factor (PGDF)-stimulated human aortic smooth muscle cells (Kim et al. 2012). These results taken together suggest that Glys have potent antioxidative properties in animal tissues and cancer cells and have the potential to be developed further as a functional dietary supplement that can reduce the damage caused by cellular oxidative stress.

Glys anti-inflammatory properties

Systemic inflammatory responses are often correlated with obesity, cardiovascular disease and type 2 diabetes (Strohacker and McFarlin 2010). In addition to antitumor, antimicrobial and antioxidant properties, Glys also exhibit anti-inflammatory properties(Kim et al. 2011a; Kim et al. 2012; Yoon et al. 2012). Glys exerted an anti-inflammatory response in a lipopolysaccharide (LPS)- induced murine macrophage cell line (RAW264.7) by suppressing the LPS-induced phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (Kim et al. 2011a). The study showed that Glys inhibited LPS-induced nitric oxide production (NO),interleukin-6 (IL-6) release and reduced the expression of inducible nitric oxide

(iNOS) and cyclooxygenase-2 (COX2) (Kim et al. 2011a). Furthermore, Glys were also found to suppress 12-*O* tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory response in mouse skin (Kim et al. 2011a). A subsequent study found that in addition to the suppression of LPS-induced gene expression, Glys also inhibited the expression of other known pro-inflammatory cytokines such as (IL-1β and IL-18 as well as tumor necrosis factor (TNF)-α, while upregulating the expression of anti-inflammatory cytokines including IL-10 in the RAW264.7 cell line (Yoon et al. 2012). The anti-inflammatory response of Glys in macrophages has also been attributed to the dosedependent inhibition of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) kinase (IKK) phosphorylation, thus blocking IκBα degradation and the formation of an NF-κB-DNA binding complex (Yoon et al. 2012). Thus, Glys show potential as therapeutic agents in the treatment of chronic diseases that are associated with systemic inflammation.

Glys effects on glucose and lipid metabolism

Recently, Glys have shown great promise in the regulation of glucose and lipid metabolism. Glys enhanced insulin-stimulated glucose uptake and decreased triglyceride accumulation in adipocyte cells (3T3-L1) (Park et al. 2010). Glys also alleviated the effects of palmitate,a long chain, saturated free fatty acid that promotes pancreatic *β*-cell dysfunction and apoptosis through induction of genes involved in endoplasmic reticulum stress in insulinoma cells (Min6) (Park et al. 2010). Glys not only improved glucose-stimulated insulin secretion (GSIS), but also decreased the expression of endoplasmic reticulum-stress genes (Park et al. 2010). In addition, Glys promoted GSIS by increasing

the secretion of glucagon-like peptide 1 (GLP-1) in enteroendocrine cells (NCI-H716), thus enhancing β-cell survival (Park et al. 2010). Another study showed that Glys decreased blood glucose levels in prediabetic rats that were given an oral dose of either 30 or 90 mg/kg of Glys (Boué et al. 2012). The same study provided evidence that Glys improved basal, insulin-stimulated and insulin-independent glucose uptake in 3T3-L1 adipocytes, an effect that was attributed to the increase in expression of glucose transporters (GLUT1 and GLUT4) (Boué et al. 2012). Furthermore, in a streptozotocin-induced mouse model of type-2 diabetes, mice fed a high fat diet (HFD) along with 10% fermented soybeans containing Glys showed the lowest peak for blood glucose and higher levels of serum insulin compared to mice fed a HFD with no soybeans or those fed a HFD containing 10% fermented soybeans without Glys (Park et al. 2012). A Glys-enriched soybean diet also dropped blood glucose levels more than control (high-fat diet without soybeans) during insulin tolerance testing. This improvement in glucose metabolism was found to be due to the potentiation of protein-kinase B (Akt), adenosine-monophosphate kinase (AMPK) and acetyl-coA carboxylase phosphorylation in the liver along with a decrease in phosphoenolpyruvate carboxykinase (PEPCK) expression, which enhanced lipid and glucose metabolism in the liver (Park et al. 2012). Thus, Glys improve glucose metabolism partly through increasing insulin secretion in the early phase and partly through the enhancement of liver insulin sensitivity. A more recent study provided evidence that Glys reduce both blood glucose and lipid levels and increase insulin sensitivity by decreasing janus-kinase (JNK) activity in insulin-resistant *db*/*db* mice (Yoon et al. 2013). The study also showed Glys' ability to decrease endoplasmic reticulum stress-induced insulin resistance by the activation of the Ca^{2+}/calmodulin-

dependent protein kinase kinase (CaMKK-AMPK) activity in skeletal muscle tissue and L6 myotubes, leading to a stimulation in glucose uptake and fatty acid oxidation in muscle cells (Yoon et al. 2013). Glys also reduced serum insulin, triglycerides and increased high-density lipoprotein (HDL) levels in *db*/*db* mice with no change in plasma low-density lipoprotein (LDL) levels (Yoon et al. 2013). Similar to this finding, Glys exhibited cholesterol-lowering activity in male Syrian hamsters that were fed a HFD along with 250 mg/kg Glys by significantly reducing total lipid content, plasma very low-density lipoprotein (VLDL) and hepatic cholesterol esters, with no change in plasma LDL, fecal bile acid or cholesterol content (Huang et al. 2013). However, no increase in plasma HDL was observed in hamsters, unlike in the *db*/*db* mice (Yoon et al. 2013). Lastly, gene expression in mammary tissues of postmenopausal cynomolgus monkeys that were fed a diet consisting of Gly-enriched soy exhibited a gene expression profile distinct from the mammary tissues of those fed a standard soy diet (Wood et al. 2012). The Gly-enriched soy diet increased the expression of lipid metabolism genes such as peroxisome proliferator-activated receptor (PPAR-γ) and adiponectin, as well as leptin, lipin 1 and lipoprotein lipase (Wood et al. 2012). In conclusion, these reports provide evidence supporting the development of Glys as dietary supplements that may alleviate hyperglycemia in type 1 and type 2 diabetes and reduce the risk of HFD-induced obesity and cardiovascular disease through the enhancement of glucose and lipid metabolism in various tissues.

Glys effects on intestinal transport and metabolism of drugs

The health-promoting effects of any dietary supplement or drug depend on its bioavailability, its systemic absorption when administered orally and its intestinal transport and metabolism. Intestinal transport of Glys was found to be rapid in human intestinal Caco-2 cells and dominated by a passive diffusion mechanism (Chimezie et al. 2014). The same study also found that Glys had no effect on the expression of P-glycoprotein (Pgp), which plays a protective role by limiting the uptake of orally consumed xenobiotics in the small intestines (Chimezie et al. 2014). Subsequent studies showed that Glys inhibited the function of two apical efflux transporters, multidrug-resistance associated protein 2 (MRP2) and breast cancer resistance protein (BCRP), with no alteration of their gene expression by Glys in Caco-2 cells (Chimezie et al. 2016; Schexnayder and Stratford 2016). Moreover, Glys appeared to inhibit the metabolism of the phytoalexin genistein, but did not inhibit the intestinal transport of aglycone, a metabolite of genistein (Chimezie et al. 2016; Schexnayder and Stratford 2016). As efflux transporters play an important role in restricting the absorption of drugs across the intestine and blood-brain barrier, these findings provide preliminary evidence for the potential of Glys to enhance the delivery of other drugs (like genistein) to cells.

Potential CNS effects

Research on Glys has provided evidence in support of its antitumor, antioxidant, antimicrobial effects as well as its effects on metabolism, and until recently, not much was known about the effects of Glys on the nervous system aside from one study that revealeds antioxidant effects of Glys in brain extracts (Kim et al. 2010b).Recent work in our lab focused on determining the effects of Glys on gene expression in the female mouse brain (Bamji et al. 2015). As initial studies highlighted the anti-estrogenic effects

of Gly and the non-ER related reports are more recent, our initial hypothesis was that Glys would act anti-estrogenically when combined with E2, but would have no independent effect on gene expression when used alone. Although we detected potentially ER-mediated anti-estrogenic effects of Glys on expression of a few genes, Glys also exhibited a gene expression profile distinct from both E2 groups (E2 and E2+Gly), indicating potential ER-independent effects on gene expression (Bamji et al. 2015). Interestingly, Glys alone upregulated expression of genes including *Nr4a1*, an orphan nuclear receptor that is involved in memory formation (Hawk and Abel 2011), and microRNA-495, which affects the expression of brain-derived neurotrophic factor (BDNF) (Mellios et al. 2008), a gene involved in synaptic plasticity and neurogenesis. On the other hand, Glys downregulated the expression of peptidylprolyl isomerase A (Ppia), which promotes neurodegeneration and apoptosis (Bamji et al. 2015; Nigro et al. 2013). These initial findings suggest that Glys may have neuroprotective effects and potentially exhibit SERM-like effects in the female mouse brain, but further research is needed to support this claim.

The aim of this review was to summarize the literature on Glys' effects *in vitro* and *in vivo*. These reports predominantly involve a mixture of Glys, but also include recent studies showing the independent effects of the different Gly isomers. Given the slight differences in structure and binding affinity of the Gly isomers to ERs, future investigations focusing on independent effects of the different Gly isomers may be useful in the development of disease-specific therapeutic interventions. From the research presented here, it is clear that Glys have the potential to be developed not only as chemotherapeutic agents, but also as modulators of glucose and lipid metabolism in

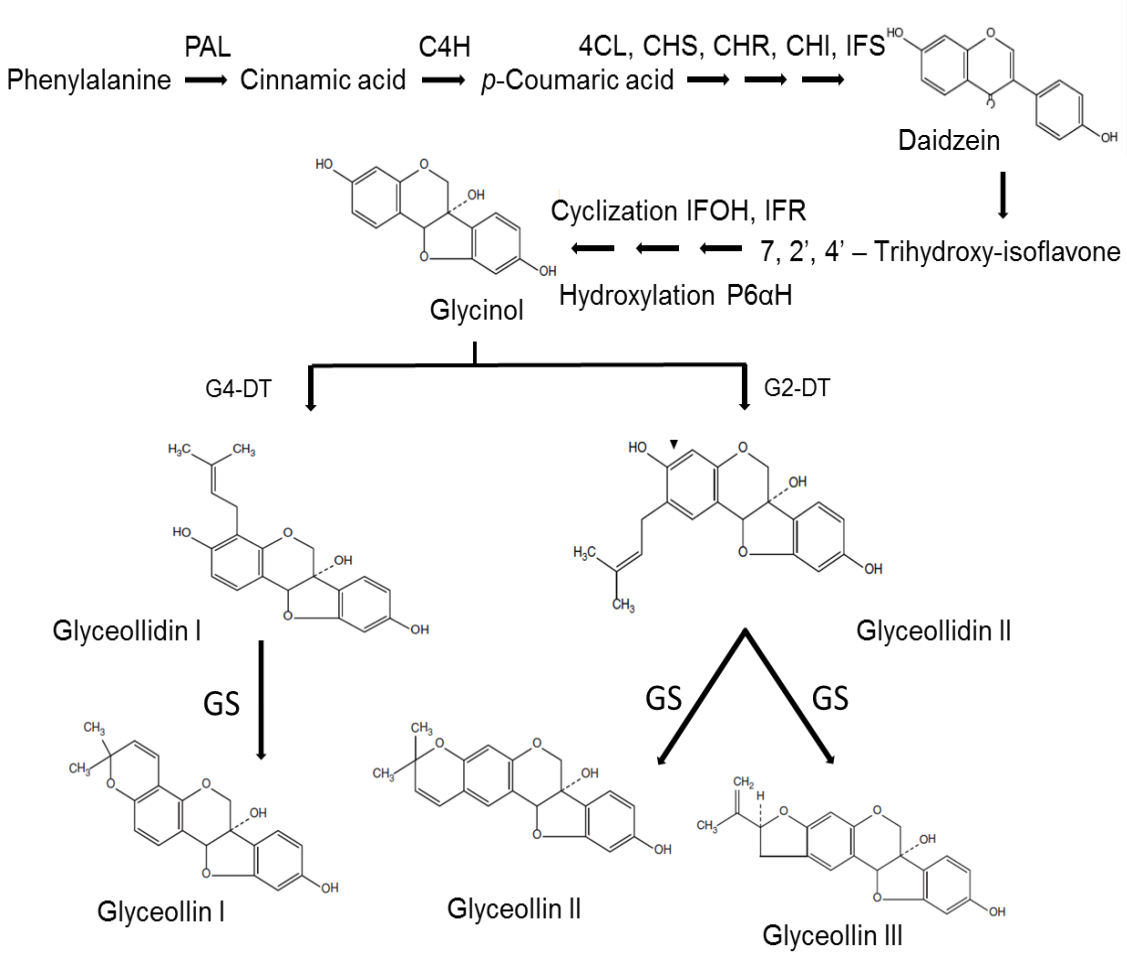
diabetic patients and as dietary supplements with potent antioxidant, antimicrobial and possibly neuroprotective functions. Further research focusing on determining the effective doses of Glys and the safety and possible side effects of Glys in non-target tissues is required to realize the potential application of Glys as dietary supplements or therapeutic agents.

Dissertation Objectives

The main purposes of this dissertation are to evaluate the effects of Glys on gene expression in the female mouse brain, make predictions about the underlying mechanisms of Gly action in the brain and relate that action to brain activity and function. Toward this end, in Chapter 2 we first examined the effects of a chronic exposure (multiple doses of Gly for 11 consecutive days) to Glys alone and in combination with E2 on gene expression in the whole female mouse brain using microarray (Bamji et al. 2015). Due to limitations with the microarray technique, especially in the detection of low-expression genes of interest, in Chapter 3 we performed a whole brain RNA-sequencing (RNA-Seq) experiment with the same samples used in Chapter 2. In Chapter 4, we tested the effects of acute (single dose of Glys followed by tissue harvest at 2, 24 and 48 hr) and chronic (multiple doses of Gly for 7 consecutive days) Glys exposure on expression of select genes in three distinct brain regions, *i.e.*, the hypothalamus, hippocampus, and cortex. We were especially interested in determining if Glys affected gene expression differently in different brain regions and if there was any difference between the acute vs. chronic dose of Glys on gene expression. Lastly, in Chapter 5 we tested five different immortalized hypothalamic cell lines (mHypoA-50, 51, 55, 59 and 63) derived from adult female mice for the presence of our select genes of interest and their responsiveness to E2, to select

one or more cell lines for future investigations evaluating if Glys affect gene expression through direct action at the neuron or through indirect peripheral effects. Results from our whole brain experiments (Chapter 2 and Chapter 3) suggest that Gly in combination with E2 can oppose E2's effects on gene expression, E2 in combination with Gly can oppose Gly's effect on gene expression, Gly can regulate gene expression similarly to E2 and can also have E2-independent effects on gene expression. In Chapter 4, we observed that both Gly treatments (Gly and E2+Gly) upregulated the expression of growth hormone (*Gh*) at the 2hr Gly exposure time in all brain regions, and prolactin (*Prl*) at the 2h Gly exposure time in the hypothalamus and cortex and at the 24h Gly exposure time in the hypothalamus. The results indicate that Gly may potentially affect neuronal processes through the regulation of genes including *Gh* and *Prl*, but the mechanism of action still remains unclear. In Chapter 5, based on the reliable detection of our genes of interest and E2-responsiveness of the cell lines, we identified two cell lines (mHypoA-55 and mHypoA-63) which could prove useful in studying the direct effects of Gly at the neuron.

Fig. 1.1: Biosynthetic pathway of glyceollins in soybeans. 4CL, 4-coumarate:coenzyme A ligase; C4H, cinnamic acid 4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; G2-DT, glycinol 2-dimethylallyl transferase; G4-DT, glycinol 4-dimethylallyl transferase; GS, glyceollin synthase; IFOH, isoflavone 2'-hdroxylase; IFR, isoflavone reductase; IFS, isoflavone synthase; P6αH, pterocarpan 6α-hydroxylase; PAL, phenylalanine ammonia-lyase.



CHAPTER 2

SOY GLYCEOLLINS REGULATE TRANSCRIPT ABUNDANCE IN THE FEMALE MOUSE BRAIN

Introduction

Glyceollins are soy-derived isoflavonoid phytoalexins that are biologically active in vertebrates(Boue et al. 2012; Burow et al. 2001; Salvo et al. 2006). Several recent studies have reported that mixtures of the isomers glyceollin I, II, and III (Gly) affect mammalian physiology *in vivo* and *in vitro*, and what has emerged is a complicated and incomplete picture of how Gly affects mammalian physiology (Fig. 2.1). Among the most exciting findings to date are results which indicate that Gly opposes the activity of 17β-estradiol (E2) in estrogen-responsive tissues, such as mammary and uterus. For example, Gly suppressed E2-mediated proliferation of MCF-7 human breast tumor cell lines *in vitro* (Burow et al. 2001). As another example, Gly inhibited E2-stimulated growth of MCF-7 breast cancer and BG-1 ovarian cancer cells, implanted as xenograft tumors in ovariectomized athymic nude mice *in vivo* (Salvo et al. 2006). Such results were reported to indicate an estrogen receptor (ER) mediated anti-estrogenic effect of Gly, mainly through its activity at ERα (Zimmermann et al. 2010). However, other studies concluded that Gly can also exhibit weak estrogenic activity via ERβ, which appears to enable Gly to promote the proliferation of MCF-7 breast tumor cells *in* vitro (Kim et al. 2010a). Thus, at present, it is unclear whether Gly is purely anti-estrogenic, or

a selective estrogen receptor modulator (SERM) whose relative estrogenic/anti-estrogenic action varies in a tissue- and/or condition- dependent manner.

Despite the promise that Gly has shown in estrogen-responsive cancer models, not all interest in Gly relates specifically to women's health. In particular, recent studies have shown that Gly decreases blood glucose levels in pre-diabetic male rats and *db/db* mice *in vivo* and increases insulin-stimulated and basal glucose uptake in 3T3-L1 adipocytes and insulin sensitivity in myotubules *in vitro* (Boue et al. 2012; Park et al. 2010; Yoon et al. 2013). Interestingly, these effects of Gly on insulin signaling are independent of classical ER-mediated effects (Boue et al. 2012), and were found to work via the CaMKK-AMPK pathway in one study (Yoon et al. 2013). Other non-ER-mediated mechanisms of Gly action have been found: Gly suppresses tumorigenesis in a xenografted triple-negative breast cancer cell line that lacks ERs (Rhodes et al. 2012), inhibits lipid peroxidation in tissue extracts of rat brain, kidney and liver (Kim et al. 2010b), upregulates expression of genes involved in lipid metabolism (Wood et al. 2012), and inhibits p70S6-mediated phosphorylation of ERα (Bratton et al. 2015). Collectively, these findings indicate that Gly may have multiple, context-dependent mechanisms of action, that E2 signaling is not the only target of Gly activity, and that in some cases E2 signaling is indirectly modified by Gly. Thus, a better understanding of how Gly affects mammalian physiology is needed before glyceollins are used as anti-cancer therapeutics and/or dietary supplements.

It is well established that compounds that interact with ERs can have effects that vary markedly between the CNS and the periphery (Arevalo et al. 2011; Halbreich and Kahn 2000; Simons et al. 2012). For example, ICI 182,720 (fulvestrant), which is purely

anti-estrogenic in the periphery, acts like a SERM in the brain (Alfinito et al. 2008). In the hypothalamus, fulvestrant exhibits anti-estrogenic activity, increasing gonadotropin releasing hormone (GnRH) pulse frequency by inhibiting estrogen's negative feedback on GnRH (Alfinito et al. 2008). Conversely, fulvestrant has estrogenic activity in the hippocampus, mimicking the effects of estradiol benzoate on place learning behavior (Alfinito et al. 2008; Steyn et al. 2007). SERMs have complicated central effects and the CNS is essential to the orchestration of both E2 and insulin signaling; therefore, it is impossible to do a rigorous cost/benefit analysis on the utility of Gly as a potential therapeutic for hormonally responsive cancers or metabolic disorders until its effects on brain physiology have been investigated. To address this question, we examined the mouse brain transcriptome using the Affymetrix Mouse Gene 1.0 ST Array after treatment of ovariectomized (ovx) mice with E2, Gly, both E2 and Gly (E2+Gly), or negative control (Con). The results show that effects of Gly and E2 interact for expression of 167 genes and that Gly also regulates gene expression independent of E2, indicating that Gly affects gene expression in mouse brain through more than one mechanism, some of which probably do not involve ERs.

Materials and Methods

Study animals, experimental manipulations, and plasma samples

Ovariectomized adult female CFW mice were purchased from Charles River Laboratories, maintained on a 12L:12D photoperiod (lights on at 6am), and given *ad libitum* access to phytoestrogen-reduced rodent chow (Teklad 2016) and tap water. The animals were allowed to acclimate for one week before a 21-day slow-release pellet of

0.1mg E2 or placebo (Innovative Research of America, Sarasota, FL) was implanted s.c. at the midline between neck and shoulders under isoflurane anesthesia followed by s.c. ketoprofen analgesia (day 1). Daily 50μl i.p. injections of 20mg/kg Gly or 1:1 DMSO/PBS vehicle began on day 1 and continued every afternoon for 11 days. The glyceollin mixture (glyceollins I, II, and III) was isolated using a procedure described previously (Salvo et al. 2006). Treatment groups were as follows (n = 3): placebo pellet + vehicle control ('Con'), placebo pellet + Gly ('Gly'), E2 pellet + vehicle ('E2'), and E2 pellet + Gly ('E2+Gly').

On the morning of day 12, mice were weighed and then euthanized by decapitation under complete isoflurane anesthesia and trunk blood was collected into heparinized tubes. Brain hemispheres were stored in RNA*later* (Ambion) overnight at 4C and subsequently stored at -20C until further processing. Blood samples were stored on ice until centrifuged and plasma was collected and stored at -20C until processed for an ELISA assay of E2 by the Ligand Assay and Analysis Core Laboratory at the University of Virginia Center for Research in Reproduction. Plasma from 'E2' and 'E2+Gly' treated mice was diluted 1:3 to fit the linear range of the ELISA (detection limits 3–300 pg/ml). Body mass (n = 24) and natural log-transformed E2 plasma levels (n = 10) were analyzed via 2 x 2 ANOVA using the model described below for the microarray data.

Ethics Statement

All protocols used for housing, handling, and euthanasia followed guidelines approved by the *Guide for the Care and Use of Laboratory Animals: Eighth Edition* (2011) and

were approved by the University of Louisville Institutional Animal Care and Use Committee (protocol number 11099).

RNA isolation and microarray platform

Total RNA was isolated from brain hemispheres of 3 mice per group using Trizol/chloroform extraction, followed by spin column purifications (Qiagen RNeasy midi kit) that included DNase (Qiagen) digestions. A Nanodrop 2000 spectrophotometer was used to assess sample purity and RNA concentration and an Agilent Bioanalyzer was used to verify sample integrity (RIN 7.9 - 9). RNA samples were then submitted to the University of Louisville Genomics Facility where they were processed according to standard Affymetrix protocols and independently hybridized to Affymetrix Mouse Gene 1.0 ST Arrays.

Microarray analysis

Low level analyses, quality control, and expression summarization

We examined all arrays at the probe level by inspecting box plots, histograms, pair-wise M v A plots of replicate GeneChips, and pseudo-images of the $\log_2$(intensity) data for each GeneChip. We then fit a probe level model (PLM) to the background adjusted and normalized probe level data and rendered pseudo-images for each GeneChip of the weights, residuals, and residual signs that were obtained by fitting the PLM (Bolstad et al. 2005). Finally, we used the gene expression estimates and standard errors obtained from the PLM to render relative log expression (RLE) and normalized unscaled standard error (NUSE) plots (Bolstad et al. 2005). Upon inspecting these quality metrics, it was

clear that two GeneChips (one E2 and one E2 + Gly) were aberrant, a conclusion that was reinforced by conducting similar quality control (QC) analyses at the transcript cluster (*i.e.*, gene) level. We therefore removed these aberrant chips and re-inspected the probe level data, which revealed no further problems. We then generated expression summaries at the transcript cluster level using the robust multiarray average (RMA) algorithm of Irizarry et al. (2003). All of these procedures were conducted using the 'oligo' package (Carvalho and Irizarry 2010) that is freely available from Bioconductor for the R statistical computing environment (R Development Core Team, 2012).

Data filtering and identification of genes differentially expressed between treatments

Because the Mouse Gene 1.0 ST Array covers 35,556 transcript clusters, there is a considerable multiple testing burden associated with analyzing gene level data from this platform. In order to minimize this burden and avoid conducting tests on genes that are unlikely to be expressed in the brain, we removed transcript clusters whose mean expression across all chips was ≤ to the mean of the first quartiles across all chips ($\log_2$(RMA) = 5.953). In addition, we removed transcript clusters that were not part of the 'main' design of the array (*i.e.*, we removed all control and unmapped transcript clusters) prior to conducting statistical analyses. Upon applying these filters, 22,056 transcript clusters were available for significance testing.

We tested each gene for differential expression using a 2 x 2 factorial ANOVA of the form $\log_2(RMA)_{ijk} = \beta_0 + G_i + E_j + (GE)_{ij} + \varepsilon_{ijk}$ where β_0= intercept, G_i = the main effect of Gly, E_j = the main effect of E2, $(GE)_{ij}$ = the interaction between Gly and E2, and ε_{ijk} = the error term associated with the *k*th individual from Gly treatment *i* and E2

treatment *j*. Differentially expressed genes (DEGs) were identified using *P*-values associated with overall model *F*-statistics and the multiple testing correction of Benjamini and Hochberg (1995; FDR = 0.20) . Once a gene was identified as differentially expressed, we then examined which terms in the model were statistically significant. When significant interaction terms were detected, we used Tukey's honest significant differences (HSD) to determine which levels (*i.e.*, Con, Gly, E2, E2+Gly) differed and did not attempt to interpret the main effects. However, when no significant interaction was detected, the main effects were interpreted unambiguously via *F*-statistics due to the fact that each main effect contained only two levels (*i.e.*, exposure vs. no exposure).

Enrichment analyses

DEGs were further analyzed using MetaCore version 6.18 software (www.portal.genego.com). The genes on 4 different DEG lists (main Gly effect only, main E2 effect only, main E2 and main Gly effects with no interaction, and significant interaction effects) were uploaded as separate files. The 'Enrichment Analysis' function was then applied to each list to identify genes that potentially affect biological pathways, maps or gene ontology (GO) terms that are brain-specific. Further, MetaCore's 'Build Network' function was used to look for any direct interactions among genes on each of the separate DEG lists. As we only used gene lists that were already deemed differentially expressed between treatment groups, we did not use *P*-value threshold options in MetaCore. Also, since the genes on our DEG lists exhibited modest differences

among treatments (see below), we did not use any fold-change cut offs when conducting analyses in MetaCore.

RT-qPCR

Genes and reaction conditions

Reference genes (*Hmg20b, Efnb2*) for reverse transcription quantitative real-time PCR (RT-qPCR) were chosen based on the degree of within and between group variation in the microarray data. Eight genes of interest (GOIs) were chosen for further investigation via RT-qPCR for a variety of reasons including: known E2 responsiveness and importance to endocrine signaling (*Prl, Gh*), identification via microarray screening (*Nr4a1*), and marginal statistical significance and/or relatively large fold change values (~ 2 fold) between two or more experimental groups in the array study (*Ngp, Fcrls, Cdh12, Slc6a4, Tph2*). The same RNA samples used for the microarray analysis were used to make cDNA via the High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems). TaqMan primers (Life Technologies, Supplementary Table 2.1) were chosen, using manufacturer recommendations, to avoid detection of non-target sequences and to have small amplicon lengths. PCRs were 10 µl reactions and were run on an ABI ViiA7 in 384 well plates. Reaction conditions were 50ºC for 2min, 95ºC for 10min, then 40 cycles of 95ºC for 15sec and 60ºC for 1min. Each PCR was technically replicated in triplicate and each plate contained three template-free controls per primer pair.

Analysis of the RT-qPCR data

We fit logistic and log-logistic models (Spiess et al. 2008) to the cycle-by-cycle fluorescence data (ΔRn) associated with each PCR using the 'qpcR' software package by Ritz and Spiess (2008). Seven, six, and five parameter logistic and log-logistic models were considered for each GOI and for each reference gene. Models were selected on a GOI-by-GOI basis via a combination of graphical inspection and consideration of model fitting metrics, such as corrected Akaike's information criterion (AICc) and R^2. Preference was given to models that could be successfully applied with high goodness of fit to as many of the amplification curves under consideration as possible. When different models were chosen for different GOIs, each respective model was independently applied to the reference gene data, to ensure that relative expression ratios (R_E) for each GOI were calculated from efficiency (E) and quantification cycle (C_q) estimates that were based on a common mathematical framework. Amplification data were smoothed using Friedman's super smoother with the span set to 0.1 as recommended in the 'qpcR' documentation. The 'uni2' option was used to test for outlier curves that lacked sigmoidal structure and reactions flagged by this test, as well as reactions where model fitting failed, the fitted model had an $R^2 < 0.99$, or $E < 1.5$, were removed prior to the calculation of R_E. R_E values were calculated according to the relative expression model of Hellemans et al. (2007). Quantification cycles (C_q) were defined as the second derivative maximum (SDM) of the logistic or log-logistic equation estimated via nonlinear regression. The reaction efficiency (E) of each PCR at C_q was determined as described in Spiess et al. (2008). Sample C_q estimates were based on the average of technical replicates that passed all QC measures, and gene-specific E estimates were based on the average across all reactions that were not flagged during QC. The calibrator

cycle for each respective gene was the average C_q estimate across all samples (Hellemans et al. 2007). Log_2 transformed R_E values (Page and Stromberg 2011) were analyzed using a 2 x 2 factorial ANOVA as described above for the microarray data.

Results

Organismal data

Body mass did not differ among groups ($F_{3,20}$ = 1.1210, P = 0.3643, R^2 = 0.1439; Fig. 2.2a); however, natural log-transformed plasma E2 levels were highly statistically significant ($F_{3,6}$ = 72.37, P < 0.0001, R^2 = 0.9731; Fig. 2.2b; raw values, mean (pg/ml) $\pm$ SEM: Con, 4.13 $\pm$ 1.13; Gly, 3.17 $\pm$ 0.003; E2, 265.65 $\pm$ 55.65; E2+Gly, 76.05 $\pm$ 21.15). The interaction between Gly and E2 was not quite statistically significant ($F_{1,6}$ = 4.6177, P = 0.0752), so post-hoc multiple pairwise comparisons were not warranted. However, given this marginally significant interaction effect, it is not surprising that the main effect of Gly was also statistically significant ($F_{1,6}$ = 7.8018, P = 0.0315). As expected, the main effect of E2 was highly statistically significant ($F_{1,6}$ = 206.6632, P < 0.0001); on the raw scale, E2-exposed animals had nearly 47 times the circulating E2 levels of non-E2 exposed animals, whose plasma E2 levels were at or near the lower detection limit of the assay. Collectively, these results show that animals in the E2 groups had significantly higher plasma E2 as compared to non-E2 exposed animals and that Gly may lower circulating E2 concentrations in animals exposed to E2.

Microarray data

Overview of the microarray data

We identified a total of 279 DEGs at an FDR of 0.20. Hierarchical clustering of the DEGs and samples enabled us to recover the four levels of the interaction between E2 and Gly and revealed that, in general, E2 and E2+Gly were most similar to each other among the four treatments, while Gly was the most distinct (*i.e.*, in general, if a gene is up regulated relative to controls by E2 and E2+Gly, then it is down regulated by Gly and *vice versa*, Fig. 2.3).

To better understand the functions of DEGs identified by different combinations of terms in our statistical model ($\alpha = 0.05$), we divided the 279 DEGs into the following categories, which are illustrated schematically in Supplementary Figure 1.1: [1] 33 DEGs with only a significant E2 main effect, [2] 5 DEGs with only a significant Gly main effect, [3] 74 DEGs with both significant Gly and E2 main effects, but no significant interaction term, and [4] 167 DEGs with significant interaction terms. For genes with significant interaction terms we further categorized genes based on patterns of significance (adjusted *P*-value < 0.05) for the six possible pair-wise comparisons obtained via Tukey's HSD. The DEGs relevant to brain function are described in Tables 1.1 to 1.4 (see Supplementary Tables 2.2-2.5 for tables listing all DEGs in each of the 4 categories).

Main effect of E2 on gene expression

Of the 33 DEGs with only a significant E2 main effect (Supplementary Table 2.2), but no interaction effect, 21 genes were upregulated in the E2 groups (*i.e.*, E2 and E2+Gly) (Supplementary Figure 2.1a) when compared to the non-E2 groups (*i.e.*, Con and Gly). Those with known brain-related functions are described in Table 1.1. Enrichment

analysis using MetaCore revealed genes involved in pathways such as 'cholesterol and sphingolipid transport' (*Arf1*), 'development/activation of astroglial cell proliferation' (*Arf1*), 'DNA damage nucleotide excision repair' (*Ercc8*), and 'development, neurogenesis and synaptogenesis' (*Apba2*). Some E2-upregulated genes also annotated to GO processes such as 'response to topologically incorrect protein' (*Hsp105, Ubc6, Ube2j2*), 'protein modification by small protein conjugation' (*Ercc8, Fbxo21, Ubc6, Ube2j2*), and 'primary microRNA processing' (*Dgcr8*). Twelve of the 33 DEGs were downregulated by E2 (Supplementary Figure 2.1b), but none of the downregulated genes annotated to any pathways or GO processes in MetaCore, nor did these have any known functions in brain.

In short, E2 upregulated several genes involved in general brain functions like maintenance of neuronal function and synaptic plasticity, neurogenesis, neurotransmitter release as well as general protein modification and miRNA processing.

Main effect of Gly on gene expression

Of the 5 genes that have only a Gly main effect (Supplementary Figure 2.1c-d, Supplementary Table 2.3), 4 have known brain-related functions (Table 2.2). Four DEGs were upregulated in Gly groups (*i.e.*, Gly and E2+Gly) as compared to the non-Gly groups (*i.e.*, Con and E2), including miR-495, which was 1.75-fold higher in Gly vs. non-Gly groups and has brain derived neurotrophic factor (*Bdnf*) as a predicted target (Wu et al. 2010). In general, Gly upregulated DEGs that are involved in neurogenesis, synaptic plasticity and tissue development and downregulated one DEG (*Ppia*, Supplementary Figure 2.1d) involved in neurodegeneration and apoptosis (Nigro et al. 2013). Only the

downregulated gene (*Ppia*) was identified by Metacore analysis for the pathway 'cholesterol and sphingolipid transport from golgi and ER to the apical membrane' and the GO process 'negative regulation of protein phosphatase type 2b activity'.

Joint main effects of E2 and Gly on gene expression in the absence of an interaction

Seventy-four DEGs, 11 of which have brain-related functions, had main effects of both E2 and Gly with no interaction term (Table 2.3). These 74 genes fell into 4 different patterns of expression (Supplementary Figure 2.1e-h, Supplementary Table 2.4) as described below.

a. Upregulated in the Gly groups (*i.e.*, Gly and E2+Gly) compared to non-Gly groups (*i.e.*, Con and E2) and downregulated in the E2 groups (*i.e.*, E2 and E2+Gly) as compared to non-E2 groups (*i.e.*, Con and Gly) (33 DEGs, Supplementary Figure 2.1e). MetaCore enrichment analysis of these revealed the pathway map 'immune response, oncostatin M-signaling via JAK-STAT in mouse cells' (*Socs3*) and the GO process 'regulation of membrane potential' (*miR-138-1*).
b. Downregulated in the Gly groups compared to non-Gly groups and upregulated in the E2 groups compared to non-E2 groups (28 DEGs, Supplementary Figure 2.1f). MetaCore enrichment analyses of these genes identified pathway maps such as 'development: epigenetic and transcriptional regulation of oligodendrocyte precursor cell differentiation and myelination' (*Hdac1* and *Olig2*), 'cholesterol and sphingolipid transport' from golgi and ER to the apical membrane (*Ppia*) and 'LRRK2 in neuronal apoptosis in Parkinson's disease' (*Prdx3*). GO processes

included ‘positive regulation of oligodendrocyte differentiation’ (*Hdac1* and Olig2), ‘negative regulation of protein phosphatase type 2b activity’ (*Ppia*), ‘negative regulation of kinase activity’ (*Prdx3*, *Socs3* and *Taf10*), and ‘regulation of membrane potential’ (*Cd9*, *Kcnip1*, *miR-138-1*, *Olig2*, *Prdx3* and *Stoml2*). MetaCore analysis also revealed involvement in process networks such as ‘response to hypoxia and oxidative stress’ (*Prdx3*), ‘development: neurogenesis in general’ (*Cd9*), ‘transcription regulation of initiation’ (*eIF4a1*), and ‘transcription by RNA polymerase II’ (*Taf10*). Note that although the gene *Ppia* appears in both the Gly main effects and in the joint E2 and Gly main effects lists, it is represented by different transcript cluster IDs in each case (Supplementary Tables 2.3 and 2.4).

c. Upregulated in Gly groups compared to non-Gly groups and also upregulated in E2 groups compared to non-E2 groups (10 DEGs, Supplementary Figure 2.1g). MetaCore analysis identified only one of these genes, *Zfyve28*, which is included in the GO process ‘negative regulation of kinase activity’.

d. Downregulated in Gly groups compared to non-Gly groups and also downregulated in E2 groups compared to non-E2 groups (3 DEGs, Supplementary Figure 2.1h). None of these genes annotated to GO processes or pathway maps on MetaCore.

Overall, the joint main effects of Gly and E2 indicate that genes upregulated in Gly groups and downregulated in E2 groups are involved in immune response, apoptosis and maintenance of synaptic plasticity, whereas genes upregulated in E2 groups and downregulated in Gly groups seem to be involved in general brain functions like

neurogenesis, apoptosis, transcriptional regulation, cholesterol and lipid transport and regulation of membrane potential as well as kinase activity. One of the 3 genes (*Prok1*) downregulated in both Gly and E2 groups compared to non-Gly and non-E2 groups, respectively, is involved in developmental neurogenesis.

E2 and Gly interaction effects on gene expression

A total of 167 DEGs were found to have statistically significant E2 x Gly interaction terms (Supplementary Table 2.5). Twenty-one of these have known brain-related functions (Table 2.4), such as embryonic brain development and neuronal differentiation. The 167 DEGs were further categorized based on the individual patterns of significance for six-possible pair-wise comparisons (Gly vs Con, E2 vs Con, E2 + Gly vs Con, E2 + Gly vs E2, E2 + Gly vs Gly, and E2 vs Gly). A combined view of all the different pairwise comparisons for each of the genes on this list with directionality taken into account reveals that these genes follow 20 unique patterns of expression. Sixty-five DEGs have one pattern of particular interest: upregulated in Gly compared to control, the E2 group not different from control, but E2+Gly gene expression significantly lower than that of Gly alone (Supplementary Figure 2.1i).

MetaCore enrichment analysis revealed that some genes on this list are involved in 5 different pathway maps: 'microRNA-dependent inhibition of epithelial to mesenchymal transition' (*miR-200a*), 'DNA damage mismatch repair' (*Mlh1*, *Msh2*), 'immune response – IFN alpha/beta signaling pathway' (*Ifnα, Pml*), 'keratan sulfate metabolism' (*B4gt4*, *Chst2*) and 'aminoacyl tRNA biosynthesis in the mitochondrion' (*Lars2, Tarsl1*). Two GO processes, 'protein demalonylation and peptidyl-lysine desuccinylation' (*Sirtuin 5*)

and ‘microglia development’ (*Itgam*), were revealed in MetaCore, which also found metabolic networks ‘L-tryptophan pathways and transport’ (*Lars2*, *Nanog*) and ‘lipid metabolism – blood group glycolipid-neo-lactoseries metabolism’ (*B4gt4*, *Chst2*), the latter especially interesting given known lipid-processing effects of Gly in the periphery (Wood et al. 2012). To summarize, genes with significant interaction effects are involved in broad functional categories like brain development, neuronal differentiation, immune response, DNA damage repair and lipid metabolism.

RT-qPCR data

The five parameter log-logistic model described by Spiess et al. (2008) was selected for all GOIs except *Prl*, for which the seven parameter logistic model was selected. In general, the correlation between the microarray data and the RT-qPCR data was strong (mean $r = 0.831$, minimum = 0.605, maximum = 0.952, $n = 8$). As can be seen in Fig. 4, we were generally able to replicate the trends in the microarray data using RT-qPCR. GOIs investigated via RT-qPCR can be broken into the following three categories based on the results of 2-way ANOVAs at the 0.05 level (Supplementary Table 2.7): no statistical significance at the overall model level (*Fcrls*, *Gh*, *Slc6a4*, *Tph2*), statistical significance at the overall model level and for the main effect of E2 (*Cdh12*, *Ngp*, *Prl*), and statistical significance at the overall model level and for the main effects of E2 and Gly (*Nr4a1*). Collectively, these results show that we were able to replicate the general trends and magnitudes of gene expression estimated via microarray using RT-qPCR for a variety of genes, many of which were not strongly differentially expressed among treatments.

Discussion

To determine potential mechanisms of action of Gly in the female mouse brain, we measured the effects of soy-derived Gly action alone and in conjunction with E2 on gene expression. Given the anti-estrogenic effects of Gly via ER-mediated mechanisms in the periphery (Ng et al. 2011; Nwachukwu et al. 2013), we expected the primary pattern of differential expression in the brain to be Gly opposition of the effects of E2 (*i.e.*, significant E2 vs Con and E2+Gly vs E2 comparisons, but not Gly vs Con comparison). However, only 4 (*Tmem79, Ssh1, Brf2, Olfr411*) of the 167 genes with a significant interaction term showed this pattern. On the other hand, 65 of the genes on this list showed the opposite pattern of expression, with E2 appearing to oppose the effects of Gly (*i.e.*, significant E2+Gly vs Gly comparison) on expression of genes that were not significantly affected by E2 compared to controls (Supplementary Figure 2.1i). Our whole brain microarray analysis showed that the Gly treatment produced the most distinct gene expression pattern, while the two E2 groups (E2 and E2 + Gly) were the most similar to each other in terms of gene expression (Fig. 2.3). We identified a total of 279 DEGs, but the fold-change values for the DEGs were modest, with *Nr4a1* exhibiting the largest change (FC = 1.89, Gly vs Con). This could be due to the fact that our microarray analysis reflects the overall expression of these genes across the whole brain. Future studies looking at expression of gene targets in specific brain regions may reveal higher fold-change effects between treatment groups in discrete brain regions.

Estrogens perform several functions in the brain related to neuroprotection, cognition, stress, food intake, etc., and ERs are distributed throughout the brain (Mitra et al. 2003). Hence it was not surprising that many of the upregulated E2 main effect genes (*e.g.*,

Calmin, *Spondin1*, *Dgcr8* and *Apba2*) are predominantly involved in pathways and processes such as neurogenesis, maintenance of synaptic plasticity, dendritic spine maintenance and axonal growth (Feinstein et al. 1999; Fenelon et al. 2013; Kirov et al. 2008; Takaishi et al. 2003). Some 'classic' E2-responsive genes (*e.g.*, estrogen or progesterone receptors, prolactin, growth hormone), however, did not make our DEG list, often due to variability within groups. For example, the average expression of *Prl* in the E2 group was 11.19 fold-change higher than that of controls, but intra-group variability prevented the identification of *Prl* as a DEG.

Average plasma E2 levels in the E2+Gly group (76.05 $\pm$ 21.15 pg/ml) were only about 30% that of the E2 treatment group (265.65 $\pm$ 55.65 pg/ml). Although the interaction term was only marginally significant (P=0.0752), this difference in plasma E2 could be biologically relevant. Regarding individual genes for which expression differed in mice treated with E2+Gly compared to E2 alone (*e.g.*, *Nr4a1, Svop*; Table 2.4, Supplementary Table 2.5), one explanation besides direct anti-estrogenic effects of Gly is that some differences in E2-driven gene expression could be due to lower plasma E2 levels in the E2+Gly group, a possibility that we cannot exclude in the current study. An interesting point for future studies is that Gly might have suppressed plasma E2 levels in the E2+Gly animals, perhaps via enhanced clearance of E2. The hormone concentrations we observed could also be due to pellet variability in maintaining plasma E2 levels (Ingberg et al. 2012).

Regardless of the source of plasma E2 variability, the difference in plasma E2 concentrations between these two groups appears to have had little overall effect on gene expression patterns, given that the two groups with E2 pellets clustered separately from

the groups that received a placebo pellet, as seen in Fig. 1.3. Furthermore, 79 DEGs had significant Gly effects (5, Gly main effect only; 74, Gly and E2 main effects with no interaction), suggesting that Gly has effects on gene expression in the brain that are independent of plasma E2 status. Three of the 5 genes upregulated by Gly alone (*i.e.*, *miR-495*, *Hmcn1* and *Tnfsf13b*) are involved in neurogenesis, tissue development and immune response functions, respectively (Farina et al. 2007; Wu et al. 2010; Xu et al. 2013) (Table 2.2). Interestingly, *Ppia*, which is downregulated in Gly groups (Tables 2.2, 2.3), is involved in apoptosis and promotes neurodegeneration by initiating a pro-inflammatory pathway leading to neuronal damage and loss of synaptic connections (Nigro et al. 2013). These results indicate that Gly by itself can regulate genes belonging to these pathways and may potentially have neuroprotective effects.

The mechanisms by which Gly affects gene expression in the brain remain unclear and need to be tested empirically, perhaps using ER knockout mice to verify which effects of Gly are indeed through ER-mediated vs. non ER-mediated mechanisms. Testing effects of Gly on gene expression in neural cell culture would also determine if the effects of Gly reported here are due to direct effects at the neuron or due to systemic effects (*e.g.*, stress, plasma E2) that affected gene expression detected at the whole brain level. Given the interest in Gly's anti-cancer and glucose handling effects in the periphery (Boue et al. 2012; Salvo et al. 2006), understanding its CNS effects is an important component of developing Gly's therapeutic potential.

Fig. 2.1 Known *in vitro* and *in vivo* effects of Gly (glyceollin I structure shown) that are ER-mediated vs non ER-mediated. See text for citations.

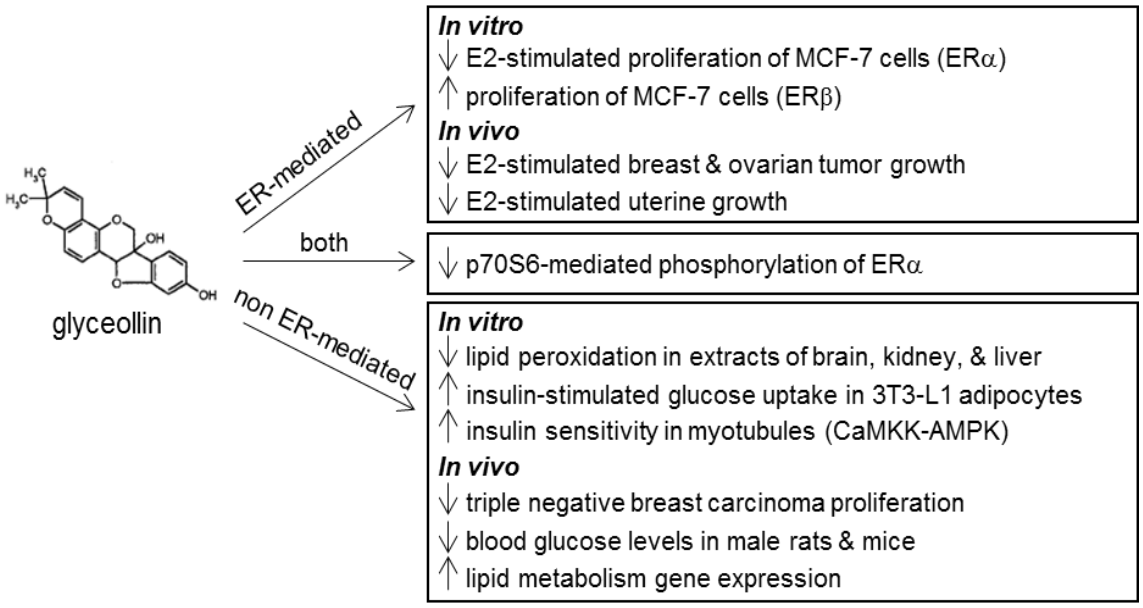


Fig. 2.2 Interaction plots showing a) body mass (mean ± SEM, ANOVA p = 0.36) b) natural log transformed plasma E2 levels (mean ± SEM, ANOVA p < 0.0001, E2 p < 0.0001, Gly p = 0.0315, E2 x Gly interaction p = 0.075). ❍ = Con, ● = E2, △ = Gly, ▲= E2 + Gly. Left columns (circles) = no Gly; right columns (triangles) = Gly. Dotted lines connect the non-E2 groups (❍ and △) and solid lines connect the two E2 groups (● and ▲).

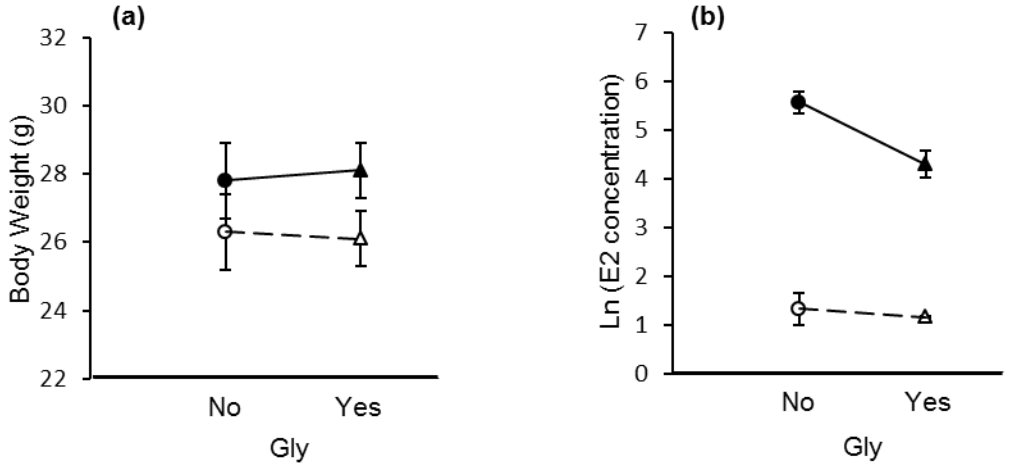


Fig. 2.3 Heat map of the 279 DEGs. Columns correspond to individual GeneChips for samples from the Gly, Con, E2, and E2 + Gly treatments, with animal IDs shown. The dendrograms on top and to the left were obtained via hierarchical clustering of a pair-wise Euclidean distance matrix.

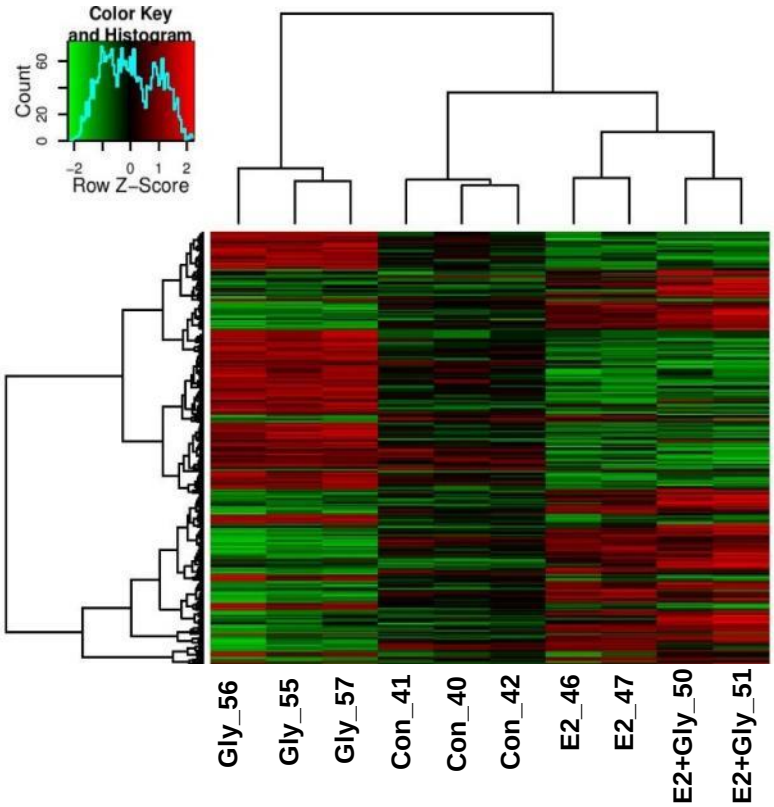


Fig. 2.4 Bar plots of the microarray (grey bars) and RT-qPCR (black bars) data for the eight genes (*Cdh12*, *Fcr1s*, *Gh*, *Prl*, *Nr4a1*, *Ngp*, *Tph2*, *Slc6a4*) whose expression levels were technically replicated. Log_2(RMA) values from the microarray experiment and log_2(R) values from the RT-qPCR experiment were centered and scaled to facilitate side-by-side comparisons.

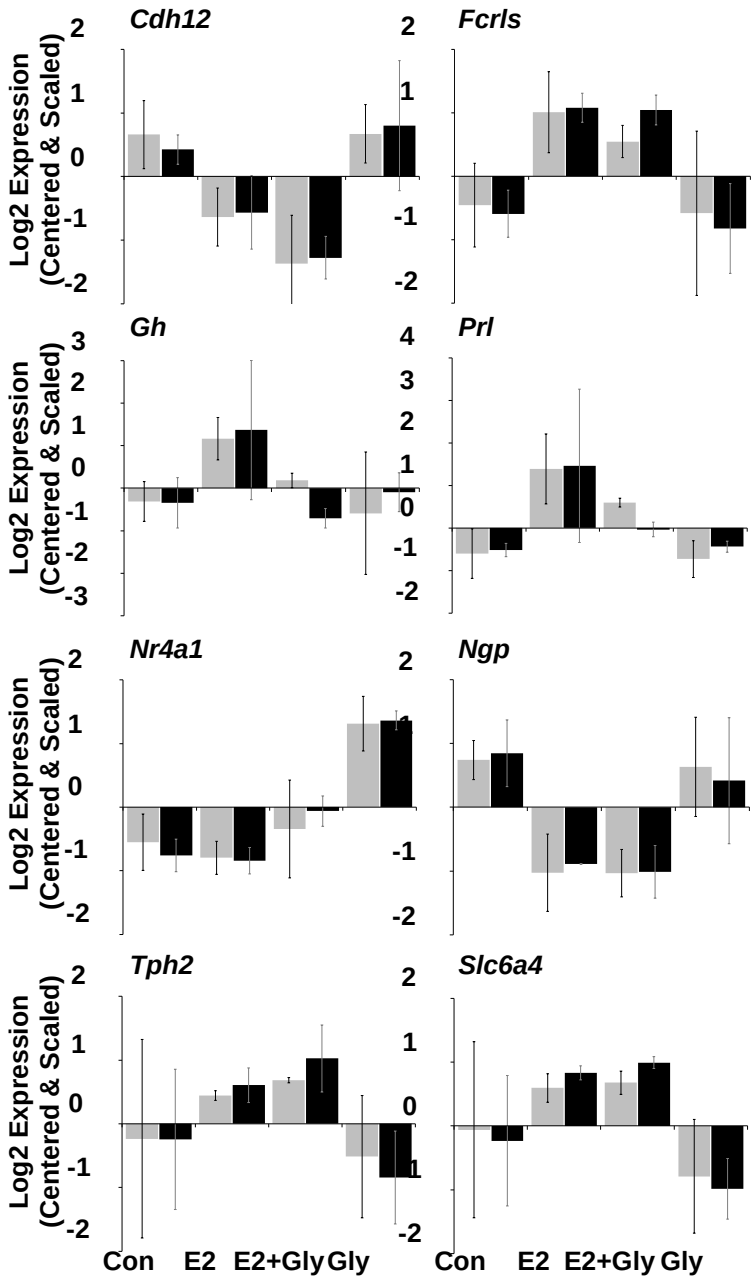


Table 2.1. Brain-related DEGs with significant main E2 effect only, no interaction term

Gene Symbol	Gene Name	Function	P-value	FC[a]	Ref
Apba2	amyloid beta (A4) precursor protein-binding, family A, member 2	neurotransmitter release	0.0002	1.14	Kirov et al, 2008
Clmn	Calmin	development & maintenance of neuronal function	0.0003	1.27	Takaishi et al, 2003
Dgcr8	DiGeorge syndrome critical region gene 8	maintenance of short & long term synaptic plasticity	0.0002	1.13	Fenelon et al, 2013
Spon1	Spondin-1	dendritic & axonal growth - hippocampus	0.0002	1.11	Feinstein et al, 1999; Ho et al, 2004

[a]FC = fold change (E2 + E2+Gly) / (Con + Gly)

Table 2.2. Brain-related DEGs with significant main Gly effect only, no interaction term

Gene Symbol	Gene Name	Function	P-value	FC[a]	References
Hmcn1	hemicentin 1	cell division & development of tissue architecture	0.0007	1.24	Xuehong et al, 2013
miR-495	microRNA 495	targets BDNF involved in synaptic plasticity & neurogenesis	0.0006	1.75	Wu et al, 2010
Ppia	peptidylprolyl isomerase A	apoptosis; promotes neurodegeneration	0.0003	0.91	Nigro et al, 2013
Tnfsf13b	tumor necrosis factor (ligand) superfamily, member 13b	innate & adaptive immune response in the CNS	0.0006	1.12	Farina et al, 2007

[a]FC = fold change (Gly + E2+Gly) / (Con + E2)

Table 2.3 Brain-related DEGs with significant Gly and significant E2 main effects, no interaction term

Gene Symbol	Gene Name	Function	P-value Gly[a]	FC Gly[a]	P-value E2[a]	FC E2[a]	References
Gly up, E2 down[a]							
Atp12a	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	mitochondrial dysfunction	0.0204	1.16	0.0004	0.76	Zhang et al, 2010
Dgkz	diacylglycerol kinase zeta	dendritic spine maintenance; neurite outgrowth	0.0247	1.11	0.0011	0.84	Seo et al,2012; Yakubchyk et al,2005
Ifitm5	interferon induced transmembrane protein 5	enriched in GABAergic neurons	0.0030	1.15	0.0031	0.88	Hardt et al, 2008
miR-211	microRNA 211	antiproliferative effects on glioma cells	0.0048	1.12	0.0005	0.83	Asuthkar et al, 2012
miR-30d	microRNA 30d	expressed in aging mouse brains	0.0044	1.09	0.0002	0.86	Li et al, 2011; Eda et al, 2011
miR-503	microRNA 503	promotes apoptosis of dendritic cells	0.0022	1.19	0.0004	0.79	Min et al, 2013
Gly down, E2 up[a]							
Ppap2b	phosphatidic acid phosphatase type 2B	neuronal differentiation precursor	$8.78E^{-06}$	0.85	0.0056	1.05	Sanchez-Sanchez, 2012
Ppia	peptidylprolyl isomerase A	apoptosis; promotes neurodegeneration	$1.63E^{-05}$	0.92	0.0103	1.02	Nigro et al, 2013
Vdac3	voltage-dependent anion channel 3	hippocampal-dependent contextual fear conditioning	0.0233	0.95	0.0003	1.13	Weeber et al, 2002
Gly up, E2 up[a]							
Lnx1	ligand of numb-protein X 1	regulates ErbB signaling in perisynaptic schwann cells	0.0005	1.09	0.0393	1.04	Young et al, 2005

Gly down, E2 down[a]							
Prok1	prokineticin 1	maintenance & proliferation of enteric neural crest cells	0.0008	0.89	0.0002	0.86	Ngan et al, 2008

[a]Gly refers to any group with glyceollin treatment (Gly or E2+Gly); E2 refers to any group with E2 treatment (E2 or E2+Gly); FC Gly = fold change (Gly + E2+Gly) / (Con + E2); FC E2 = fold change (E2 + E2+Gly) / (Con + Gly)

Table 2.4 Brain-related DEGs with significant E2 x Gly interaction effects

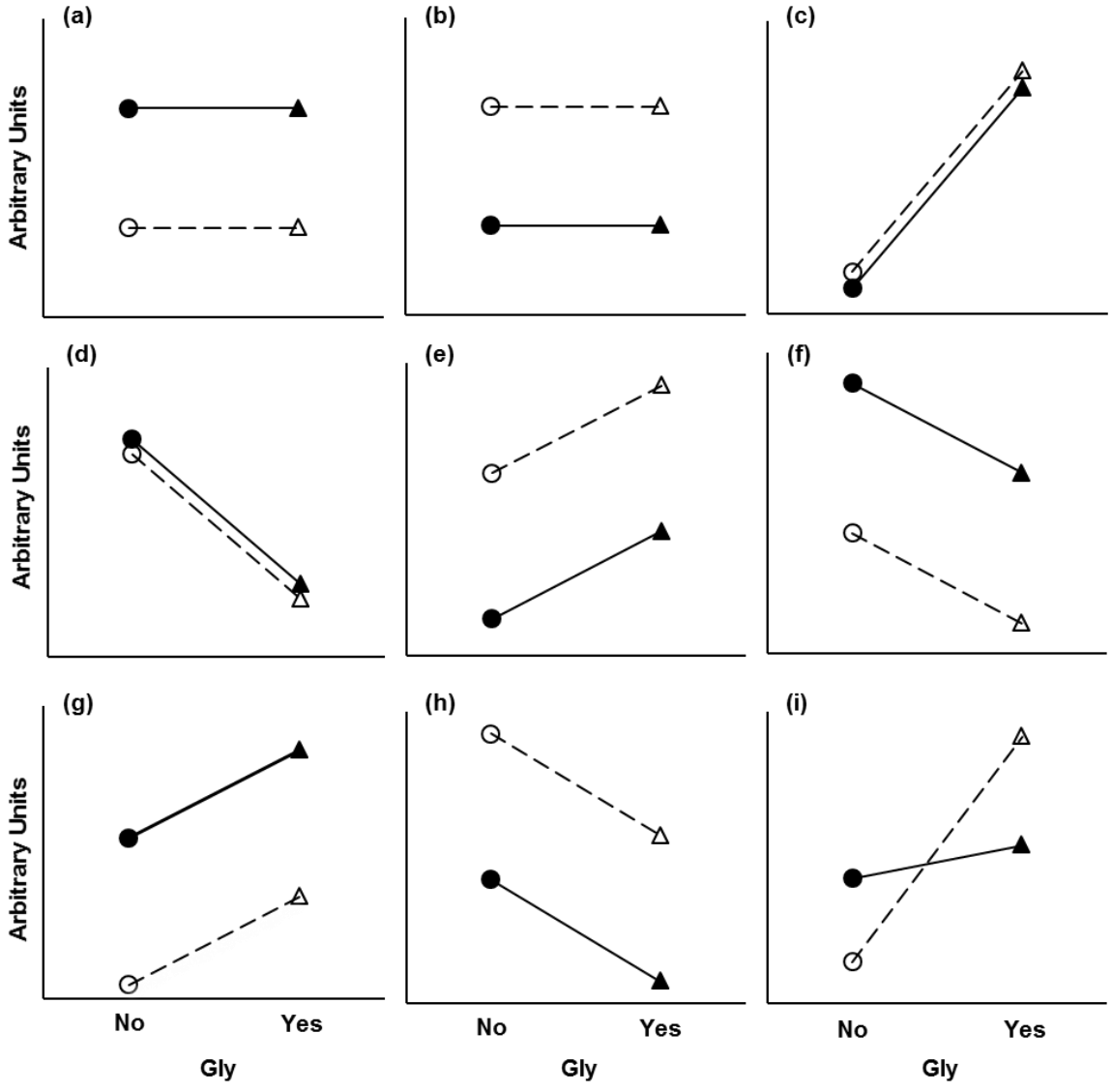
Gene Symbol	Gene Name	Function	Int P-value[a]	FC[a] GvC	FC EvC	FC EGvC	FC EGvG	FC EGvE	FC EvG	References
Alkbh3	alkB, alkylation repair homolog 3 (E. coli)	RNA metabolism & mRNA export	0.0127	1.02	1.03	1.23	1.21	1.19	1.01	Zheng et al, 2013
Alkbh5	alkB, alkylation repair homolog 5 (E. coli)	RNA metabolism & mRNA export	0.0172	0.94	1.10	1.22	1.29	1.11	1.16	Zheng et al, 2013
Bcs1l	BCS1-like (yeast)	expressed in neuronal processes during neuronal development	0.0019	0.86	1.04	1.27	1.48	1.22	1.22	Kotarsky et al, 2007
Coro7	coronin 7	expressed in the developing mouse brain at early embryonic stages	0.0265	1.06	1.04	1.26	1.19	1.21	0.98	Rybakin et al, 2004
Derl2	Der1-like domain family, member 2	degradation of misfolded glycoproteins in the ER	0.0212	0.81	1.03	1.00	1.24	0.97	1.28	Oda et al, 2006
Grfa3	glial cell line derived neurotrophic factor family receptor alpha 3	neurotrophic factor family receptor found in cerebellum & spinal cord	0.0048	1.12	0.94	0.87	0.78	0.93	0.84	Masure et al, 1998
Igsf1	immunoglobulin superfamily, member 1	expressed in pituitary & hypothalamus	0.0089	0.77	1.04	1.06	1.38	1.02	1.35	Bischof et al, 2005
Kif6	kinesin family	neuronal migration	0.0012	1.18	1.06	0.98	0.83	0.93	0.89	Falnikar et al,

	member 6									2013
Manf	mesencephalic astrocyte-derived neurotrophic factor	expressed in purkinje cells in cortex, hippocampus & cerebellum	0.0225	0.81	1.04	0.98	1.21	0.94	1.29	Lindholm et al, 2008
miR-210	microRNA 210	hypoxia-induced apoptosis of neuroblastoma cells	0.0057	1.19	0.96	0.98	0.82	1.02	0.81	Chio et al, 2013
miR-23a	microRNA 23a	oligodendrocyte differentiation & myelin synthesis	0.0035	1.16	0.93	0.96	0.82	1.03	0.80	Lin et al, 2013; Smirnova et al, 2005
Nlrc5	NLR family, CARD domain containing 5	innate immune system responses	0.0031	1.20	0.95	0.88	0.73	0.93	0.78	Tong et al, 2012
Nme1	nucleoside diphosphate kinase 1	involved in glial-neuronal cell fate determination	0.0207	0.90	1.07	1.11	1.23	1.03	1.19	Owlanj et al, 2012
Nr4a1	nuclear receptor subfamily 4, group A, member 1	cytokine & metabolic pathways, long-term memory	0.0028	1.89	0.97	1.23	0.65	1.26	0.52	Zhao et al, 2010; McNulty et al, 2012
Ryr1	ryanodine receptor 1, skeletal muscle	promotes calcium release from central neurons	0.0297	1.04	1.06	1.18	1.14	1.11	1.02	Kakizawa et al, 2011
Smarcal 1	SWI/SNF related matrix associated, actin dependent regulator of chromatin, subfamily a-like 1	replication stress-response	0.0115	0.90	1.07	1.14	1.28	1.07	1.20	Bansbach et al, 2009
Spg11	spastic paraplegia 11	expressed in cortical & spinal motor neurons	0.0008	0.99	0.99	1.18	1.19	1.19	1.00	Murmu et al, 2011

Svop	SV2 related protein	expressed in adult mouse hippocampus & cerebellum, sugar-transporter	0.0478	1.05	1.12	1.34	1.27	1.20	1.06	Cho et al, 2009
Usp22	ubiquitin specific peptidase 22	expressed in the developing mouse brain at early embryonic stages	0.0063	0.98	1.04	1.26	1.28	1.21	1.06	Lee et al, 2006
Wnt7b	wingless-related MMTV integration site 7B	expressed in the developing mouse brain	0.0147	0.87	1.04	1.03	1.18	1.00	1.19	Abu-Khalil et al, 2004
Zbtb7a	zinc finger and BTB domain containing 7a	oligodendrocyte differentiation	0.0005	0.83	1.04	1.05	1.25	1.00	1.25	Dobson et al, 2012

[a]Int P-value = E2 x Gly interaction term P-value; FC = fold change

Supplementary Figure 2.1 Interaction plots showing representative patterns of expression of DEGs. a-b) DEGs with main E2 effect only, no interaction effect (n=33); c-d) DEGs with main Gly effect only, no interaction effect (n=5); e-h) DEGs with main E2 and main Gly effects, no interaction effect (n=74); e) Gly up, E2 down; f) Gly down, E2 up; g) Gly up, E2 up; h) Gly down, E2 down; i) One of the 20 significant interaction effects patterns, which indicates possible E2 opposition of Gly effects on gene expression (65 of 167 DEGs, see text). ❍ = Con, ● = E2, △ = Gly, ▲= E2 + Gly. Left columns (circles) = no Gly; right columns (triangles) = Gly. Dotted lines connect the non-E2 groups (❍ and △), solid lines connect the two E2 groups (● and ▲)



Supplementary Table 2.1. Genes measured with RT-PCR, and Life Technologies assay IDs for TaqMan primers.

Gene code	Gene name	Gene ID	Assay ID
Efnb2	Ephrin B2	NM_010111	Mm01215897_m1
Hmg20b	High mobility group 20B	NM_010440	Mm00468918_m1
Prl	Prolactin	NM_011164	Mm00599950_m1
Ngp	Neutrophilic granule protein	NM_008694	Mm00476389_m1
Fcrls	Fc receptor-like S	NM_030707	Mm00472833_m1
Nr4a1	Nuclear receptor subfamily 4	NM_010444	Mm01300401_m1
Gh	Growth hormone	NM_008117	Mm00433590_g1
Cdh12	Cadherin 12	NM_001008420	Mm01165359_m1
Slc6a4	Solute carrier family 6	NM_010484	Mm00439391_m1
Tph2	Tryptophan hydroxylase 2	NM_173391	Mm00557715_m1

Supplementary Table 2.2. DEGs with significant main E2 effect only, no interaction effect

Cluster ID	Gene Name	Gene Symbol	E2 *P*-value	FC - E2/No E2
10402473	calmin	Clmn	0.0002778	1.272591485
10444927	nurim (nuclear envelope membrane protein)	Nrm	0.0003073	1.272512378
10420237	ubiquitin-conjugating enzyme E2, J2 homolog (yeast)	Ube2j2	0.0004253	1.250372284
10518494	patched domain containing 2	Ptchd2	0.0004024	1.24588567
10498296	COMM domain containing 2	Commd2	0.0003058	1.240037953
10382701	SAP30 binding protein	Sap30bp	0.0002989	1.198836286
10475830	mitochondrial ribosomal protein S5	Mrps5	0.0004473	1.198301029
10424060	Smg-5 homolog, nonsense mediated mRNA decay factor pseudogene	A930017M01Rik	0.0002298	1.194212595
10394429	RIKEN cDNA 1110057K04 gene	1110057K04Rik	0.0002304	1.189768918
10535637	pentatricopeptide repeat domain 1	Ptcd1	0.000214	1.188513774
10563583	serum amyloid A-like 1	Saal1	0.0001505	1.174200366
10407057	excision repaiross-complementing rodent repair deficiency, complementation group 8	Ercc8	0.0003957	1.15482094
10535904	heat shock 105kDa/110kDa protein 1	Hsph1	4.74E-05	1.149153469
10524941	F-box protein 21	Fbxo21	0.0002588	1.14900239
10598389	WD repeat domain 45	Wdr45	0.0003289	1.144614549
10553917	amyloid beta (A4) precursor protein-binding, family A, member 2	Apba2	0.0002452	1.136367819
10580169	coiled-coil domain containing 130	Ccdc130	0.0004312	1.133102262
10453792	THO complex 1	Thoc1	0.0003816	1.13075786
10438313	DiGeorge syndrome critical region gene 8	Dgcr8	0.0002161	1.127223329
10386370	ADP-ribosylation factor 1	Arf1	0.0001687	1.126823468
10556509	spondin 1, (f-spondin) extracellular matrix protein	Spon1	0.0001597	1.114214783
10551953	predicted gene 1082	Gm1082	0.0002641	0.889650316

10526718	sperm motility kinase 3A	Smok3a	0.0002448	0.871153708
10558698	outer dense fiber of sperm tails 3	Odf3	0.0001787	0.85634709
10493903	late cornified envelope 3C	Lce3c	0.0005959	0.841790711
10589625	protease, serine, 43	Prss43	0.0002384	0.82190624
10357696	predicted gene 10188	Gm10188	0.0003313	0.820374562
10364155	RIKEN cDNA 2610028H24 gene	2610028H24Rik	0.0003097	0.817654509
10569896	CD209g antigen	Cd209g	0.0002588	0.801977346
10566595	olfactory receptor 697	Olfr697	0.0002991	0.775205364

Supplementary Table 2.3. DEGs with significant main Gly effect only, no interaction effect

Cluster ID	Gene Name	Gene Symbol	Gly *P*-value	FC - Gly/No Gly
10482507	peptidylprolyl isomerase A	Ppia	0.00027473	0.914567569
10398402	microRNA 495	Mir495	0.00057025	1.751643961
10576951	tumor necrosis factor (ligand) superfamily, member 13b	Tnfsf13b	0.00058411	1.116875689
10358611	hemicentin 1	Hmcn1	0.00066053	1.24250467
10447977	WD repeat domain 27	Wdr27	0.00082034	1.203776944

Supplementary Table 2.4. DEGs with significant Gly and significant E2 main effects, no interaction effects

Cluster ID	Gene Name	Gene Symbol	E2 *P*-value	Gly *P*-value	FC-E2/No E2	FC - Gly/No Gly
10424555	---		0.002195973	0.00124968	0.69027004	1.505967514
10504121	predicted gene 3893	Gm3893	0.036336185	0.00065509	0.88785647	1.323358338
10504148	RIKEN cDNA 4933409K07 gene	4933409K07Rik	0.031914404	0.00086073	0.8880316	1.296291092
10504169	RIKEN cDNA 4933409K07 gene	4933409K07Rik	0.02651595	0.00079573	0.88575532	1.292134123
10504172	RIKEN cDNA 4933409K07 gene	4933409K07Rik	0.033764379	0.00068863	0.89660009	1.287345527
10392699	---		0.009216708	0.00071232	0.86586133	1.278074156
10590462	microRNA 138-1	Mir138-1	0.005892133	0.00212107	0.83509886	1.26151245
10369927	---		0.00092109	0.0111436	0.7579822	1.212772051
10392834	predicted gene 11711	Gm11711	0.006612927	0.00149895	0.87931996	1.199375158
10559172	keratin associated protein 5-3	Krtap5-3	0.000426792	0.00830389	0.75050392	1.196567733
10604612	microRNA 503	Mir503	0.000374233	0.00224243	0.78507007	1.189765774
10529385	zinc finger, FYVE domain containing 28	Zfyve28	0.014657347	0.00069105	1.08962178	1.17398773
10606389	---		0.000978379	0.01017972	0.79228767	1.161735161
10420316	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	Atp12a	0.000390177	0.02043303	0.76292553	1.158240903
10507961	metal response element binding transcription factor 1	Mtf1	0.005048835	0.00044364	1.081455	1.148285719
10569011	interferon induced transmembrane protein 5	Ifitm5	0.00309811	0.00302969	0.88148091	1.145363656
10575074	transmembrane and coiled-coil domains 7	Tmco7	0.000425722	0.00070862	1.1730721	1.14500354
10363970	RAB36, member RAS oncogene family	Rab36	0.002111967	0.00117055	1.1370113	1.143333248
10535747	predicted gene 10858	Gm10858	0.000283642	0.00717405	0.7764139	1.143208962
10520948	phospholipase B1	Plb1	0.00108551	0.0026923	0.8636325	1.140101235

10559175	keratin associated protein 5-4	Krtap5-4	0.000280936	0.00243769	0.8424084	1.131668739
10553895	microRNA 211	Mir211	0.000496499	0.00484091	0.83467245	1.124439293
10375553	olfactory receptor 1389	Olfr1389	0.001122493	0.00151768	0.88472251	1.120751727
10449596	---		0.001537814	0.00276721	0.89066975	1.1191586
10515242	NOL1/NOP2/Sun domain family, member 4	Nsun4	0.000391448	0.00976323	1.2412183	1.118227032
10434754	cDNA sequence BC106179	BC106179	0.000316209	0.01806873	0.77665781	1.117170896
10451481	cDNA sequence BC032203	BC032203	0.000577131	0.00287683	1.15990174	1.115019833
10474004	diacylglycerol kinase zeta	Dgkz	0.001056113	0.02468176	0.83982308	1.11256167
10505779	alkaline ceramidase 2	Acer2	0.000446772	0.02432106	1.29264789	1.108196309
10481262	ficolin B	Fcnb	3.71E-05	0.00028789	0.87402403	1.099320058
10414772	predicted gene 10890	Gm10890	0.000454958	0.01973874	0.82201726	1.095959985
10429199	microRNA 30d	Mir30d	0.000230439	0.00440432	0.86355439	1.09204143
10530666	ligand of numb-protein X 1	Lnx1	0.03934717	0.00052707	1.03502155	1.090166633
10391555	pancreatic polypeptide	Ppy	0.000868686	0.01393784	0.87676346	1.083460407
10399387	mesogenin 1	Msgn1	0.000183581	0.00217594	0.87968247	1.081950768
10513592	WD repeat domain 31	Wdr31	0.000305246	0.02162895	1.18557831	1.075204956
10507671	guanylate cyclase activator 2a (guanylin)	Guca2a	0.000476461	0.01459981	0.88490962	1.073229988
10559989	aurora kinase C	Aurkc	0.000164036	0.00622595	0.8880848	1.067976341
10397428	RIKEN cDNA 1700020O03 gene	1700020O03Rik	6.58E-05	0.0101924	1.18652492	1.06389794
10551435	RIKEN cDNA 9530053A07 gene	9530053A07Rik	6.19E-05	0.0409589	0.83033149	1.063634937
10383010	suppressor of cytokine signaling 3	Socs3	7.80E-05	0.03374494	0.81265095	1.062245274
10507719	schlafen like 1	Slfnl1	0.000994175	0.03603581	0.9036554	1.056997435
10560237	G protein-coupled receptor 77	Gpr77	0.00014766	0.0391774	0.82201276	0.953302203

10454984	WD repeat domain 55	Wdr55	1.95E-05	0.00663639	1.16794343	0.952327991
10577534	voltage-dependent anion channel 3	Vdac3	0.000333508	0.02332508	1.1261931	0.948005801
10387545	eukaryotic translation initiation factor 4A1	Eif4a1	0.004091706	0.0016437	1.05044103	0.947087953
10427772	threonyl-tRNA synthetase	Tars	0.000309922	0.00841852	1.13240599	0.935387376
10452525	NADH dehydrogenase (ubiquinone) flavoprotein 2	Ndufv2	0.005686588	0.00132796	1.04964645	0.934488745
10566525	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor	Taf10	3.51E-05	0.0018658	1.15786613	0.929120738
10447004	histone deacetylase 1	Hdac1	0.000143653	0.0002311	1.08889386	0.927286391
10463704	arsenic (+3 oxidation state) methyltransferase	As3mt	0.000857271	0.02811834	1.15974357	0.926548707
10468869	peroxiredoxin 3	Prdx3	0.000717133	0.02349014	1.17408008	0.925094664
10441902	SPARC related modular calcium binding 2	Smoc2	0.000929691	0.00602154	1.11529782	0.92171861
10567574	peptidylprolyl isomerase A	Ppia	0.01025328	1.63E-05	1.02426757	0.919112335
10368935	glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1	Qrsl1	0.000373931	0.01444949	1.19353599	0.913499024
10561140	mitochondrial ribosomal protein L41	Mrpl41	0.000788042	0.00808273	1.14801946	0.911506194
10512443	stomatin (Epb7.2)-like 2	Stoml2	0.000693955	0.04051634	1.24198993	0.91113441
10560548	gem (nuclear organelle) associated protein 7	Gemin7	0.000828162	0.02259287	1.19516696	0.910974675
10516652	IQ motif containing C	Iqcc	0.002498413	0.00080395	1.07724756	0.909791887
10351491	olfactomedin-like 2B	Olfml2b	0.001460787	0.00539464	1.13607036	0.9020669
10345926	RIKEN cDNA 1500015O10 gene	1500015O10Rik	0.001418663	0.00371689	1.1240296	0.901512304
10501084	prokineticin 1	Prok1	0.000223595	0.00076156	0.86064223	0.887671109
10385096	Kv channel-	Kcnip1	0.025177799	0.00068519	1.05909392	0.880520552

	interacting protein 1					
10510197	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	Ppp2r5a	0.005412457	0.00059226	1.08823765	0.879242806
10548030	CD9 antigen	Cd9	0.00193229	0.00171101	1.13442297	0.874130093
10516778	zinc finger, CCHC domain containing 17	Zcchc17	0.001020832	0.00444588	1.20274783	0.86794695
10540275	glucoside xylosyltransferase 2	Gxylt2	0.004810183	0.00180108	0.89207655	0.8655777
10506496	phosphatidic acid phosphatase type 2B	Ppap2b	0.005596524	8.78E-06	1.04668334	0.852024197
10436823	oligodendrocyte transcription factor 2	Olig2	0.010171793	0.0015143	1.11431626	0.835815164
10423505	carboxymethylenebut enolidase-like (Pseudomonas)	Cmbl	0.028033234	0.00127775	1.08991123	0.826956277
10569368	---		0.002858606	0.00039387	1.14747467	0.806451477
10552311	---		0.002248153	0.00257408	1.23216774	0.803644701
10356457	DnaJ (Hsp40) homolog, subfamily B, member 3	Dnajb3	0.002000887	0.0004311	1.17134659	0.794770847

Supplementary Table 2.5. DEGs with significant interaction effects

Cluster ID	Gene Name	Gene Symbol	Int *P*-value
10555039	potassium channel tetramerisation domain containing 21	Kctd21	0.00081238
10414911	T cell receptor alpha variable region 13-3	Trav13-3	0.00432031
10551169	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	B3gnt8	0.00491566
10431912	solute carrier family 38, member 2	Slc38a2	0.00930295
10497920	ankyrin repeat domain 50	Ankrd50	0.01594006
10402570	predicted gene 10427	Gm10427	0.02333913
10541524	Nanog homeobox	Nanog	0.02512536
10600301	signal sequence receptor, delta	Ssr4	0.0303531
10414594	methyltransferase 11 domain containing 1	Mett11d1	0.00111753
10595718	carbohydrate sulfotransferase 2	Chst2	0.00126477
10566213	olfactory receptor 605	Olfr605	0.04246859
10380285	transmembrane protein 100	Tmem100	0.00531559
10435724	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4	B4galt4	0.00042491
10573342	microRNA 23a	Mir23a	0.00349054
10388728	predicted gene 11190	Gm11190	0.00790087
10605943	PDZ domain containing 11	Pdzd11	0.0105884
10598287	LOC100270707	LOC100270707	0.03367059
10346780	eukaryotic translation initiation factor 4A1	Eif4a1	0.0314693
10519266	microRNA 200a	Mir200a	0.00038099
10533474	RIKEN cDNA 1500011H22 gene	1500011H22Rik	0.0000612
10567380	uromodulin	Umod	0.00025501
10399038	zinc finger protein 386 (Kruppel-like)	Zfp386	0.00048782
10378082	gamma-glutamyltransferase 6	Ggt6	0.00069264
10505888	interferon alpha 5	Ifna5	0.0006976
10594053	promyelocytic leukemia	Pml	0.00078797
10347417	BCS1-like (yeast)	Bcs1l	0.00187542
10427035	nuclear receptor subfamily 4, group A, member 1	Nr4a1	0.00281126
10463448	progressive external ophthalmoplegia 1 (human)	Peo1	0.00109516
10488185	beaded filament structural protein 1, in lens-CP94	Bfsp1	0.00162073
10500218	threonyl-tRNA synthetase 2, mitochondrial (putative)	Tars2	0.00172527
10450296	superkiller viralicidic activity 2-like (S. cerevisiae)	Skiv2l	0.00205481
10369264	oncoprotein induced transcript 3	Oit3	0.00220297
10497968	predicted gene 16508	Gm16508	0.00232913
10458183	glial cell line derived neurotrophic factor family receptor alpha 3	Gfra3	0.00476846
10495781	breast cancer anti-estrogen resistance 3	Bcar3	0.00872323
10557862	integrin alpha M	Itgam	0.00894082
10570119	carbohydrate kinase domain containing	Carkd	0.00958363
10546944	transmembrane protein 111	Tmem111	0.01069165
10347254	SWI/SNF related matrix associated, actin dependent	Smarcal1	0.01150968

	regulator of chromatin, subfamily a-like 1		
10440131	G protein-coupled receptor 15	Gpr15	0.01696801
10593903	COMM domain containing 4	Commd4	0.01861085
10566564	mitochondrial ribosomal protein L17	Mrpl17	0.02876081
10569308	keratin associated protein 5-1	Krtap5-1	0.03114893
10561748	cation channel, sperm-associated, gamma 2	Catsperg2	0.00734316
10545337	peptidylprolyl isomerase A	Ppia	0.04245722
10364984	zinc finger and BTB domain containing 7a	Zbtb7a	0.00051312
10445855	kinesin family member 6	Kif6	0.00119466
10414751	T cell receptor alpha variable 13N-1	Trav13n-1	0.00156947
10351515	U1b1 small nuclear RNA	Rnu1b1	0.00161936
10514323	LOC100037413	LOC100037413	0.00212262
10494413	U1b1 small nuclear RNA	Rnu1b1	0.00230563
10574100	NLR family, CARD domain containing 5	Nlrc5	0.00306202
10476590	MACRO domain containing 2	Macrod2	0.00317966
10405001	bicaudal D homolog 2 (Drosophila)	Bicd2	0.00363827
10520010	peptidase (mitochondrial processing) beta	Pmpcb	0.0036612
10396231	translocase of outer mitochondrial membrane 20 homolog (yeast)-like	Tomm20l	0.00381057
10373642	olfactory receptor 810	Olfr810	0.00393616
10534343	elastin	Eln	0.00426135
10571302	transmembrane protein 66	Tmem66	0.00438588
10570587	defensin beta 34	Defb34	0.00446013
10538466	growth hormone releasing hormone receptor	Ghrhr	0.00462289
10569100	microRNA 210	Mir210	0.00567214
10604761	microRNA 504	Mir504	0.00642339
10441610	radial spoke 3A homolog (Chlamydomonas)	Rsph3a	0.00750799
10414775	predicted gene 17006	Gm17006	0.00782767
10387516	sex hormone binding globulin	Shbg	0.00796872
10490982	cDNA sequence BC002189	BC002189	0.00866363
10452639	myosin, light chain 12B, regulatory	Myl12b	0.00866468
10411508	pentatricopeptide repeat domain 2	Ptcd2	0.00870187
10390907	keratin associated protein 1-3	Krtap1-3	0.00873647
10604473	immunoglobulin superfamily, member 1	Igsf1	0.00891861
10447619	radial spoke 3A homolog (Chlamydomonas)	Rsph3a	0.00906843
10597748	golgi reassembly stacking protein 1	Gorasp1	0.00925192
10485198	tetraspanin 18	Tspan18	0.01092499
10414906	predicted gene 13978	Gm13978	0.01142163
10427063	1700011A15Rik	1700011A15Rik	0.01240035
10398332	microRNA 673	Mir673	0.01328989
10368893	lactation elevated 1	Lace1	0.01346342
10431210	wingless-related MMTV integration site 7B	Wnt7b	0.01472102
10591177	olfactory receptor 24	Olfr24	0.01955383

10389865	non-metastatic cells 1, protein (NM23A) expressed in	Nme1	0.02072888
10388033	Der1-like domain family, member 2	Derl2	0.02117396
10570178	A230072I06Rik	A230072I06Rik	0.02175148
10578262	microRNA 383	Mir383	0.02215785
10591735	elongation factor 1 homolog (ELF1, S. cerevisiae)	Elof1	0.02233775
10596575	mesencephalic astrocyte-derived neurotrophic factor	Manf	0.02253612
10505436	microRNA 455	Mir455	0.02311658
10436372	discoidin, CUB and LCCL domain containing 2	Dcbld2	0.02476946
10569288	keratin associated protein 5-4	Krtap5-4	0.02555693
10366407	predicted gene 10752	Gm10752	0.02728883
10582006	predicted gene 10620	Gm10620	0.02924252
10390352	karyopherin (importin) beta 1	Kpnb1	0.02955975
10465411	solute carrier family 22 (organic anion/cation transporter), member 12	Slc22a12	0.03021274
10440717	keratin associated protein 16-8	Krtap16-8	0.03215136
10449061	rhomboid, veinlet-like 1 (Drosophila)	Rhbdl1	0.03396643
10390961	keratin associated protein 17-1	Krtap17-1	0.03611369
10468213	2310034G01Rik	2310034G01Rik	0.03858789
10576940	family with sequence similarity 155, member A	Fam155a	0.03906579
10434930	1700025H01Rik	1700025H01Rik	0.04189886
10606941	ripply1 homolog (zebrafish)	Ripply1	0.04819616
10570671	defensin, alpha, 22	Defa22	0.00083945
10499348	transmembrane protein 79	Tmem79	0.00229643
10532767	slingshot homolog 1 (Drosophila)	Ssh1	0.00822885
10577948	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like	Brf2	0.0094499
10581523	telomeric repeat binding factor 2	Terf2	0.00572701
10388308	olfactory receptor 411	Olfr411	0.00112187
10437698	thioredoxin domain containing 11	Txndc11	0.00189391
10565735	A630091E08Rik	A630091E08Rik	0.00369184
10549889	aurora kinase C	Aurkc	0.01059349
10574159	NLR family, CARD domain containing 5	Nlrc5	0.01137177
10376685	alkB, alkylation repair homolog 5 (E. coli)	Alkbh5	0.01716566
10397083	RNA binding motif protein 25	Rbm25	0.02287385
10572497	interleukin 12 receptor, beta 1	Il12rb1	0.02350707
10542395	activating transcription factor 7 interacting protein	Atf7ip	0.0254685
10447395	mutS homolog 2 (E. coli)	Msh2	0.0265917
10458782	protein geranylgeranyltransferase type I, beta subunit	Pggt1b	0.0309373
10486710	leucine carboxyl methyltransferase 2	Lcmt2	0.03242389
10480379	mitochondrial ribosomal protein S5	Mrps5	0.03519811
10588482	POC1 centriolar protein homolog A (Chlamydomonas)	Poc1a	0.01234184
10409629	kelch-like 3 (Drosophila)	Klhl3	0.00044438
10486898	spastic paraplegia 11	Spg11	0.00075123

10597288	mutL homolog 1 (E. coli)	Mlh1	0.0008876
10462276	predicted gene 815	Gm815	0.00132461
10490690	uridine-cytidine kinase 1-like 1	Uckl1	0.00245731
10575291	zinc finger protein 821	Zfp821	0.00253378
10380591	SNF8, ESCRT-II complex subunit, homolog (S. cerevisiae)	Snf8	0.00274997
10556244	small nucleolar RNA, H/ACA box 23	Snora23	0.00275466
10486154	A430105I19Rik	A430105I19Rik	0.00278218
10425000	leucine rich repeat containing 14	Lrrc14	0.00278311
10464153	NHL repeat containing 2	Nhlrc2	0.00347658
10550978	pleckstrin homology-like domain, family B, member 3	Phldb3	0.00535724
10386636	ubiquitin specific peptidase 22	Usp22	0.00631343
10385022	EF-hand calcium binding domain 9	Efcab9	0.01263034
10485282	alkB, alkylation repair homolog 3 (E. coli)	Alkbh3	0.01266648
10404815	sirtuin 5 (silent mating type information regulation 2 homolog) 5 (S. cerevisiae)	Sirt5	0.01312765
10486403	phospholipase A2, group IVE	Pla2g4e	0.0197829
10437399	coronin 7	Coro7	0.02650838
10405125	F-box and WD-40 domain protein 17	Fbxw17	0.02729384
10561561	ryanodine receptor 1, skeletal muscle	Ryr1	0.02965675
10590563	leucyl-tRNA synthetase, mitochondrial	Lars2	0.03945992
10426782	amiloride-sensitive cation channel 2, neuronal	Accn2	0.04727799
10532784	SV2 related protein	Svop	0.04780432
10486754	diphosphoinositol pentakisphosphate kinase 1	Ppip5k1	0.04931709
10394245	DnaJ (Hsp40) homolog, subfamily C, member 27	Dnajc27	0.03392871

Supplementary Table 2.6. Body weight (g) and plasma E2 (pg/ml) measures on day 12 (mean $\pm$ SEM).

Measure	Con	E2	Gly	E2+Gly
body wt (g)	26.3 $\pm$ 1.1	27.8 $\pm$ 1.1	26.1 $\pm$ 0.8	28.1 $\pm$ 0.8
plasma E2 (pg/ml)	4.13 $\pm$ 1.13[a]	265.65 $\pm$ 55.65 [b]	3.17 $\pm$ 0.03 [a]	76.05 $\pm$ 21.15 [b]

[a,b] Different superscripts, $p < 0.001$; same superscripts, $p > 0.05$

Supplementary Table 2.7. RT-qPCR statistical outcomes

GOI	Gene name	Overall F (df)	Overall P	R^2	E2 F (df)	E2 P	Gly F (df)	Gly P	Int F (df)	Int P
Cdh12	Cadherin 12	8.114 (3, 6)	0.016	0.802	22.526 (1, 6)	0.003	1.057 (1, 6)	0.344	1.122 (1, 6)	0.330
Fcrls	Fc receptor-like S	1.823 (3, 6)	0.243	0.477	5.166 (1,6)	0.063	0.265 (1, 6)	0.625	0.087 (1, 6)	0.778
Gh	Growth hormone	1.684 (3, 6)	0.269	0.457	3.730 (1,6)	0.102	1.168 (1, 6)	0.321	0.361 (1, 6)	0.570
Ngp	Neutrophilic granule protein	7.425 (3, 6)	0.019	0.788	22.2196 (1, 6)	0.003	0.025 (1, 6)	0.880	0.019 (1, 6)	0.894
Nr4a1	Nuclear receptor subfamily 4	10.64 (3,6)	0.008	0.842	9.088 (1, 6)	0.024	13.566 (1, 6)	0.010	5.047 (1, 6)	0.066
Prl	Prolactin	8.339 (3, 6)	0.015	0.807	22.763 (1, 6)	0.003	1.751 (1, 6)	0.234	0.919 (1, 6)	0.375
Slc6a4	Solute carrier family 6	1.285 (3, 6)	0.362	0.391	2.976 (1, 6)	0.135	0.273 (1, 6)	0.620	0.434 (1, 6)	0.534
Tph2	Tryptophan hydroxylase 2	0.684 (3, 6)	0.594	0.255	1.895 (1, 6)	0.218	0.0008 (1, 6)	0.978	0.145 (1, 6)	0.716

CHAPTER 3

NEXT GENERATION SEQUENCING ANALYSIS OF SOY GLYCEOLLINS AND 17-β ESTRADIOL: EFFECTS ON TRANSCRIPT ABUNDANCE IN THE FEMALE MOUSE BRAIN

Introduction

Glyceollins (Glys) are produced by soy plants in response to stressful conditions including fungal infections, UV exposure or changes in temperature. Research on Glys was initially focused on its anti-estrogenic actions through estrogen receptors (ERs), mainly ERα, suppressing estrogen-responsive breast and ovarian tumors both *in vitro* in cancer cell lines (Burow et al. 2001) and *in vivo* in mice (Salvo et al. 2006). Although some studies have shown that Glys can exhibit weak estrogenic activity at ERβ, thus promoting breast tumor proliferation (Kim et al. 2010a), Glys also exhibit non ER-mediated effects, suppressing triple-negative tumors that lack ERs (Rhodes et al. 2012), increasing insulin-stimulated glucose uptake in adipocytes (Boue et al. 2012; Park et al. 2010) and upregulating lipid metabolism genes in mammary tissue (Wood et al. 2012). A recent study showed that different isomers of Glys (*e.g.*, Gly I, II and III) can possess antagonistic/agonistic actions at different human ERs, depending on the prenylation pattern of the isomers, the structure of the backbone and the length of the compound (van de Schans et al. 2016). For example, Gly I, II and III exhibit agonistic activity at ERβ, Gly II and III exhibit antagonistic activity at ERα, while Gly I exhibits selective estrogen-

receptor (SERM)-like activity at ERα (van de Schans et al. 2016). Thus, Glys may not be purely anti-estrogenic but may possess SERM-like activity in a tissue- and/or condition-dependent manner.

Prior research did not focus on the effects of Glys in the brain, the mechanism of which is still unclear. We recently published data from a microarray experiment describing the effects of Glys on gene expression in the female mouse brain (Bamji et al. 2015). Ovariectomized (ovx) mice were treated with 17-β estradiol (E2), Gly, both E2 and Gly (E2+Gly), or negative control (Con). Differential gene expression analysis among treatment groups revealed that the gene expression patterns of the two E2 treatments were the most similar, with Gly exhibiting a gene expression profile distinct from both E2 groups (Bamji et al. 2015). However, we did not observe statistically significant differences in expression among treatment groups for some of the genes of interest such as estrogen receptors (ERs), growth hormone (*Gh*) and prolactin (*Prl*) that are commonly affected by E2 treatment in the brain.

Although microarrays have been powerful tools to study differential gene expression, there are limitations to the technique. The requirement of transcript-specific probes in microarrays inhibits the detection of novel gene transcripts, with the method relying heavily on prior knowledge of the genome sequence (Wang et al. 2009). Another issue is the differences in hybridization events or cross-hybridization among array probes, leading to high background signal for low expression transcripts and signal saturation for highly expressed transcripts (Fu et al. 2009; Mortazavi et al. 2008). Due to these limitations, RNA-Seq technology has gained momentum, offering a more robust comparison of the transcriptome, with low background signal and high sensitivity and

efficiency of detecting differential gene expression, especially for transcripts that are either very high or very low in expression within cells. Moreover, RNA-Seq technology has been used to quantify transcript expression in the adult mouse brain, to evaluate parental biases in the expression of imprinted genes (Gregg et al. 2010), to detect differential gene expression in the mouse striatum (Bottomly et al. 2011) or to classify specific cell types in the mouse cortex and hippocampus (Zeisel et al. 2015). Given the clear benefits of using RNA-Seq technology over microarrays, we evaluated the usefulness of this technique to identify differential gene expression between treatments and hypothesized that Gly would oppose the effect of E2 on gene expression in the female mouse brain. Toward this end, we re-examined the mouse brain transcriptome using paired-end RNA sequencing performed on the Illumina GAIIx platform after treatment of ovx mice with E2, Gly, both E2 and Gly (E2+Gly), or negative control (Con), the same samples used in the prior microarray study. Overall, the genes regulated by E2 and Gly, independently and in combination, were involved in similar pathway maps and networks in both RNA-Seq and microarray experiments. Gly in some cases may oppose the effect of E2 on gene expression and vice versa, but Gly can also regulate genes similarly to E2 (*e.g.*, if E2 upregulates the expression of a gene, Gly also upregulates the gene, although the magnitude of the effect may differ). Thus, Gly may not be a pure anti-estrogen, but may act as a selective estrogen receptor modulator (SERM) in the brain.

Materials and Methods

Direct link to deposited data

The raw reads and gene expression data have been deposited in the National Center for Biotechnology Information (accession no. GSE81336). (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mtkpkkusrxmzfob&acc=GSE81336)

Study animals and experimental treatments

Ovariectomized adult female Swiss Webster (CFW) mice were purchased from Charles River Laboratories, maintained on a 12L:12D photoperiod (lights on at 6:00 AM), and given *ad libitum* access to phytoestrogen-reduced rodent chow (Teklad 2016, phytoestrogen levels range from less than detectable to 20ppm) and tap water. The animals were allowed to acclimate for one week before a 21-day slow-release pellet of 0.1mg E2 or placebo (Innovative Research of America, Sarasota, FL) was implanted s.c. at the midline between neck and shoulders under isoflurane anesthesia followed by s.c. ketoprofen analgesia (day 1). Daily 50µl i.p. injections of 20mg/kg Gly or 1:1 DMSO/PBS vehicle began on day 1 and continued every afternoon for 11 days. The Gly mixture (Gly I, II, and III) was isolated using a procedure described previously (Salvo et al. 2006). Three mice were in each of four treatment groups, which were as follows: placebo pellet + vehicle control (Con), placebo pellet + Gly (Gly), E2 pellet + vehicle (E2), and E2 pellet + Gly (E2+Gly).

On the morning of day 12, mice were weighed and then euthanized by decapitation under complete isoflurane anesthesia. Brain hemispheres were stored in RNA*later* (Ambion) overnight at 4°C and subsequently stored at -20°C until further processing.

Ethics Statement

All protocols used for housing, handling, and euthanasia followed guidelines approved by the *Guide for the Care and Use of Laboratory Animals: Eighth Edition* (2011) (Council 2011) and were approved by the University of Louisville Institutional Animal Care and Use Committee (protocol number 11099).

RNA isolation and microarray platform

Total RNA was isolated from brain hemispheres of 3 mice per group using Trizol/chloroform extraction, followed by spin column purifications (Qiagen RNeasy midi kit) that included DNase (Qiagen) digestions. A Nanodrop 2000 spectrophotometer was used to assess sample purity and RNA concentration, and an Agilent Bioanalyzer was used to verify sample integrity (RIN 7.9 - 9). RNA from mice in each treatment group was pooled into one sample each per treatment. The outliers detected in the microarray experiment through quality control analyses (Bamji et al. 2015) were not included in the pooled RNA samples, so RNA from only two biological replicates were pooled for the E2 and E2+Gly treatments. RNA samples were sent to Cofactor Genomics (St. Louis, MO) for processing and sequencing. Paired-end RNA sequencing was performed using the Illumina GAIIx platform (sequencer ID @HWI-ST522), generating 50-bp raw sequence data.

RNA-Seq Analysis

Aligned RNA-Seq reads were assembled according to the mm10.gtf annotation file (downloaded from ENSEMBL using cufflinks, version 2.2.1). For the pairwise comparisons of all treatment groups (E2 vs Con, Gly vs Con, E2+Gly vs Con, E2+Gly vs

E2, E2+Gly vs Gly, Gly vs E2), both cufflinks assemblies were merged, and the resulting merged gtf file served as the transcript input for use in cufflinks and cuffdiff. For the comparisons of both E2 groups vs. non-E2 groups (E2 and E2+Gly vs Con and Gly) and both Gly groups vs. non-Gly groups (E2+Gly and Gly vs E2 and Con), both cufflinks assemblies were merged, and the resulting merged gtf file served as the transcript input for use in cufflinks and cuffdiff.

Differentially expressed genes (DEGs) were then identified using a cut off $\log_2 FC \geq 4$ by comparing the treated condition with the control using the tuxedo suite of programs included in cufflinks and cuffdiff (version 2.2.1). The DEGs in each treatment that had an FPKM value 0 in one treatment and an FPKM value < 1 in the other treatment, were eliminated from any further analysis. After the sorting, all 0 values in any treatment were replaced by the minimum FPKM value observed across all genes for that treatment (Con – 1.01×10^{-6}, E2 – 2.2×10^{-6}, Gly – 2.6×10^{-7}, E2+Gly – 4.72×10^{-7}) in order to avoid a zero in the denominator when calculating FC.

Enrichment analyses

DEGs in the 8 different treatment comparisons were uploaded as separate files and analyzed using MetaCore version 6.18 software (www.portal.genego.com). The 'Enrichment Analysis' function was then applied to each DEG list to identify genes that potentially affect biological pathways, maps or gene ontology (GO) terms. As we only used gene lists that were already deemed differentially expressed between treatment groups, we did not use any *P*-value or fold change threshold options in MetaCore.

Results

Overview of the RNA-Seq data

We identified a total of 58,059 DEGs in 8 different treatment comparisons (E2 vs Con; Gly vs Con; E2+Gly vs Con; E2+Gly vs E2; E2+Gly vs Gly; E2 vs Gly; E2 and E2+Gly vs Con and Gly; Gly and E2+Gly vs E2 and Con) (Table 3.1). A combined view of the different treatments revealed that not only were the two E2 treatments (E2 and E2+Gly) most similar to each other in terms of gene expression, but also that Gly was similar to E2 in several cases (Fig.3.1). Some of the interesting patterns include: (1) Gly exhibiting a gene expression pattern similar to control, but opposing the E2 effect on gene expression in the E2+Gly group (*i.e.*, a 'classic' anti-estrogenic response); (2) E2 affecting gene expression similarly to control, but opposing the Gly effect on gene expression in the E2+Gly treatment, (3) Gly, E2 and E2+Gly showing similar gene expression patterns that are all different from control and (4) Gly and E2 patterns of gene expression being similar to each other and differing from control, but in combination (E2+Gly) being similar to control.

Effect of E2 on gene expression

To evaluate the overall effect of E2 on gene expression, we observed patterns in three different comparisons: (i) E2 vs Con (*i.e.*, effect of E2 alone); (ii) E2+Gly vs Gly (*i.e.*, effect of E2 on Gly); and (iii) Both E2 groups (E2 and E2+Gly) vs non-E2 groups (Gly and Con).

Of the 8,223 DEGs that were identified in the E2 vs Con comparison, 5,505 genes were upregulated by E2 (Table 3.1). Enrichment analysis using MetaCore revealed that the genes upregulated by E2 compared to control were involved in signaling pathways including nociceptin receptor, angiotensin and neurohormone signaling. E2 also upregulated genes that play a role in general cystoskeletal remodeling, epigenetic regulation of gene expression, protein folding, cell cycle regulation and inflammation (Supplementary Table 3.1a). E2 downregulated 2,718 genes compared to control (Table 3.1) which are involved in signaling pathways including K-Ras, ERK1/2, STK3/4 and YAP/TAZ, PTMs in BAFF-induced signaling and cholecystokinin signaling (Supplementary Table 3.1a). E2 also downregulated genes that regulate cell adhesion, spermatogenesis, sperm motility and copulation (Supplementary Table 3.1a).

Further, 8,705 genes were identified in the E2+Gly vs Gly comparison (Table 3.1). E2+Gly upregulated 4,647 DEGs compared to Gly, which are involved in pathways such as cytoskeletal remodeling, LRRK2 signaling, ESR1 signaling and neurogenesis (Supplementary Table 3.1b). E2+Gly downregulated 4,058 DEGs compared to Gly (Table 3.1), which are involved in immune responses, signal transduction pathways, cytoskeletal functions, protein folding and cell adhesion (Supplementary Table 3.1b).

In addition, 4,514 DEGs were identified in a comparison of both the E2 groups (E2 and E2+Gly) vs. the non-E2 groups (Gly and Con) (Table 3.1). Of these, 2,703 were upregulated in the E2 groups, some of which were enriched for pathways such as nociception receptor signaling and various neurophysiological processes (Supplementary Table 3.1c). On the other hand, both E2 groups downregulated the expression of 1,811

DEGs, involved in signal transduction, immune response and neurophysiological pathways (Supplementary Table 3.1c).

Overall, E2 alone or in combination with Gly upregulated genes involved in nociception receptor signaling and several neurophysiological processes like regulation of nerve impulse, hormone signaling, cytoskeletal remodeling, inflammation and cell cycle regulation. In addition, E2 downregulated genes involved in the LRRK2, Hippo and ERK1/2 pathways as well as genes involved in immune response and inflammatory pathways.

Effects of Gly on gene expression

To evaluate the overall effect of Gly on gene expression, we observed patterns in three different comparisons: (i) Gly vs Con (*i.e.*, effect of Gly alone); (ii) E2+Gly vs E2 (*i.e.*, effect of Gly on E2); and (iii) Both Gly groups (Gly and E2+Gly) vs non-Gly groups (E2 and Con).

In the Gly vs. Con comparison, we identified a total of 9,931 DEGs, of which 5,707 were upregulated by Gly (Table 3.1). Enrichment analyses using MetaCore revealed that the genes upregulated by Gly vs. Con were involved in PKA signaling, renal water reabsorption, protein folding, cytoskeleton remodeling (PKA), immune responses and regulation of lipid metabolism (Supplementary Table 3.2a). Gly downregulated 4,224 DEGs compared to Con, which are involved in pathways such as HBV signaling, cytoskeleton remodeling (TGF/WNT), Endothelin1/EDNRA signaling, PTEN signal transduction and neurogenesis (Supplementary Table 3.2a).

Additionally, 6,079 DEGs were identified in the E2+Gly vs. E2 comparison, *i.e.*, the effect of Gly on E2 signaling (Table 3.1). Of these, 2,520 DEGs were upregulated in the E2+Gly treatment, which were enriched for pathways such as ERK1/2, adenosine A2A receptor and G-protein (RAS family), FSH beta and amphoterin signaling (Supplementary Table 3.2a). Further, E2+Gly downregulated the expression of 3,559 DEGs compared to E2. These downregulated DEGs were involved in pathways including immune responses, WNT signaling, neurogenesis and neurophysiological processes and cell adhesion (Supplementary Table 3.2b).

Lastly, 3,857 DEGs were identified when the two Gly groups (Gly and E2+Gly) were compared to the non Gly groups (E2 and Con) (Table 3.1). There were 1,782 DEGs upregulated in the two Gly groups, which enriched for pathways including cytoskeleton remodeling, immune response, ESR1 signaling and neurophysiological processes (Supplementary Table 3.2c). Both Gly groups downregulated the expression of 2,075 genes, some of which play a role in pathways such as Flt3 signaling, cytoskeleton remodeling, JNK signaling, cell adhesion and transcriptional regulation (Supplementary Table 3.2c).

In summary, Gly upregulated genes belonging to signal transduction pathways (PKA, ESR1), regulation of nerve impulse, cytoskeletal remodeling, hormone signaling as well as genes involved in immune responses and lipid metabolism. In addition, Gly downregulated genes involved in developmental neurogenesis, synaptogenesis, cell adhesion (synaptic contact) as well as genes involved in the PTEN signaling pathway.

Discussion

The objective of this study was to determine the potential mechanisms of action of Gly alone and in combination with E2 by examining the mRNA transcriptome of female mice in the whole brain, using RNA-Seq technology and comparing results with those obtained from our whole brain microarray analysis. Even though our whole brain microarray study indicated that Gly might affect gene expression through both ER-mediated and non ER-mediated mechanisms, we did not observe any statistically significant differential expression for some of our low expression genes of interest (*e.g.*, ERs, *Prl* and *Gh*). ERs (*Esr1*, *Esr2* and *Gper1*) are expressed throughout the brain and are all generally downregulated by E2. *Gh* and *Prl* are also widely distributed in the brain and are usually upregulated after E2 treatment. The lack of differential expression among treatments for ERs, *Gh* and *Prl* in the microarray analysis could possibly be due to the limitations of the technique in detection of low expression genes. We anticipated that with higher specificity and sensitivity in detection of differential expression, the RNA-Seq technique would help us detect differences in expression of ERs, *Gh* and *Prl*, if any, among treatments. Additionally, we expected that RNA-Seq would give us a broader, more detailed perspective on the whole transcriptome effects of Gly on gene expression in the female mouse brain.

Given the known anti-estrogenic effects of Gly in peripheral tissues, the initial hypothesis was that Gly by itself would have no effect compared to control, but would oppose the E2 effect on gene expression in the brain (*i.e.*, significant differences in E2 vs Con and E2+Gly vs E2 comparison, but not the Gly vs Con comparison). Results from the microarray data revealed that there was Gly opposition of E2 effects on expression of

a few genes, as expected, but with an unexpected E2 opposition of the independent effects of Gly on gene expression as well (Bamji et al. 2015).

The microarray analysis also indicated that Gly had the most distinct gene expression pattern, with both E2 and E2+Gly effects on gene expression being the most similar. The RNA-Seq analysis also indicated that both E2 groups had the most similar gene expression patterns. However, the RNA-Seq experiment additionally showed that for several genes, Gly affects gene expression similarly to E2 (in most cases both E2 and Gly upregulate the expression of genes) when compared to control, although the magnitude of the effect may differ. This difference in overall expression patterns in the microarray vs. the RNA-Seq results can be attributed to a difference in analytical methods, the presence/absence of biological replicates and the differences in gene lists between the two experiments. Thus, it is important to note that the microarray analysis was performed using a two-factor ANOVA with biological replicates (N=2-3 per treatment) whereas the RNA-Seq analysis was performed using a one-factor ANOVA design with pooled RNA from biological replicates (N=1 per treatment). Further, we are comparing a list of 279 DEGs identified as significant in the microarray experiment with a list of over 58,000 DEGs identified in the RNA-Seq experiment. Nevertheless, we observed some similarities between the RNA-Seq and microarray experiments. First, both methods identified Gly's opposition of the E2 effect on gene expression and E2 opposing the Gly effect on gene expression. Second, a comparison of the E2-regulated and Gly-regulated genes from the microarray and RNA-Seq analyses revealed common DEGs that were identified in both experiments. Sixty-four DEGs with significant E2 effects and 50 DEGs with significant Gly effects were common to both RNA-Seq and microarray experiments.

Finally, the most highly upregulated gene by Gly in our microarray data, *Nr4a1* (FC = 1.89), was similarly upregulated by Gly compared to Con in the RNA-Seq data (FC = 2.32), with E2 opposition of the Gly upregulation, but this effect was not found to be statistically significant. Thus, while there was some agreement of the RNA-Seq results with those of the microarray, the RNA-Seq experiment revealed Gly regulation of gene expression that was more similar to E2 than the microarray results suggested.

Estrogens regulate numerous functions in the brain including reproduction (Wintermantel et al. 2006), food intake (Brown and Clegg 2010), cognition (Daniel 2013) neuronal synaptic plasticity (Li et al. 2004) and pain perception (nociception) (Craft et al. 2004) through ERs distributed throughout the brain (Mitra et al. 2003). Hence, unsurprisingly, E2 upregulated the expression of genes involved in hormone signaling, nociception receptor signaling, cytoskeletal remodeling, regulation of nerve impulse, inflammation and cell cycle regulation (Supplementary Table 3.1a-c). Interestingly, E2 downregulated genes involved in the Hippo (YAP/TAZ) pathway that regulates organ growth, cell function and regeneration as well as tumor suppression (Johnson and Halder 2014), which is unsurprising given the ability of E2 to promote tumor proliferation. Previous work has shown that E2 often downregulates the expression of its classical receptors, *Esr1* and *Esr2*, but the effects may differ by brain region, timing, sex and species (Lauber et al. 1991; Nomura et al. 2003; Patisaul et al. 1999; Simerly and Young 1991). Neither of the classical ERs were found to be differentially expressed in the RNA-Seq experiment, which is similar to our microarray results (Bamji et al. 2015). E2 also exerts rapid effects such as kinase activation, calcium mobilization and rapid transcriptional activation of genes through action at membrane-associated ERs like *Gper1*

(Prossnitz and Maggiolini 2009). Like the classical ERs, *Gper1* was also not differentially expressed in any of the treatment comparisons in the RNA-Seq experiment. Considering that the expression of the different ERs is highly brain-region specific and that we are measuring gene expression across the whole brain, this result is perhaps to be expected. Future studies focused on brain-region specific effects on gene expression may reveal a clearer picture of the effects of Gly alone and in combination with E2 on expression of ERs in the mouse brain.

In addition to ERs, other E2-responsive genes (*Gh*, *Prl*) were detected as differentially expressed in RNA-Seq, unlike in the microarray study. E2 is known to upregulate *Gh* and *Prl* expression in the rodent brain (Addison and Rissman 2012; Torner et al. 1999), and we expected to see Gly opposition of this E2 upregulation. Unsurprisingly, E2 upregulated both *Gh* and *Prl* compared to Con (Table 3.2). It should be noted here that the high FC values observed for both *Gh* and *Prl* are due to the fact that there was little to no expression (FPKM values < 0.0018) of either of these genes in the Con treatment. Thus, the small increases in FPKM values in the treated (E2) condition (*e.g.*, *Prl* FPKM = 1.15 and *Gh* FPKM = 7.77 in the E2 treatment) led to high FC values that are misleading regarding absolute expression. Surprisingly, Gly alone slightly upregulated *Gh* compared to Con, but when combined with E2 (E2+Gly), *Gh* expression did not reach that stimulated by E2 alone compared to Con (Table 3.2). On the other hand, Gly downregulated *Prl* compared to Con, and in combination with E2, completely opposed the E2 upregulation of *Prl* in the E2+Gly treatment (Table 3.2). Neither *Gh* nor *Prl* were differentially expressed in the Gly vs. Con comparison, and the FC values in the E2+Gly vs. Con comparison were reduced to 0.8% of E2 vs Con for *Gh*

and 0.15% of E2 vs Con for *Prl* expression. Therefore, Gly alone had no pronounced effect on *Gh* or *Prl* gene expression compared to Con, but in combination with E2, Gly exhibited an anti-estrogenic effect. This result is in agreement with Gly's anti-estrogenic activity in other cell types, *e.g.* MCF-7 breast cancer cells (Burow et al. 2001; Salvo et al. 2006).

The RNA-Seq experiment revealed a pattern of Gly regulation of genes in the female mouse brain that was not observed in the microarray study. Gly upregulated genes that are involved in hormone signaling, cytoskeletal remodeling, regulation of nerve impulse and immune responses (Supplementary Table 3.2a-c), which was similar to the pathway maps/networks regulated by E2 in the brain. For example, the brain-specific insulin gene *Ins2* was upregulated by both E2 (FC = 1,227,422) and Gly (1,600,791) compared to Con and is potentially involved in learning and memory as well as food intake (Mehran et al. 2012). As mentioned earlier for *Gh* and *Prl*, the high FC values observed for *Ins2* are also due to little to no expression of this gene in the Con treatment. Gly also upregulated genes involved in lipid metabolism, specifically insulin's regulation of glycogen metabolism, which is not true of E2 treatment. This is interesting as neuronal glycogen accumulation is associated with neurodegeneration and glycogen metabolism is important, especially under stressful conditions in the mouse brain (Duran and Guinovart 2015). Moreover, this finding is in agreement with a prior study that showed upregulation of lipid metabolism genes in mammary gland tissues of postmenopausal female cynomolgus monkeys that were fed a Gly-rich soy diet (Wood et al. 2012). Interestingly, Gly downregulated some pro-apoptotic genes in the PTEN-signaling pathway (*e.g.*, *Bad* and *Foxo3*), suggesting a potential neuroprotective role of Gly in the brain. A prior study

showed that neuronal apoptosis in the developing rat brain after hypoxia-ischemia (HI) involves the PTEN-Akt-FOXO3 signaling pathway (Li et al. 2009). The study suggested that PTEN inhibition followed by downregulation of bcl2-interacting-mediator of cell death (Bim) attenuated apoptosis in rat neurons and may play a neuroprotective role in the rat brain after HI (Li et al. 2009). Gly also downregulated genes involved in synaptogenesis and neurogenesis. One example of a Gly-downregulated gene is synaptophysin (*Syp*), which is involved in formation, exocytosis and endocytosis of synaptic vesicles (Kwon and Chapman 2011) and is usually upregulated by E2 (Frick et al. 2002). Of importance is the fact that, like the microarray data, the RNA-Seq data also revealed that Gly by itself, compared to Con, regulates the expression of many more genes than when the two Gly groups (Gly and E2+Gly) are compared to the non-Gly groups (E2 and Con); the difference in these two sets of comparisons is the addition of E2 treatment in the latter (Table 3.1). Thus, in some cases, Gly may act similarly to E2, while in other cases, Gly exhibits either anti-estrogenic or non E2-like effects on gene expression in the brain.

Although 3 biological replicates of whole brain homogenates were pooled in the RNA-Seq experiment and thus a direct comparison of the RNA-Seq and microarray results cannot be made, the genes regulated by E2 and Gly independently and in combination were enriched for similar pathway maps and networks in both studies. Moreover, unlike the microarray study, RNA-Seq enabled the detection of differential expression of low expression genes like *Prl* and *Gh*, both of which are expressed in the brain (Aramburo et al. 2014; Marano and Ben-Jonathan 2014), upregulated by E2

(Addison and Rissman 2012; Torner et al. 1999) and in the current study serve as examples of Gly's anti-estrogenic effects in the brain.

The mechanism by which Gly affects gene expression in the brain is still unclear. To gain a deeper understanding of Gly's mechanisms of action in the female mouse brain, future studies should focus on 1) evaluating the effects of Gly on gene expression in specific brain regions, 2) using brain-specific ER-knockout mice to identify which of Gly's effects are ER-mediated vs. non ER-mediated and 3) using a neuronal cell culture system to test if Gly affects gene expression directly at the neuron or through an indirect peripheral mechanism of action.

Figure Caption

Fig. 3.1: Heat map of 15,000 DEGs (rows) with $\log_2FC \geq 4$ for at least one comparison and FPKM ≥ 1. Columns correspond to pooled treatment groups (L-R): Control, Gly, E2 and E2+Gly. Black to red = downregulation of a given gene (row); orange = average expression of a gene; yellow to white = upregulation relative to the average expression for the gene.

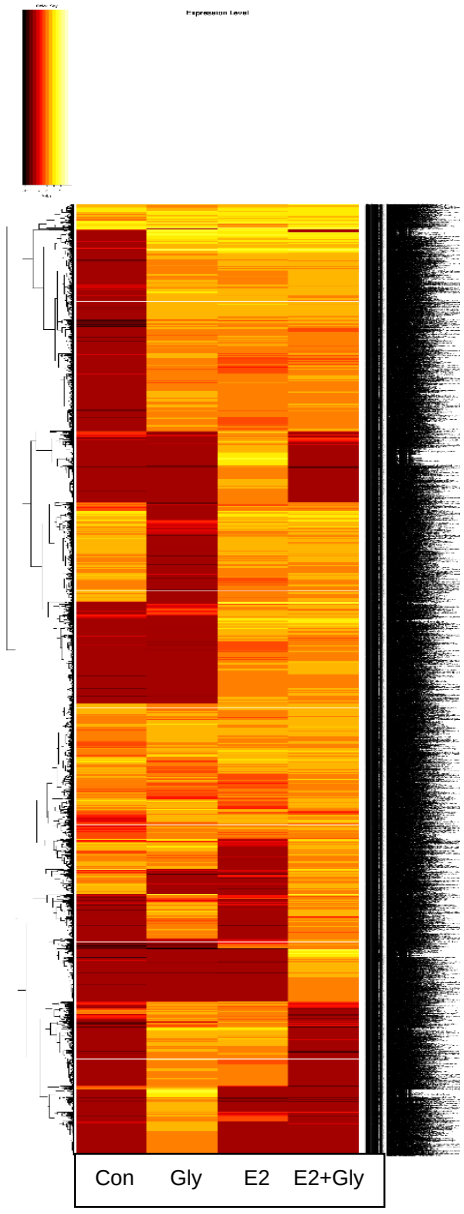


Table 3.1. Number of DEGs identified in 8 different treatment comparisons using a $\log_2FC \geq 4$ cut off in the RNA-Seq experiment.

Comparisons	Total	Upregulated	Downregulated
E2 vs Control	8223	5505	2718
Gly vs Control	9931	5707	4224
E2+Gly vs Control	9900	5968	3932
E2+Gly vs E2	6079	2520	3559
E2+Gly vs Gly	8705	4647	4058
Gly vs E2	6850	4237	2613
E2 groups vs Non-E2 groups	4514	2703	1811
Gly groups vs Non-Gly groups	3857	1782	2075

Differentially expressed genes (DEGs) identified using $\log_2FC \geq 4$ cut off. E2 groups (E2 and E2+Gly); Non-E2 groups (Gly and Con); Gly groups (Gly and E2+Gly); Non-Gly groups (E2 and Con)

Table 3.2. Fold change values for *Gh* and *Prl* compared to Con.

	E2 vs Con	Gly vs Con	E2+Gly vs Con
Gh	*7,685,409	1.66	64870
Prl	*648	0.58	1

* indicates significant differential expression between treatments

Supplementary Table 3.1a. E2 vs Con effects on gene expression in the whole female mouse brain. RNA samples from different animals within each treatment were pooled into one sample per treatment group. Pathway maps/process networks were obtained using the "enrichment analysis" function in MetaCore 6.18 and only ones with the top 5 highest z-scores are reported.

Direction	Pathway maps/ Process networks	Genes
Upregulated	Nociception – Nociceptin receptor signaling	*Adrbk1*, *Arrb1*, *Cacna1b*, *Cacng5*, *Fos*, *Gnao1*, *Jak1*, *Jak2*, *Limk1*, *Limk2*, *Mapk1*, *Mapk9*, *Pla2g4c*, *Prkar1a*, *Prkcg*, *Rps6ka1, Src*
	Development – Angiotensin signaling via beta-Arrestin	*Adrbk1*, *Ap2b1*, *Arrb1*, *Arrb2*, *Calm2*, *Map3k5*, *Mapk1*, *Mapk10*, *Mdm2*, *Prkcg, Src*
	Cytoskeleton remodeling	*Actn4*, *Csnk2b*, *Eif4a2*, *Eif4g1*, *Eif4g3*, *Ilk*, *Itgav*, *Limk1*, *Limk2*, *Mapk1*, *Myh10*, *Myl1*, *Myl9*, *Paxn*, *Pik3r2*, *Pip5k1c, Ptk2*, *Smad3*, *Src*
	Transcription – Epigenetic regulation of gene expression	*Ehmt2*, *H3f3a*, *Hdac6*, *Hdac8*, *Kat2a*, *Kat2b*, *Kat6b*, *Kat7*, *Kdm3a*, *Kdm4c*, *Kdm5c*, *Kmt2a*, *Prmt5*, *Sirt3*, *Smyd2*
	Neurophysiological process – ACM regulation of nerve impulse	*Cacna1a*, *Cacna1b*, *Cacna1c*, *Cacna1g*, *Cacna1h*, *Calm2*, *Fkbp1a*, *Gnao1, Plcb4*, *Ppp3cb*, *Prkar1b*, *Prkcg*, *Trpc3*
	Protein folding and maturation – insulin processing	*Ins2*
	Cell cycle G1-S – Growth factor regulation	*Akt1*, *Akt2*, *Akt3*, *Ccnd3*, *E2f1*, *Erbb2*, *Fgfr1*, *Fos*, *Foxn3*, *Gsk3a*, *Ikbkb*, *Jak2*, *Mapk1*, *Mapk9, Prkcg*, *Ptk2*, *Src*
	Inflammation – Amphoterin signaling	*Akt1*, *Akt2*, *Akt3*, *Ccl12*, *Hmgb1*, *Ikbkb*, *Ikbkg*, *Irak1*, *Irak2*, *Itgam*, *Limk1*, *Limk2*, *Mapk1*, *Mapk8*, *Mapk9*, *Mapk10*, *Myl1*, *Myl9*
	Reproduction, feeding and neurohormone signaling	*Adam9*, *Akt2*, *Calm2*, *Ccnd3*, *Cpeb1*, *Csnk2b*, *Fos*, *Gnao1*, *H3f3a, Hspa9*, *Ide*, *Itgam*, *Ptk2*, *Slpi*, *Src*, *Stx2*
Downregulated	K-RAS signaling in pancreatic cancer	*Akt2*, *Braf*, *Cdkn1a*, *Chuk*, *Exoc2*, *Kras*, *Map2k2*, *Pdpk1*, *Pten*, *Rac1*, *Raf1*, *Ralgds*, *Tiam1*, *Vegfa*
	Signal Transduction – ERK1/2 signaling pathway	*Araf*, *Braf*, *Grb10*, *Kras*, *Map2k2*, *Mras*, *Prkcd, Raf1*, *Rapgef3*, *Rapgef4*
	Development – Positive regulation of STK3/4 (Hippo) pathway and	*Actb, Actg1*, *Adcy3*, *Adcy7*, *Amotl2*, *Crb3*, *Ctnnb1*, *Fbxw11*, *Inadl*, *Mob1a*, *Prkacb*,

negative regulation of YAP/TAZ function	*Prkab1*, *Prkar1a*, *Prkar1b*, *Raf1*, *Rassf5*, *Stk4*, *Stk11*, *Yap1*
Signal Transduction – PTMs in BAFF-induced signaling	*Akt2*, *Bcl2l11*, *Foxo3*, *Map2k2*, *Map2k4*, *Map2k7*, *Mapk14*, *Pdpk1*, *Pim2*, *Prkcd,* *Raf1*, *Tnfrsf13b*, *Trim2*
Signal transduction – Cholecystokinin signaling	*Adcy3*, *Adcy7*, *Akt2*, *Chuk*, *Fgfr2*, *Gnag*, *Map2k2*, *Mras*, *Oprl1*, *Oprm1*, *Plce1*, *Prkar1b*, *Prkacb*, *Ptk2b*, *Rac1*, *Raf1*, *Sp1,* *Src*
Cell adhesion – Cell junctions	*Actb*, *Actg1*, *Actn4, Arhgef2*, *Cask*, *Cdh4*, *Cgn*, *Crb3*, *Csnk2a1*, *Ctnnb1*, *Ctnnd1*, *Epb41*, *Erbb2ip*, *Fer*, *Git1*, *Git2*, *Gjc1*, *Inadl*, *Jup*, *Mpdz*, *Mtdh*, *Ocln*, *Plcg1*, *Prkcd*, *Prkci*, *Prkd3*, *Ptn*, *Rac1*, *Sp1*, *Src*, *Tiam1*, *Zeb2*
Reproduction - spermatogenesis, copulation and motility	*Adcy7*, *Akt2*, *Bmpr1a*, *Brd2*, *Brd7*, *Cacna1i*, *Csnk2a1*, *Daxx*, *Ddc*, *Dnmt3a*, *Fancg*, *Fkbp4*, *Ggt1*, *Gpx4*, *Hmga1*, *Hspa4*, *Mapk14*, *Nr6a1*, *Prkacb*, *Prkar1b*, *Prkcd*, *Prkci*, *Prkd3*, *Raf1*, *Src*, *Rapgef3*, *Rapgef4*, *Sox5*, *Sp1*, *Spag9*, *Spin2c*, *Tbpl1*, *Tle3,* *Xrn2*, *Ybx2*

Supplementary Table 3.1b. E2+Gly vs Gly effects on gene expression in the whole female mouse brain. RNA samples from different animals within each treatment were pooled into one sample per treatment group. Pathway maps/process networks were obtained using the "enrichment analysis" function in MetaCore 6.18 and only ones with the top 5 highest z-scores are reported.

Direction	Pathway maps/ Process networks	Genes
Upregulated	Cytoskeleton remodeling - TGF, WNT and cytoskeletal remodeling	*Actn4*, *Akt1*, *Axin2*, *Cav1*, *Ccnd1*, *Col4a1*, *Ctnnb1*, *Dvl1*, *Grb2*, *Gsk3b*, *Ilk*, *Limk2*, *Map2k1*, *Map2k2*, *Map3k7*, *Mdm2*, *Myl12a*, *Mylpf*, *Pik3cd*, *Pik3r2*, *Ppard*, *Rac1*, *Raf1*, *Sos2*, *Sp1*, *Src*, *Tcf7l1*, *Tcf7l2*, *Tln1*, *Vegfa*
	LRRK2 in neurons in Parkinson's disease	*Actg1*, *Ap2a1*, *Ap2b1*, *Cdk5rap2*, *Cltc*, *Gsk3b*, *Hsp90ab1*, *Lrrk2*, *Map2k1*, *Mapk1*, *Mapk3*, *Mapt, Mark2*, *Nsf*, *Prkacb*, *Rac1*
	Signal Transduction – ESR1 signaling pathway	*Adcy3*, *Bcas3*, *Bcl11a*, *Bloc1s1*, *Cav1*, *Ccnd1*, *Ccng2, Cdc25b*, *Ddx5*, *Erbb4*, *Gsk3b*, *Hdac1*, *Isl1*, *Kmt2d*, *Maob*, *Map2k1*, *Map2k2*, *Mapk1*, *Med1*, *Msh2*, *Mta1*, *Mta2*, *Ncoa2*, *Ncoa5*, *Ncoa6*, *Ncor1*, *Nrip1*, *Pelp1*, *Phb2*, *Pik3ca*, *Pik3r2*, *Ppargc1b*, *Prkacb*, *Prkar1a*, *Prmt2*, *Raf1*, *Rara*, *Rexo4*, *Rps6ka1*, *Rxra*, *Safb2*, *Smad3*, *Smd4*, *Socs3*, *Sos2*, *Sp1*, *Src*, *Stat5b*, *Tbp*, *Tcf20*, *Ube2d2a*, *Vegfa*
	Development Neurogenesis – Axonal guidance	*Ablim1*, *Actb*, *Actg1*, *Adam10*, *Adam17*, *Adam23*, *Adcyap1r1*, *Arhgap26*, *Baiap2*, *Braf*, *Calm1*, *Dctn1*, *Efna1*, *Efnb1*, *Epha7*, *Ephb2, Evl*, *Fez1*, *Gas7*, *Gda*, *Itgb2*, *Itpr2*, *L1cam*, *Limk2*, *Map2k1*, *Map2k2*, *Mapk1*, *Mical1*, *Myl1*, *Ngf*, *Nrp2*, *Pik3cd*, *Plcb4*, *Plxnd1*, *Ppp1cc*, *Ppp1r1b*, *Ppp3ca, Prkar1a*, *Prkca*, *Rac1*, *Rapgef3*, *Rapgef4*, *Rps6ka3*, *Rtn4*, *Sdcbp*, *Sema6d*, *Sema4d*, *Sepp1*, *Unc5c*, *Vasp*, *Wasf2*
Downregulated	Immune response – Platelet activating factor/ PTAFR pathway signaling	*Actb*, *Chuk*, *Il1b, Jak2*, *Map3k5*, *Mapk14*, *Nfatc2*, *Stat2*
	Signal transduction – Erk Interactions: Inhibition of Erk	*Dusp1*, *Dusp3*, *Dusp7*, *Dusp16*, *Prkcd*, *Prkce*, *Prkcz*, *Ptpn5*, *Ptprr*, *Vrk3*
	Signal transduction – PTMs (phosphorylation in TNF-alpha induced NF-kB signaling	*Akt1*, *Azi2*, *Chuk*, *Csnk2a1*, *Ikbkg, Map3k14*, *Pik3r2*, *Prkcd*, *Prkcec*, *Tab2*, *Tnfrsf1a*
	Cytoskeleton – Actin filaments	*Ablim1*, *Actb*, *Ank3*, *Cdc42bpa*, *Cttn*, *Diap1*, *Epb41*, *Fermt2*, *Flna*, *Klhl20*, *Kptn*, *Lasp1*, *Limk1*, *Myh10*, *Myl1, Mylk3*, *Myo1b*, *Myo1c*, *Myo6*, *Nebl*, *Pacsin2*, *Pacsin3*, *Pfn2*, *Plec, Src*, *Tiam1*, *Tnnt1*, *Tnnt2*, *Tpm1*, *Twf1*, *Wasf2*,

	Znhit3
Protein folding – Protein folding nucleus	*Csnk2a1*, *Ctnnb1*, *Dnajc1*, *Faf1*, *Fkbp1a*, *Fkbp8, Hcfc1*, *Hdac1*, *Hdac2*, *Hnrnpd*, *Hsp90aa1*, *Hsp90ab1*, *Hsp90b1*, *Hspa8*, *Hyou1*, *Nfya*, *Npm1*, *Park7*, *Ppp3ca*, *Rbbp4*, *Rbbp7*, *Ruvbl2*, *Taf1*, *Taf3*, *Tbp*, *Trap1*
Cell adhesion – Synaptic contact	*Actb*, *Anxa4*, *Ctnnb1, Ctnnd1*, *Cttn*, *Dlgap4*, *Dnm2*, *Epb41*, *Grik2*, *Grip1*, *Grm1*, *Homer3*, *Itsn1, Kcnj3*, *Kcnj13*, *Lin7a*, *Nrxn1*, *Ocln*, *Opcml*, *Pfn2*, *Shank3*, *Sharpin*, *Stx8*, *Stx16*, *Synpo*, *Syt3*, *Syt7*, *Syt9*, *Tanc1*, *Tjp1*

Supplementary Table 3.1c. Gene expression in E2 groups (E2, E2+Gly) vs Non-E2 (Gly, Con) groups in the whole female mouse brain. RNA samples from different animals within each treatment were pooled into one sample per treatment group. Pathway maps/process networks were obtained using the "enrichment analysis" function in MetaCore 6.18 and only ones with the top 5 highest z-scores are reported.

Direction	Pathway maps/ Process networks	Genes
Upregulated	Nociception – Nociceptin receptor signaling	*Adrbk1*, *Arrb1*, *Cacna1a*, *Cacna2d1*, *Cacnb1*, *Cacng5*, *Gna15*, *Gnao1*, *Limk2*, *Mapk9*, *Plcb1*, *Prkacb*, *Prkar1a*, *Prkcg*, *Rac1*, *Th*
	Neurophysiological process – ACM regulation of nerve impulse	*Cacna1a*, *Cacna1g, Fkbp1a*, *Gnao1*, *Itpr2*, *Plcb1*, *Ppp3cb*, *Prkacb*, *Prkar1a*, *Trpc3*
	Neurophysiological process – Constitutive and regulated NMDA receptor trafficking	*Cask*, *Dlg1*, *Dlg3*, *Dlg4*, *Exoc4*, *Gnao1*, *Itpr2, Plcb1*, *Ppp1r1b*, *Ppp3cb*, *Prkacb*, *Prkar1a*
	Neurophysiological process – Synaptic vesicle fusion and recycling in nerve terminals	*Ap2b1*, *Ap2m1*, *Cacna1a*, *Dnm1*, *Nsf, Pip5k1c*, *Ppp3cb*, *Snap91*, *Snapin*, *Syp*, *Syt1*
	Neurophysiological process – Activity-dependent synaptic AMPA receptor removal	*Ap2a1*, *Ap2b1, Ap2m1*, *Dlg4*, *Dnm2*, *Gria1*, *Itpr2*, *Nsf*, *Pip5k1c*, *Plcb1*, *Ppp3cb*
	Neurophysiological process – Dynein-dynactin motor complex in axonal transport in neurons	*Actr1b*, *Dctn1, Dync1i1*, *Dync1i2*, *Dynlt3*, *Kpna1*, *Ntrk3*, *Osbpl1a*, *Snapin*
Downregulated	Signal transduction – Additional pathways of NF-kB activation (in the cytoplasm)	*Adcy3, Akt1*, *Akt2*, *Akt3*, *Map3k3*, *Pdpk1*, *Pik3r2*, *Prkcd*, *Prkce*, *Prkci*, *Rps6ka1*, *Src*
	Immune response – IL-15 signaling	*Adam17*, *Akt1*, *Akt2*, *Akt3*, *Il15ra*, *Mknk1*, *Pdpk1*, *Pik3r2*, *Prkce, Rps6kb2*, *Src*
	Signal transduction – PTMs (phosphorylation) in TNF alpha-induced NF-kB signaling	*Adam17*, *Adcy3, Akt1*, *Pdpk1*, *Pik3r2*, *Prkcd*, *Prkce, Src*
	Neurophysiological process – NMDA-dependent postsynaptic long term potentiation in CA1 hippocampal neurons	*Akt1*, *Akt2*, *Akt3, Braf*, *Grin2a*, *Grm1*, *Mnk1*, *Pdpk1*, *Ptk2b*, *Rps6ka1*, *Ryr2*, *Src*

Supplementary Table 3.2a. Gly vs Con effects on gene expression in the whole female mouse brain. RNA samples from different animals within each treatment were pooled into one sample per treatment group. Pathway maps/process networks were obtained using the "enrichment analysis" function in MetaCore 6.18 and only ones with the top 5 highest z-scores are reported.

Direction	Pathway maps/ Process networks	Genes
Upregulated	Signal transduction – PKA signaling	*Akap2, Akap7, Akap11, Akap13, Gnao1, Gnas, Pde4a, Pdpk1, Phkg1, Phkg2, Pkig, Ppp1r1b, Ppp2r2c, Ppp2r4, Ppp2r5b, Prkar2b, Rps6ka1, Smad3*
	Transport – Role of AVP in regulation of Aquaporin 2 and renal water reabsorption	*Akap7, Calm2, Fos, Gnas, Mapk14, Mylk3, Prkar2b, Sipa1, Stx4a, Tpm1*
	Protein folding and maturation – Insulin processing	*Ins2*
	Cytoskeleton remodeling – Role pf PKA in cytoskeleton reorganization	*Abl1, Add1, Akap13, Calm2, Gnas, Lasp1, Limk1, Mylk3, Pak1, Pxn, Vasp*
	Immune response – Platelet activating factor/ PTAFR pathway signaling	*Akt1, Arrb1, Chuk, Gnao1, Il1b, Jak2, Map2k3, Map3k5, Mapk14, Nfatc2, Nfkb1*
	Regulation of lipid metabolism – Insulin regulation of glycogen metabolism	*Akt1, Akt2, Calm2, Gck, Gys2, Ins2, Pdpk1, Phkg1, Phkg2, Pygm, Rps6ka1*
Downregulated	HBV signaling via protein kinases leading to HCC	*Hras, Igf2, Jak1, Map2k2, Map2k4, Map3k1, Mapk1, Mapk8, Mapk10, Prkca, Prkci, Prkd2, Prkd3, Ptk2b, Raf1, Sos2, Sp1, Src*
	Cytoskeleton remodeling – TGF, WNT and cytoskeletal remodeling	*Actg1, Actn4, Cav1, Cdkn1a, Csnk2a1, Ctnnb1, Dvl1, Foxo3, Gsk3b, Hras, Limk2, Map2k2, Map3k7, Mapk1, Mdm2, Mknk1, Myc, Myl12a, Mylpf, Pik3cd, Pik3r3, Rac1, Raf1, Sos2, Sp1, Src, Tcf7, Tcf7l1, Tcf7l2, Vegfa, Wnt5b*
	Development – Endothelin-1/EDNRA signaling	*Adcy3, Adcy7, Ccnd1, Ctnnb1, Gnaq, Gsk3b, Hras, Map2k2, Map2k4, Map3k1, Mapk1, Mapk8, Pik3cd, Pik3r2, Ptk2b, Rac1, Raf1, Sos2, Src*
	Signal transduction – PTEN pathway	*Bad, Cdkn1a, Ctnnb1, Foxo3, Gsk3b, Hras, Igf1, Magi2, Map2k2, Mapk1, Mdm2, Pcna, Pik3cd, Pik3r3, Raf1, Sos2, Src, Tcf7, Tcf7l1, Tcf7l2*
	Development – Neurogenesis and synaptogenesis	*Actg1, Adarb1, Agrn, Atn1, Dlg3, Dlgap2, Fgf7, Kcng2, Nlgn2, Nos1, Nrg4, Park2, Ppfia1, Prkca, Rims3, Sema4f, Sh3gl2, Snta1, Sntb1, Syp*

Supplementary Table 3.2b. E2+Gly vs E2 effects on gene expression in the whole female mouse brain. RNA samples from different animals within each treatment were pooled into one sample per treatment group. Pathway maps/process networks were obtained using the "enrichment analysis" function in MetaCore 6.18 and only ones with the top 5 highest z-scores are reported.

Direction	Pathway maps/ Process networks	Genes
Upregulated	Signal transduction – ERK1/2 signaling pathway	*Araf*, *Braf*, *Grb10*, *Map2k1*, *Map2k2*, *Mapk3*, *Mras*, *Raf1*, *Rapgef3*, *Rapgef4*, *Sos2*
	Development – Adenosine A2A receptor signaling	*Braf, Chuk, Gnas, Ikbkb, Map2k1, Map2k2, Mapk3, Mapk14, Nfkb1, Pak1, Prkacb, Prkar1b, Prkcz, Rapgef3, Rps6ka3*
	Cytoskeleton remodeling	*Col4a1, Csnk2a1, Ctnnb1, Eif4g1, Flna, Map2k2, Map2k3, Mapk3, Mapk14, Mknk1, Myl12a, Mylpf, Pak1, Pip5k1c, Pten, Rac1, Raf1, Smad3, Sos2, Tln2, Vegfa*
	G-protein signaling – Ras family GTPases in kinase cascades (schema)	*Braf, Map2k1, Map2k2, Map2k3, Map2k4, Mapk3, Mapk14, Pak1, Rac1*
	Reproduction – FSH beta signaling pathway	*Acvr1, Adcy3, Chuk, Ctgf, Etv4, Gnas, Map2k1, Map2k2, Map2k3, Mapk3, Nfkb1, Ppp2r1a, Prkacb, Raf1, Ror2, Rps6ka3, Sp1, Tgm2, Vegfa*
	Inflammation – Amphoterin signaling	*Chuk, Ikbkb, Itgb2, Map2k1, Map2k2, Mapk3, Nfkb1, Pak1, Rac1, Raf1, Sp1*
Downregulated	Immune response – Platelet activating factor/ PTAFR pathway signaling	*Actb, Akt1, Akt2, Arrb1, Arrb2, Ccl12, Gnao1, Gnb2, Jak2, Lsp1, Map3k5, Pik3r2, Plcb4, Ppp3ca, Src, Stat2, Stat5a*
	Development – WNT signaling pathway, degradation of beta catenin in the absence of WNT signaling	*Apc, Csnk1d*, *Csnk1e*, *Ctbp2*, *Fbxw11*, *Lef1, Tcf7l2*
	Neurophysiological process – Dynein-dynactin motor complex in axonal transport in neurons	*Akt1, Akt2, Dync1i1, Dync1i2, Hap1, Hdac6, Kpna4, Mapk8ip3, Mapk10, Nde1, Ntf3, Ntrk3, Osbpl1a, Prnp*
	Cell adhesion – Synaptic contact	*Actb*, *Actn4*, *Dlg1, Dlgap4*, *Dnm2*, *Epb41*, *Grip1*, *Grm1*, *Kalrn*, *Kcnj13*, *Kcnj16*, *L1cam, Lin7a*, *Nlgn2, Nrxn1*, *Nrxn3*, *Shank3*, *Sptan1*, *Stx16*, *Synpo*, *Tanc1*, *Tip1*
	Development – Neurogenesis and synaptogenesis	*Actb*, *Dlg1*, *Dvl3*, *Fgfr2*, *Grip1*, *Grm1*, *Lin7a*, *Lrp8*, *Mog*, *Nlgn2*, *Nrg2*, *Nrxn1*, *Nrxn3*, *Nsf*, *Ntf3*, *Ntrk3*, *Park2*, *Ppfia3*, *Shank3, Stx16*, *Synpo*, *Syp*, *Tbr1*, *Wnt5a*
	Regulation of CFTR activity (normal and CF)	*Adcy3, Flna*, *Gnas*, *Ppp2r1a*, *Ppp2r4*, *Ppm1d*, *Prkab1*, *Prkce*, *S100a10, Snap23, Stxbp1*

Supplementary Table 3.2c. Gene expression in Gly groups (Gly, E2+Gly) vs Non-Gly (E2, Con) groups in the whole female mouse brain. RNA samples from different animals within each treatment were pooled into one sample per treatment group. Pathway maps/process networks were obtained using the "enrichment analysis" function in MetaCore 6.18 and only ones with the top 5 highest z-scores are reported.

Direction	Pathway maps/ Process networks	Genes
Upregulated	Cytoskeleton remodeling	*Ctnnb1*, *Eif4a1*, *Eif4g1*, *Eif4g3*, *Flna*, *Mknk1*, *Myl2*, *Mylpf*, *Pak1*, *Pik3r2*, *Pip5k1c*, *Rac1*, *Smad3*
	Immune response – CD28 signaling	*Akt1, Nfatc2*, *Nfkb1*, *Pak1*, *Pik3r2*, *Pip5k1c*, *Rac1*
	Mitogenic action of Estradiol/ ESR1 (nuclear) in breast cancer	*Carm1*, *Ccne*, *Chd8*, *Nfya, Pou2f1*, *Ppm1d*
	Immune response – Phagosome in antigen presentation	*Derl2*, *Hsp90b1*, *Nfkb1*, *Pak1*, *Pik3r2, Psme4*, *Rac1*, *Ralgds*, *Vasp*
	Neurophysiological process – Transmission of nerve impulse	*Agtr2*, *Dlg4*, *Gnas*, *Gria1*, *Gria3*, *Grik2*, *Homer3*, *Kcnma1, Trim28*
Downregulated	Development – Flt3 signaling	*Atf2*, *Bad, Flt3l*, *Hras*, *Map2k3*, *Map2k7*, *Map3k1*, *Mapk8*, *Mapk14*, , *Pdpk1*, *Src*
	Cytoskeleton remodeling – Reverse signaling by ephrin B	*Actg1, Ephb4*, *Gnb1*, *Gnb2*, *Gnb4*, *Gng7*, *Gngt2*, *Hras*, *Ilk*, *Mapt*, *Pxn*, *Rgs3*, *Src*, *Wipf1*
	Signal transduction – JNK pathway	*Atf2, Daxx*, *Hras*, *Map2k7*, *Map3k7*, *Map3k1*, *Map3k12*, *Map4k2*, *Mapk8*, *Tnfrsf1a*
	Neuroprotective action of lithium	*Atf2, Bdnf*, *Grin2a*, *Hspa5*, *Map2k3*, *Map2k7*, *Mapk8*, *Mapk14*, *Mapt*, *Ptk2b*, *Src*
	Cell adhesion – Synaptic contact	*Actg1*, *Actn4*, *Dlg1*, *Dlgap4*, *Dnm2*, *Epb41*, *Itsn1*, *Kalrn*, *Kcnj16*, *Nrxn3*, *Ntm, Sdcbp*, *Shank1*, *Sharpin*, *Sptan1*, *Syt3*, *Syt6*, *Tanc1*
	Reproduction – Spermatogenesis, motility and copulation	*Arsa*, *Atf2, Brd2*, *Cacna1i*, *Crem*, *Daxx*, *Dbh*, *Fkbp4*, *Gpx4*, *H2afz*, *Hras*, *Hspa5, Map3k7*, *Mapk14*, *Mkks*, *Myc*, *Ncoa4*, *Odf2l*, *Sp1*, *Spag4*, *Spag9*, *Src*, *Xrn2*
	Transcription – Nuclear receptors transcriptional regulation	*Hmgb1*, *Hras*, *Map2k3*, *Map3k1*, *Map3k7*, *Mapk14*, *Ncoa4*, *Ncor1, Nr2f1*, *Thrap3*

CHAPTER 4

ACUTE VS. CHRONIC EXPOSURE TO SOY GLYCEOLLINS AFFECTS MRNA EXPRESSION OF ESTROGEN RECEPTOR–REGULATED GENES IN DISTINCT BRAIN REGIONS OF THE FEMALE MOUSE

Introduction

Glyceollins (Gly) are phytoalexins produced by soy plants in response to stressful stimuli including fungal infections, exposure to UV or changes in temperature. Research on Gly gained momentum mainly because, unlike other soy compounds such as genistein and daidzein that exhibit estrogenic properties, Gly exhibited anti-estrogenic effects. Gly opposes the effects of 17-β estradiol (E2) *in vitro* in cancer cell lines as well as *in vivo* in peripheral tissues through action at estrogen receptors (ERs), mainly ERα (Zimmermann et al. 2010). For example, Gly suppresses E2-responsive breast (MCF-7) and ovarian (BG-1) tumors both *in vitro* and *in vivo* (Burow et al. 2001; Salvo et al. 2006) and decreases E2-stimulated uterine growth *in vivo* (Salvo et al. 2006). However, other studies suggested that Gly can have weak estrogenic activity at ERβ, thereby promoting proliferation of MCF-7 breast tumors *in vitro* (Kim et al. 2010a). A recent study showed that different Gly isomers (*e.g.*, Gly I, II and III) possess antagonistic/agonistic actions at different human ERs, depending on the prenylation pattern of the isomers, the structure of the backbone and the length of the compound, with the effects being selective estrogen receptor modulator (SERM)-like at ERα (Gly I) and agonistic at ERβ (Gly I, II and III)

(van de Schans et al. 2016). Thus it is likely that Gly acts like a SERM, with antagonistic/agonistic action at ERs in a tissue- and/or condition-dependent manner.

Although Gly is well known for its ER-mediated effects, Glys also exhibit non ER-mediated effects. Some examples include suppression of triple-negative breast carcinomas (which lack ERs) (Rhodes et al. 2012); increasing insulin-stimulated glucose uptake in 3T3-L1 adipocytes (Boue et al. 2012; Park et al. 2010); decreasing blood glucose levels in rodents (Park et al. 2012); increasing lipid metabolism gene expression (Wood et al. 2012); inhibiting lipid peroxidation in rat brain, kidney and liver extracts (Kim et al. 2010b); and inhibiting melanogenic enzymes in B16 melanoma cells (Lee et al. 2010).

The antitumor, antifungal, antioxidant and nutrient metabolism effects of Gly are well established (see Chapter 1); however, the effects of Gly on brain physiology and function remain unclear. Compounds that act at ERs may exhibit varied effects in the periphery and the central nervous system. One example is ICI 182,720 (fulvestrant), which is purely anti-estrogenic in the periphery but acts more like a SERM in the brain (Alfinito et al. 2008). Fulvestrant inhibits E2's negative feedback on GnRH, thus acting as an anti-estrogen in the hypothalamus, whereas it mimics the effect of E2 on place-learning behavior in the hippocampus (Alfinito et al. 2008; Steyn et al. 2007). In order to evaluate the effect of Gly in the brain, a microarray experiment was performed on whole brain samples of female mice. The goal of the study was to determine if 11-days of chronic treatment with Gly alone affected gene expression compared to control, and whether Gly, when in combination with E2 (E2+Gly), opposed the E2 effect on gene expression. Our whole brain microarray indicated that Gly opposed the E2 effect on

expression of a few genes, but exhibited a gene expression pattern distinct from both E2 groups (E2 and E2+Gly) (Bamji et al. 2015). In addition, an RNA-Sequencing (RNA-Seq) experiment was performed on pooled whole brain homogenate samples from the 4 treatment groups to provide a genome-wide view of Gly's actions on gene expression in the brain and to examine Gly's effects on some of the low expression genes of interest. Our RNA-Seq results indicated that, depending on the gene, Gly can oppose the effect of E2, act similarly to E2 or regulate genes in a manner different from E2 action (Chapter 3). Thus, our microarray and RNA-Seq experiments suggested that Gly potentially exhibits a SERM-like effect in the female mouse brain.

One limitation of the previous experiments was that they did not take into account the heterogeneity of the brain. Not only do different brain regions perform distinct and unique functions, but also gene expression can be highly brain-region specific (Merchenthaler et al. 2004). In order to assess the effects of Gly on gene expression in specific brain regions, in the present study we focused on: 1) hypothalamus — the neuroendocrine center that regulates the release of hormones from the pituitary, sexual behavior and reproduction, food and water intake, temperature regulation, circadian rhythms, emotional responses and stress; 2) hippocampus — responsible for learning, short term, long term and spatial memory, and plays an important role in hypothalamic-pituitary-adrenal (HPA) axis negative feedback; 3) cortex — involved in general cognition, executive function, motor control and processing of multisensory functions (Kandel et al. 2000). Additionally, we evaluated the effects of a chronic 7-day vs. acute (2hr, 24hr and 48hr) Gly treatment on gene expression in the above mentioned brain regions.

Genes of interest were selected based on their known expression in the brain, their responsiveness to E2, and results of the previous whole brain microarray (Bamji et al. 2015) and RNA-Seq studies (Chapter 3). It is well known that ERα (*Esr1*), G-protein coupled estrogen receptor 1 (*Gper1*), an orphan nuclear receptor (*Nr4a1*), growth hormone (*Gh*) and prolactin (*Prl*) are expressed in the hypothalamus, hippocampus and cortex and are all regulated by E2 (Addison and Rissman 2012; Brown et al. 2010; Furigo et al. 2014; Hazell et al. 2009; Lundholm et al. 2008; Mitra et al. 2003; Saucedo-Cardenas and Conneely 1996; Simerly and Young 1991). We evaluated the mRNA abundance of all genes of interest using quantitative real-time polymerase chain reaction (qPCR). Our results suggest that unlike E2, Gly does not seem to have brain-region specific effects on the expression of *Esr1*, *Gper1* or *Nr4a1* in the hypothalamus, hippocampus or cortex but similar to known E2 regulation, affects the expression of *Gh* in all three brain regions and *Prl* in the hypothalamus and cortex after only a single acute dose (2hr or 24hr Gly exposure). Thus Gly, through its effects on *Gh* and *Prl*, can potentially regulate neuronal processes involved in food intake, stress and cognition in the female mouse brain, but the exact mechanism of Gly action is still unclear.

Materials and Methods

Study animals

All protocols for housing, handling and euthanasia followed guidelines approved by the *Guide for the Care and Use of Laboratory Animals: Eighth Edition* (Council 2011) and were approved by the University of Louisville Institutional Animal Care and Use

Committee (protocol number 13013). Adult female Swiss Webster (CFW) mice (approximately 31-35 days old) were purchased from Charles River Laboratories and given *ad libitum* access to phytoestrogen-reduced chow (Teklad 2016) and tap water. Animals were group housed, 3 mice/cage and maintained on a 12L:12D photoperiod (lights on at 6 AM).

Ovariectomy, hormone replacement, experimental manipulations and plasma samples

Animals were allowed to acclimate for 1 week after arrival before they were ovariectomized (ovx) under isoflurane anesthesia and implanted s.c. with Silastic capsules (Dow Corning; effective length 14mm, 1.57mm inner diameter x 3.18mm outer diameter), filled with either 35µl sesame oil (placebo) or 35 µl of 36 µg/ml E2 dissolved in sesame oil. One-week post ovx, mice were injected i.p. with 50 µl of either 1:1 DMSO: PBS (vehicle) or Gly (20 mg/kg/day) dissolved in 1:1 DMSO: PBS. Our four treatment groups were as follows (n = 96, 6 per treatment group per time point): negative controls (Con; Silastic placebo + vehicle injection), estrogen only (E2; Silastic E2 + vehicle injection), Gly only (Gly; Silastic placebo + Gly injection) and E2+Gly (Silastic E2 + Gly injection). Mice were euthanized at 2hr, 24hr or 48hr after a single dose of Gly or vehicle (acute exposure) or after 7 daily doses of Gly or vehicle (chronic exposure). Mice were weighed weekly: at arrival, at ovx, one week post-ovx (7d only), and at euthanasia (2hr, 24hr, 48 hr, or 7d after Gly treatment started). After decapitation under heavy isoflurane anesthesia, brains were rapidly extracted and placed in RNALater for 24h at 4C before storage at -20C. Trunk blood samples were stored on ice until centrifuged and plasma was collected and stored at -20°C until processed for an ELISA assay of E2 by the Ligand Assay and Analysis Core Laboratory at the University of Virginia Center for

Research in Reproduction. Plasma from E2 and E2+Gly-treated mice were diluted 1:2 to fit the linear range of the ELISA (detection limits 3-300pg/ml). Only a few samples from the non-E2 groups (Con, n = 2 and Gly, n = 4) were sent for analysis. Samples with a %CV > 30 or with values outside the detection range of the assay were excluded (5 E2 samples, 2 E2+Gly samples and 3 Gly samples).

RNA extraction, cDNA amplification and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from brain regions using the RNeasy Micro Kit (Qiagen) for the hypothalamus tissue and the PureLink RNA Mini Kit (Life Technologies) for hippocampus and cortex tissues. A nanodrop 2000 spectrophotometer was used to assess sample purity and RNA concentration. Extracted RNA samples (1ug/ml) were then used to make cDNA using the High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, 4368814) according to manufacturer's instructions. All cDNA samples were diluted 1:20 before use in qPCR reactions.

Taqman primers were selected, using the manufacturer's recommendations, to avoid the detection of non-target sequences (Supplementary Table 4.1). All qPCR reactions were 10 µl reactions and were run on ABI ViiA7 in 384-well plates. Reaction conditions were 50°C for 2 mins, 95°C for 10 min followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. Each qPCR reaction was technically replicated in triplicate and each plate contained three template-free controls per primer pair for each of the genes of interest: *Esr1*, *Gper1* (7 day Gly exposure – hypothalamus only), *Nr4a1, Gh* and *Prl* (Supplementary Table 4.1).

Data analysis

Body weight and plasma E2 data: Body weight data were analyzed via two-factor repeated measures ANOVA with treatment and week as factors using SigmaStat 3.1, with acute and chronic groups analyzed separately. Plasma E2 data (E2 groups vs non-E2 groups) was analyzed using a two-sample F-test for variances followed by a two-sample T-test assuming unequal variances in Excel.

Estimation of initial target concentrations: Raw fluorescence data was collected from the Applied Biosystems ViiA7 Real-Time PCR System and the x1m1 filter values were used in the estimation procedure. Initial target concentrations in micromoles/L were estimated for each sample using the kinetic model of Cobbs (Cobbs 2012). The kinetic model was adapted for use with the taqman detection system by modeling hybridization kinetics of the taqman probe with the same model used for hybridization kinetics of the primers (Cobbs 2012) but with unique equilibrium constants for both probe and primers. Baseline fluorescence was modeled as a linear trend, and intercept and slope parameters for baseline fluorescence were included in the model. The model was fit to data using the nlsLM function in the minpack.lm package of R which gives least squares estimates of parameter values using the Levenberg-Marquardt algorithm (Elzhov et al. 2013). Parameter starting values for each fit were obtained by searching a grid of 50625 parameter vectors, and the one with the lowest sum of squared deviations between the observed and predicted fluorescence values was used as the starting vector for the nlsLM function. Each fit was evaluated by examination of the mean square residual and by visual inspection of a plot of the observed and fitted fluorescence values vs. cycle number to determine if they were in good agreement. Samples giving poor fit or which failed to

amplify were eliminated from any further analysis. Among the 4968 samples tested that were not water controls, 446 failed to amplify and 2 were found to be unusable because of poor fit to the model. The rest all showed good fit to the model and the estimated initial target concentration was used in the analysis.

Statistical analysis of gene expression: Base 10 logarithm of initial target concentration was the response variable in all analyses. All analyses modeled technical replicate values as a random effect nested in biological replicate values. The biological replicate value was modeled as a random effect and nested in the fixed factors in the model. The fixed effect factors (Brain region, Exposure time, E2, and Gly) were crossed and interaction effects were dropped only if found to be non-significant by p-value and if their removal resulted in a decrease in AIC. Model residuals were found in all cases to satisfy the assumptions of normality and homogeneity of variance. All analyses were done with the lmer function of the lme4 package of R (Bates et al. 2014). Variance inflation factors (VIF) were used to indicate any problems with multi-collinearity and were obtained using the vif function in the car package of R (Fox and Weisberg 2011). Linear contrasts were tested with the glht function in the multcomp package of R version 3.0.2 (Hothorn et al. 2008).

Results

Effects of E2 and Gly on Body Weight and Plasma E2

We observed a significant interaction effect between treatment and week on body weight at the acute time points ($P = 0.041$; Fig. 4.1a) and the chronic Gly exposure time ($P = 0.039$; Fig. 4.1b). However, there was no significant main effect of treatment for

either acute or chronic Gly exposure times on body weight (Fig. 4.1a-b). As expected, there was a highly significant difference ($P < 0.001$) in plasma E2 levels in the E2 groups (E2 and E2+Gly; 32.67 $\pm$ 3.44 pg/ml) vs. the non-E2 groups (Con and Gly; 8.83 $\pm$ 0.66 pg/ml).

Effects of brain region and exposure time within treatments

Although the primary objective of this study is to compare effects of treatments (see below), we also explored expression patterns within the different treatment groups for each gene of interest (Fig. 4.2, Table 4.1). Overall, we observed moderate (*Esr1*, *Nr4a1*, *Gper1*) to low (*Gh*, *Prl*) levels of mRNA expression in all brain regions (*Gper1* only tested in hypothalamus, $\log_{10}$ initial target concentration range -11.49 to -11.22μM, which corresponds to 3.23×10^{-12} to 6.02×10^{-12}μM, not shown in Fig. 4.2). There was a highly significant brain region and exposure time interaction effect on *Esr1* expression in the Con ($P < 0.0001$), Gly ($P = 0.0004$) and E2+Gly treatments ($P < 0.0001$; Fig. 4.2a, 4.2c-d). There was also a significant main effect of exposure time ($P = 0.012$) and brain region ($P < 0.0001$) on *Esr1* expression after E2 treatment (Fig. 4.2b). Initial target concentration of *Esr1* was the highest in the hypothalamus, compared to the hippocampus and cortex, irrespective of time point or treatment group. *Esr1* levels were similar to each other in hippocampus and cortex at all time points in the Gly group; for the other treatment groups, *Esr1* levels were also similar within treatments at the 2hr and 7d time points but diverged at 24 and 48 hr time points, such that *Esr1* concentration was the lowest in the cortex, with expression in the hippocampus being intermediate to the hypothalamus at both time points (Fig. 4.2a-d). In the case of *Nr4a1*, there was a highly significant brain region and exposure time interaction effect in the Con group ($P = 0.006$;

Fig. 4.2e), a significant exposure time main effect in the E2 (P = 0.002; Fig. 4.2f) and E2+Gly treatments (P = 0.046; Fig. 4.2h) and a significant brain region main effect in the Gly (P < 0.0001; Fig. 4.2g), E2 (P < 0.0001; Fig. 4.2f) and E2+Gly (P < 0.0001; Fig. 4.2h) treatments. *Nr4a1* had a relatively lower initial target concentration in the hypothalamus compared to the other two regions, irrespective of exposure time or treatment, except at the 24hr time point in the control treatment, for which expression was similar in hypothalamus and cortex (Fig. 4.2e-h). Further, *Nr4a1* had the highest concentration in the hippocampus at all acute exposure times, particularly in the Con group (Fig. 4.2e-h). At the chronic exposure time, however, expression levels in the hippocampus are as low as in the hypothalamus, with cortex *Nr4a1* expression being the highest at this time point, irrespective of treatment (Fig. 4.2e-h). In contrast to *Esr1* and *Nr4a1*, no significant interaction was observed between brain region and exposure time on *Gh* expression in any of the treatments (Fig. 4.2i-l). However, there was a significant main effect of exposure time in the E2 (P = 0.008; Fig. 4.2j) and E2+Gly (P = 0.038; Fig. 4.2l) treatments, and a significant main effect of brain region in the Gly (P = 0.04; Fig. 4.2k) and E2+Gly (P = 0.044; Fig. 4.2l) treatment groups on *Gh* expression. The initial target concentration of *Gh* was higher at the 48hr Gly time point compared to the 2hr in the hypothalamus after E2 treatment (Fig. 4.2j) and was higher in all brain regions at 48hr compared to the 24hr time point in the E2+Gly treatment (Fig. 4.2l), explaining the exposure time main effects. The brain region main effects on *Gh* expression are due to higher *Gh* expression in the hypothalamus compared to the hippocampus and cortex at the 24hr time point in the Gly treatment (Fig. 4.2k) and due to higher hypothalamic *Gh* levels compared to the hippocampus at 24hr in the E2+Gly treatment (Fig. 4.2l). Similar

to *Gh*, there was no interaction of brain region and exposure time in any treatment condition on *Prl* expression (Fig. 4.2m-p), but it should be noted that we do not have *Prl* data for hippocampus or cortex at the 7d time point. We did observe a significant main effect of exposure time in the Con (P = 0.007; Fig. 4.2m) and E2 (P = 0.039; Fig. 4.2n) treatments and a significant main effect of brain region in the Con (*P* < 0.0001; Fig. 4,2m), Gly (P = 0.034; Fig. 4.2o) and E2+Gly (P = 0.002; Fig. 4.2p) treatments on *Prl* expression. *Prl* initial target concentration was higher at the 24hr and 48hr time points compared to the 2hr time point in the Con treatment in both the cortex and hippocampus (Fig. 4.2m) and was higher in the 48hr compared to the 2hr time point in the E2 treatment in all brain regions (Fig. 4.2n). Further, *Prl* target concentration was higher at all acute Gly exposure times in the hippocampus compared to the cortex and hypothalamus within the Con treatment (Fig. 4.2m); was higher in the hippocampus and hypothalamus compared to the cortex at the 24hr time point within the Gly treatment (Fig. 4.2o) and was higher in the hypothalamus compared to the cortex and hippocampus at 24h and 48h time points in the E2+Gly treatment (Fig. 4.2p).

Effects of E2 and Gly treatments on gene expression in the hypothalamus

Both E2 groups (E2 and E2+Gly) downregulated the expression of *Esr1* compared to non E2 groups (Con and Gly) at the 2hr time point, but the E2 main effect was only marginally significant (FDR-P = 0.069; Fig. 4.3a). There was no significant E2 main effect on expression of *Gper1*, *Nr4a1*, *Gh* or *Prl* in the hypothalamus. There was a significant Gly main effect on expression of hypothalamic *Gh* and *Prl*, with both Gly groups upregulating *Gh* (FDR-P = 0.009; Fig. 4.3b) and *Prl* (FDR-P = 0.035; Fig. 4.3c) at the 2hr time point. There was also a significant Gly main effect at the 24hr time point,

with both Gly groups upregulating *Prl* expression (FDR-P = 0.041; Fig. 4.3d). There was no significant Gly main effect on expression of *Esr1*, *Gper1* or *Nr4a1* in the hypothalamus. No significant E2 and Gly interaction effects were observed for *Esr1*, *Gper1*, *Nr4a1*, *Gh* or *Prl* in the hypothalamus.

Effects of E2 and Gly treatments on gene expression in the cortex

Both E2 groups upregulated *Prl* expression in the cortex at the 24hr time point, although the E2 main effect was only marginally significant (FDR-P = 0.069; Fig. 4.3g). There was no significant main effect of E2 on *Esr1*, *Nr4a1* or *Gh* expression in the cortex. Similar to the hypothalamus, we observed a significant Gly main effect at the 2hr time point, with both Gly groups upregulating the expression of both *Gh* (FDR-P = 0.049; Fig. 4.3e) and *Prl* (FDR-P = 0.049; Fig. 4.3f) in the cortex. No significant Gly main effect was observed for *Esr1* or *Nr4a1* in the cortex. We did not observe any significant E2 and Gly interaction effects for the expression of *Esr1, Nr4a1, Gh* or *Prl* in the cortex.

Effects of E2 and Gly treatments on gene expression in the hippocampus

There was no significant E2 main effect on the expression of any of the genes of interest in the hippocampus. Both Gly groups downregulated *Esr1* expression at the 24hr time point in the hippocampus, although the Gly main effect was only marginally significant (P = 0.06; Fig. 4.3h). A significant Gly main effect on *Gh* expression was observed at the 2hr time point, with both Gly groups upregulating hippocampal *Gh* expression (P = 0.049; Fig. 4.3i). There was no significant Gly main effect on the expression of either *Nr4a1* or *Prl* in the hippocampus. No significant E2 and Gly

interaction effect was observed for the expression of *Esr1*, *Nr4a1*, *Gh* or *Prl* in the hippocampus.

Discussion

The main goals of this study were to better understand the effect of Gly on gene expression in the brain by a) evaluating the effects of acute vs. chronic Gly exposure on expression of *Esr1*, *Gper1*, *Nr4a1*, *Gh* and *Prl* in three different brain regions: hypothalamus, hippocampus and cortex, and b) comparing the results from this study with the data obtained from the whole brain microarray and RNA-Seq experiments. In general, the strongest treatment effects were due to Gly exposure, with no statistically significant interaction effects of Gly and E2. We found that all effects of Gly on gene expression occurred at acute (2hr, 24hr) exposures rather than chronic.

Given the well-known anti-estrogenic effects of Gly in peripheral tissues, our initial expectation was that Gly, by itself, would not have any effect compared to Con, but in combination with E2, would oppose effects of E2 on gene expression in the brain. Both microarray and RNA-Seq analyses revealed that depending on the gene, Gly regulates expression independently of E2 action, similarly to E2, or opposes E2's effects, thus exhibiting potential SERM-like effects in the female mouse brain. Although we obtained valuable information from the whole brain microarray and RNA-Seq analyses, there are a few limitations. Since the brain is a heterogeneous organ, the whole brain analyses were unable to detect differential expression between treatment groups of some genes of interest, *e.g.*, ERs like *Esr1* and *Gper1* that are distributed throughout the brain, perhaps because different ERs have distinct expression patterns in different brain regions

(Merchenthaler et al. 2004). Additionally, the whole brain experiments were limited to one chronic time point (11 consecutive days of Gly exposure), which excludes the evaluation of acute Gly effects on gene expression of our select genes. Thus, in the current study, we evaluated the effects of acute vs chronic exposure to Gly on expression of those select genes in 3 brain regions.

Effects of E2 and/or Gly on expression of ERs

Firstly, we evaluated the effect of E2 and/or Gly (acute and chronic doses) on *Esr1* gene expression in the hypothalamus, hippocampus and cortex. *Esr1* expression was of particular interest as Gly was initially known to exhibit its anti-estrogenic effects mainly through ERα (Zimmermann et al. 2010). Although we did not find any significant E2 or Gly effects on *Esr1* expression in our whole brain experiments, we were interested in determining if the effect of E2 and Gly, if any, would be more pronounced in different brain regions. Prior studies have shown that *Esr1* is the most abundant ER in the hypothalamus, especially in the medial preoptic area (MPOA), periventricular nucleus (PVN), dorsomedial nucleus and the venterolateral hypothalamic nucleus (Merchenthaler et al. 2004; Mitra et al. 2003). Unsurprisingly, *Esr1* not only had a relatively higher initial target concentration in the hypothalamus compared to other brain regions but was also the most highly expressed gene relative to our other genes of interest in this brain region, irrespective of treatment (Fig. 4.2a-d). Also, ERs are often downregulated by E2 in the mouse brain, an effect that can vary depending on the brain region, timing and dose of E2 administration and sex of the experimental mice (Lauber et al. 1991; Simerly and Young 1991). As *Esr1* expression can vary in different hypothalamic nuclei, it was not

surprising to find only a marginally significant E2 main effect on *Esr1* expression, with a downregulation of *Esr1* expression in both E2 groups (E2 and E2+Gly) compared to non-E2 groups (Con and Gly) at the 2hr time point in the hypothalamus (Fig. 4.3a). In the hippocampus, *Esr1* expression is generally scattered and relatively low compared to the hypothalamus (Merchenthaler et al. 2004; Mitra et al. 2003), with *Esr1* levels declining postnatally, specifically as mice approach puberty (Koboldt et al. 2013). As expected, we observed relatively low *Esr1* expression in the hippocampus compared to the hypothalamus (Fig. 4.2a-d). Further, the basal expression of *Esr1* in the cortex is known to be low, especially in adult mice (Merchenthaler et al. 2004; Mitra et al. 2003; Wilson et al. 2011), which explains our relatively lower *Esr1* expression in the cortex compared to the hypothalamus, irrespective of treatment and Gly exposure time (Fig.2a-d).

Overall, similar to our whole brain experiment, we did not find any significant E2 or Gly main effects on *Esr1* expression in any of the brain regions tested. It should be noted though that the lack of chronic Gly exposure effects in this study make it difficult to compare results from this study to those from the whole brain experiments (Chapters 2 and 3). In addition to genomic ERs including *Esr1*, E2 is also known to stimulate gene expression through rapid, non-genomic mechanisms involving membrane ERs. Therefore, we evaluated the effects of E2 and acute Gly on the expression of hypothalamic *Gper1*, a known membrane-associated ER, which is highly expressed in the arcuate nucleus (ARC), PVN, paraventricular, suprachiasmatic and supraoptic nuclei (SON) and the ventromedial hypothalamus (Brailoiu et al. 2007; Hazell et al. 2009). Like *Esr1*, neither E2 nor Gly affected *Gper1* expression in the hypothalamus. *Gper1* expression was much lower ($\log_{10}$ initial target concentration ranging from -11.49 to -

11.22μM) in different treatments) than *Esr1* in the hypothalamus (log_{10} initial target concentration ranging from -9.77 to -9.45μM).

Effects of E2 and/or Gly on Gh and Prl expression

Next, we evaluated the effects of E2 and/or Gly (acute and chronic doses) on the expression of E2-regulated *Gh* and *Prl* in the hypothalamus, hippocampus and cortex. Both *Gh* and *Prl* were upregulated by E2 treatment compared to Con in the microarray (Bamji et al. 2015) and RNA-Seq (Chapter 3) whole brain experiments, but the effect was significant only in the RNA-Seq experiment. Gly did not significantly affect the expression of *Gh* or *Prl* compared to Con in the RNA-Seq analysis, but in combination with E2 (E2+Gly), inhibited the E2 upregulation of both genes. Thus, the RNA-Seq experiment revealed a classic anti-estrogenic effect of Gly on expression of *Gh* and *Prl*. We were interested in determining the brain-region specific effects of Gly, if any, on expression of *Gh* and *Prl*.

Gh is expressed in several hypothalamic nuclei (Addison and Rissman 2012), is influenced by gonadal sex and the sex chromosome complement in the hypothalamus and can regulate food intake and changes in appetite (Bohlooly-Y et al. 2004; Chan et al. 1996; Quinnies et al. 2015). *Gh* is highly upregulated by E2 treatment in the MPOA and ARC regions of the mouse hypothalamus (Addison and Rissman 2012). We found a similar E2 upregulation of *Gh* in our whole brain RNA-Seq experiment (Chapter 3). Unexpectedly, there was no significant E2 main effect on the expression of *Gh*, but there was a significant Gly main effect on *Gh* expression at the 2hr Gly exposure time point in the hypothalamus (FDR-P = 0.009), with both Gly groups (Gly and E2+Gly)

upregulating *Gh* expression compared to non-Gly groups (Con and E2; Fig. 4.3b). As prior studies have shown upregulation of *Gh* post E2-treatment in very specific hypothalamic regions (MPOA and ARC) (Addison and Rissman 2012), the lack of a significant E2 main effect could possibly be due to the fact that we evaluated *Gh* expression in the hypothalamus as a whole. Further, *Gh* is endogenously produced in the hippocampus (Nyberg and Burman 1996; Sun et al.), is upregulated by E2 (Donahue et al. 2006) and plays a role in neuronal processes related to memory formation, mood and cognition in rats (Donahue et al. 2002; Donahue et al. 2006; Sonntag et al. 2005; Sytze van Dam and Aleman 2004). In the cortex, on the other hand, *Gh* is generally expressed in moderate levels (Addison and Rissman 2012), and E2 regulation of *Gh* in this brain region is not well established. We observed moderate to low levels of *Gh* expression in the hippocampus and cortex. Interestingly, just as in the hypothalamus, we did not see any significant E2 upregulation of *Gh* in the hippocampus or cortex. A previous study found increased *Gh* mRNA in the hippocampus 24 hours after two consecutive days of s.c. E2 injections (Donahue et al. 2006). Thus, the lack of an E2 main effect in the hippocampus in the current study could possibly be due to the fact that *Gh* expression was determined after 7 days (2hr, 24hr and 48hr Gly exposure animals) or 14 days (7 day Gly exposure animals) of exposure to E2. However, like in the hypothalamus, there was a significant Gly main effect on *Gh* expression at the 2hr time point, with an upregulation of *Gh* expression in both Gly groups compared to non-Gly groups in the hippocampus and cortex (FDR-P = 0.049; Fig. 4.3e and 4.3i). Similar to our whole brain experiments, *Gh* expression was almost undetectable in the Con mice, resulting in elimination of many Con replicates from the analysis, meaning that the detected increases in *Gh* expression in

the Gly group are underestimated. Also, the Gly main effect at 2hr post Gly exposure is mostly driven by the high levels of *Gh* observed in approximately half (2-3) of the 6 total mice in both the Gly and E2+Gly treatments in all three brain regions. Although E2 was reported to increase *Gh* expression in other studies, the addition of E2 is not driving this effect in the current study, as plasma E2 levels and *Gh* expression were not correlated and half the Gly-only treatment animals also showed the same increase.

The effects of E2 and/or Gly on *Prl* expression were similar to *Gh* in the current study. There was a significant Gly main effect on *Prl* expression at 2hr in the hypothalamus and cortex, similar to *Gh*, as well as at 24hr in the hypothalamus, with Gly groups (Gly and E2+Gly) upregulating *Prl* expression compared to non-Gly groups (Con and E2; Fig. 4.3c-d and 3f). Also, similar to *Gh*, the main effects of Gly on *Prl* expression must be interpreted carefully, due to undetectable *Prl* expression in most control mice (*i.e.*, those samples eliminated from analysis) and only half the Gly and E2+Gly treated animals driving the significant Gly main effect. *Prl* was of interest as it is expressed in several hypothalamic nuclei, is generally upregulated by E2 and is involved in neurogenesis, maternal behavior, food intake and stress (Marano and Ben-Jonathan 2014; Torner et al. 2004; Torner et al. 1999). Furthermore, *Prl* immunoreactive neurons are found in the rat cerebral cortex (DeVito 1988), with E2 having no effect on *Prl* mRNA or protein expression in the cortex of hypophysectomized rats (DeVito et al. 1992). In contrast to our whole brain RNA-Seq data, there was no significant main effect of E2 on *Prl* expression in the hypothalamus or hippocampus, with only a marginally significant E2 effect at the 24hr time point in the cortex (Fig. 4.3g). *Prl* expression was not affected by E2 or Gly in the hippocampus. These findings suggest that a single, acute dose of Gly

(2hr or 24hr) can affect the expression of genes including *Gh* and *Prl*, involved in neuronal processes that regulate food intake, stress and cognition, independent of an E2 effect. Thus, Gly may regulate these pathways, but via a mechanism that remains to be determined.

Effects of E2 and/or Gly on Nr4a1 expression

Lastly, we evaluated the expression of *Nr4a1*, an orphan nuclear receptor, which was of interest as it was the most highly upregulated gene by Gly compared to Con in our whole brain microarray study (Bamji et al. 2015). However, this Gly upregulation of *Nr4a1* was not observed in the RNA-Seq experiment. We were interested to determine Gly's brain-region specific effects on *Nr4a1* expression in the hypothalamus, hippocampus and cortex. *Nr4a1* is moderately expressed in various hypothalamic nuclei (Saucedo-Cardenas and Conneely 1996). *Nr4a1* is downregulated by E2 in the mouse hypothalamus (Lundholm et al. 2008) and plays a functional role in stress responses (Helbling et al. 2014), food intake (Chen et al. 2015), and cognition (McNulty et al. 2012). *Nr4a1* is also highly expressed in the hippocampus, with highest expression in the CA1 region (McNulty et al. 2012; Saucedo-Cardenas and Conneely 1996). *Nr4a1* plays a role in memory formation (Hawk and Abel 2011), specifically object location memory (McNulty et al. 2012). Thus, it was not surprising to observe relatively higher *Nr4a1* expression in the hippocampus, compared to the hypothalamus and cortex. As it is an immediate early-response gene and is upregulated 2 hrs after object location memory training in the CA1 region of the mouse hippocampus (McNulty et al. 2012), we expected that acute Gly (2hr, 24hr or 48hr), would increase *Nr4a1* expression, especially in the hippocampus. However, in contrast to the microarray study, we did not detect significant

upregulation of *Nr4a1* expression by Gly in any of the brain regions tested, nor did we detect E2 downregulation of *Nr4a1* in the hypothalamus.

In summary, we evaluated the effects of Gly on gene expression in 3 grossly dissected brain regions: the hypothalamus — central regulator of reproduction, food intake and stress; the hippocampus — involved in learning, memory consolidation and cognition; and the cortex — involved in movement, executive function, sensory perception and accurate recognition of visual and auditory stimuli in the mouse brain (Kandel et al. 2000). Moreover, we focused on expression of genes involved in reproduction (*Esr1*, *Gper1*, *Prl*), food intake (*Esr1*, *Gh*, *Prl*), stress (*Nr4a1*, *Gh* and *Prl*) and cognition (*Esr1*, *Gh* and *Nr4a1*) to gain a clearer understanding of Gly's effects in the female mouse brain. Our results suggest that Gly does not have brain-region specific effects on the expression of *Esr1*, *Gper1* or *Nr4a1* in the hypothalamus, hippocampus or cortex. On the other hand, Gly upregulates *Gh* expression in the hypothalamus, hippocampus and cortex as well as *Prl* expression in the hypothalamus and cortex. In contrast to prior studies, we did not see any E2 effect on *Gh* or *Prl* expression. It should be noted that the E2 effect on gene expression at the acute Gly exposure time points is a 7-day E2 effect (E2 replacements were done a week before tissue harvesting) compared to the 2h or 24h effects of a single Gly dose on *Gh* and *Prl* expression, which limits a direct comparison of E2 and Gly effects observed in the whole brain microarray (Bamji et al. 2015) and RNA-Seq (Chapter 3) experiments with the results of this study.. Therefore, we cannot rule out the possibility that the significant Gly main effects on *Gh* and *Prl* at the acute exposure times may be through rapid Gly action at either classical or genomic ERs (like *Esr1*) or through a membrane-associated ER (like *Gper1*), even though Gly by

itself did not regulate the expression of these ERs in the current study. Although the exact mechanism of Gly action on *Gh* and *Prl* expression remains unclear, this was an exciting finding, as Gly, through its effects on *Gh* and *Prl*, may regulate neuronal processes involved in food intake, stress and cognition in the female mouse brain. Future studies measuring expression of our select genes through the use of ER knockout experiments would help elucidate whether Gly's effect on expression of our genes of interest is through an ER-dependent or ER-independent mechanism in the female mouse brain. Furthermore, testing the effects of Gly on a neuronal cell culture model may also help determine

whether the effects observed in this study are due to direct effects at the neuron or through some indirect peripheral effects on gene expression. Given the interest in Gly's antitumor, antimicrobial and nutrient metabolism (specifically glucose and lipid) effects, understanding its effects in the central nervous system may be important in developing Gly's therapeutic potential.

Table 4.1. Brain region and Gly exposure time interaction effect and main effect P-values within each treatment group for *Esr1*, *Nr4a1*, *Gh* and *Prl* obtained using a generalized linear mixed model.

Gene Name	Treatment	Interaction effect P-value	Exposure time main effect P-value	Brain region main effect P-value
Esr1	Control	**<0.001	*0.043	**<0.001
	E2	0.066	*0.012	**<0.001
	Gly	**<0.001	**<0.001	**<0.001
	E2+Gly	**<0.001	*0.014	**<0.001
Nr4a1	Control	*0.006	*0.036	**<0.001
	E2	0.357	*0.002	**<0.001
	Gly	0.807	0.596	**<0.001
	E2+Gly	0.095	*0.046	**<0.001
Gh	Control	0.649	0.076	0.087
	E2	0.994	*0.008	0.304
	Gly	0.564	0.421	*0.040
	E2+Gly	0.700	*0.038	*0.044
Prl	Control	0.752	*0.007	**<0.001
	E2	0.971	*0.039	0.088
	Gly	0.315	0.592	*0.033
	E2+Gly	0.835	0.445	*0.002

Fig. 4.1. Average weekly body weight (g) (mean ± SEM) of animals exposed to a) a single acute dose of Gly (2hr, 24hr and 48hr) and b) 7 consecutive days (chronic dose) of Gly, measured one week before ovariectomy (B), at ovariectomy (O), one week after ovariectomy and beginning of Gly injections (I, chronic only) and at euthanasia (E), separated by treatment. Treatments are represented as follows: ❍ = Con, ● = E2, △ = Gly, ▲= E2 + Gly. Dotted lines connect the data points within each of the non-E2 groups (❍ and △) and solid lines connect the data points within each of the E2 groups (● and ▲).

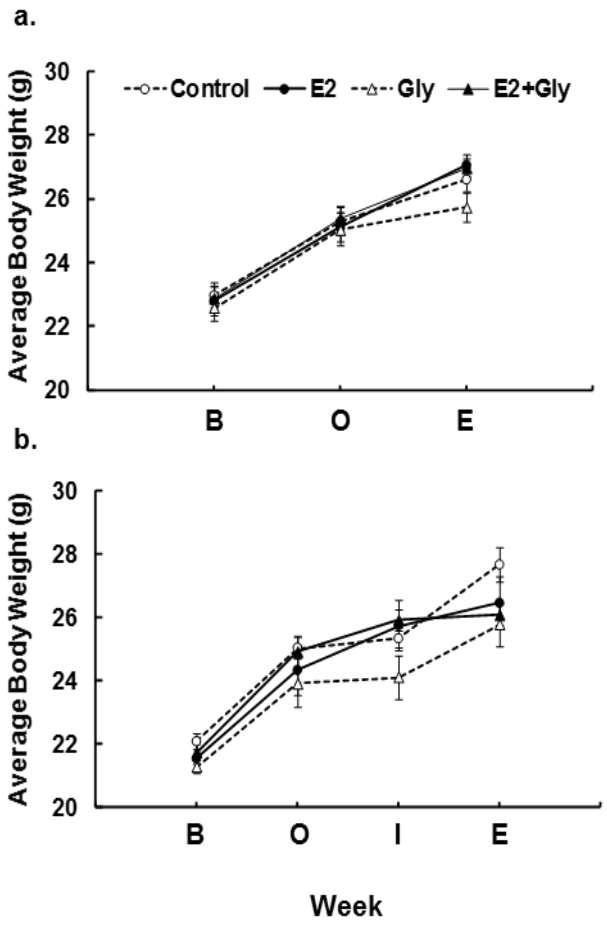


Fig. 4.2. Brain region and exposure time (hrs) interaction plots within each treatment, expressed as $\log_{10}$ initial target concentration (μM) (mean ± SEM). Rows (top to bottom) represent genes *Esr1*, *Nr4a1*, *Gh* and *Prl*. Columns (L-R) represent treatments: Control, E2, Gly and E2+Gly. Brain regions in each plot are represented as follows: hypothalamus (green), hippocampus (orange) and cortex (purple).

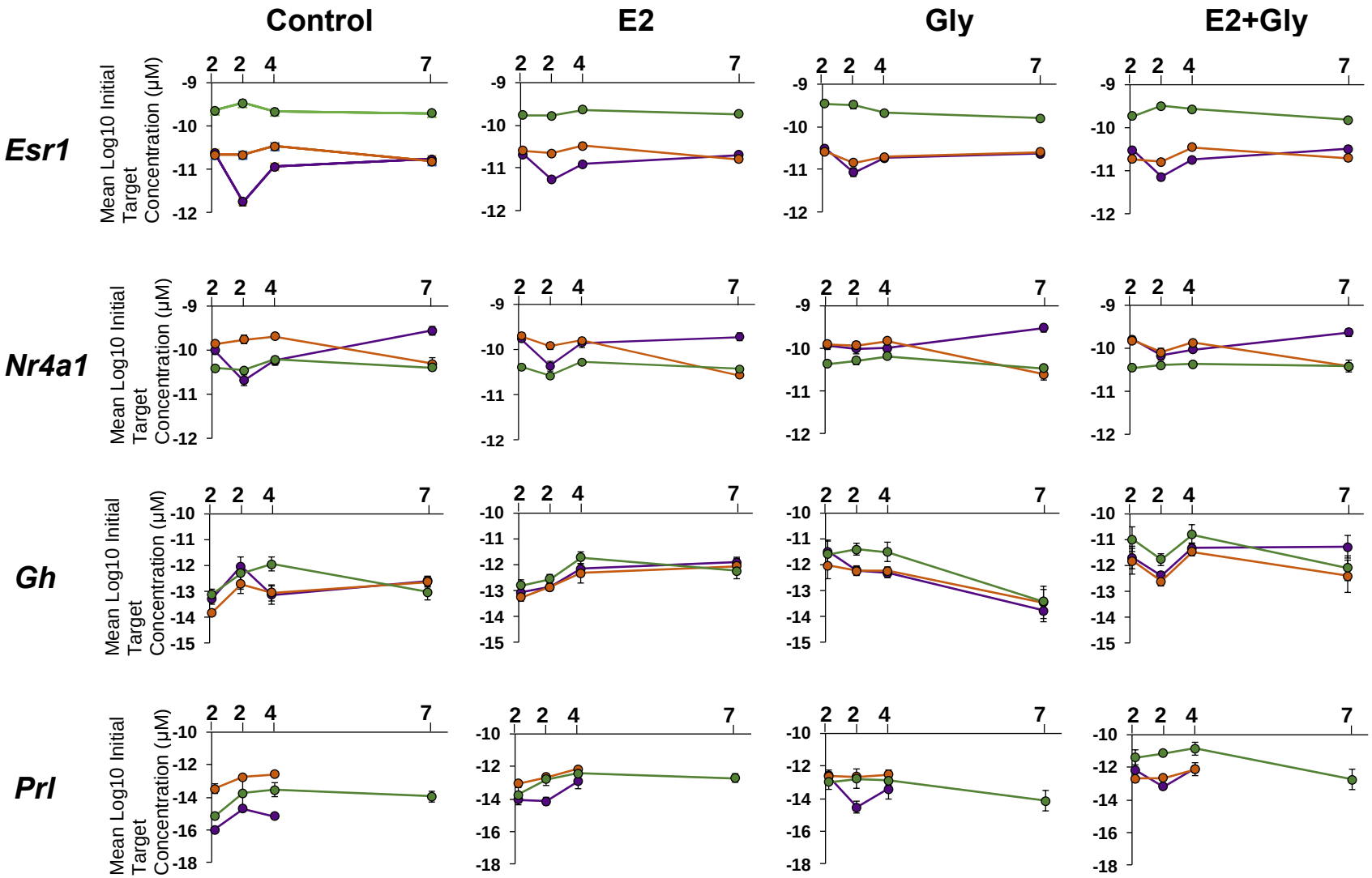
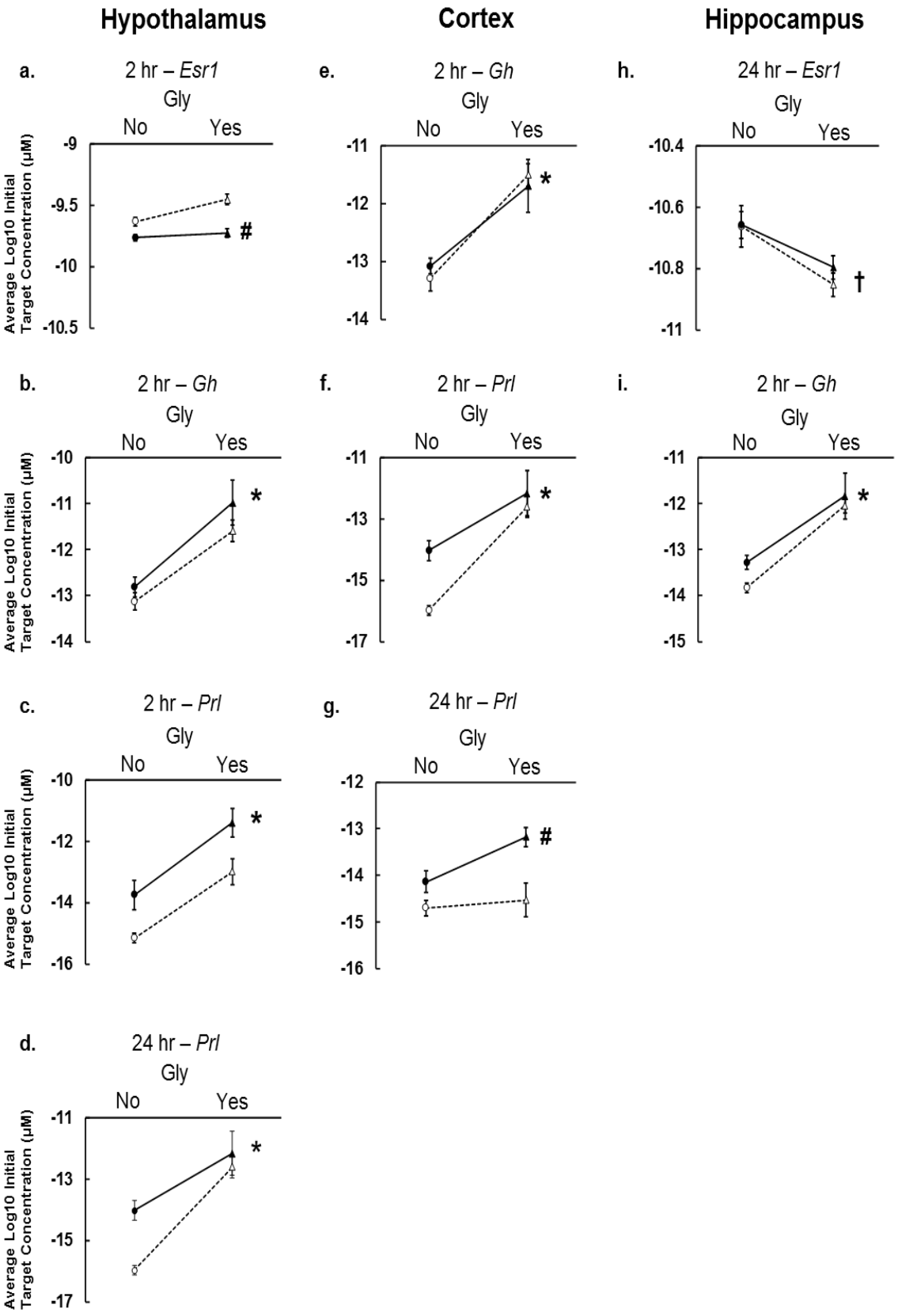

Control
E2
Gly
E2+Gly
Esr1
Nr4a1
Gh
Prl
Mean Log10 Initial Target Concentration (µM)
2 2 4 7
-9
-10
-11
-12
-13
-14
-15
-16
-18

Fig. 4.3. E2 and Gly treatment interaction plots showing $\log_{10}$ initial target concentration (μM) (mean ± SEM). Columns (L-R) represent brain regions: hypothalamus, cortex and hippocampus, respectively. * significant Gly main effects (FDR-P-value < 0.05); † marginally significant Gly main effect (FDR-P-value = 0.06); # marginally significant E2 main effect (FDR-P-value = 0.069). Treatments are represented as follows: ❍ = Con, ● = E2, △ = Gly, ▲= E2 + Gly. Left columns (circles) = no Gly; right columns (triangles) = Gly. Dotted lines connect the non-E2 groups (❍ and △) and solid lines connect the two E2 groups (● and ▲).


Hypothalamus
Cortex
Hippocampus
a.
2 hr – Esr1
b.
2 hr – Gh
c.
2 hr – Prl
d.
24 hr – Prl
e.
2 hr – Gh
f.
2 hr – Prl
g.
24 hr – Prl
h.
24 hr – Esr1
i.
2 hr – Gh
Gly
No
Yes
Average Log10 Initial Target Concentration (μM)
-9
-9.5
-10
-10.5
-10
-11
-12
-13
-14
-10
-11
-12
-13
-14
-10
-12
-14
-16
-11
-13
-15
-17
-11
-12
-13
-15
-17
-10.4
-10.6
-10.8
-11
-11
-12
-13
-14
-15
-12
-13
-14
-15
-16
*
#
†

Supplementary Table 4.1 Genes measured with qPCR and assay IDs for TaqMan primers (Life Technologies)

Gene Code	Gene Name	Gene ID (NCBI)	Assay ID
Esr1	Estrogen receptor 1	13982	Mm00433149_m1
Gper1	G-protein coupled estrogen receptor	76854	Mm02620446_s1
Nr4a1	Nuclear receptor, subfamily 4, group A, member 1	15370	Mm01300401_m1
Gh	Growth hormone	14599	Mm00433590_g1
Prl	Prolactin	19109	Mm00599950_m1

CHAPTER 5

NEUROENDOCRINE CHARACTERIZATION OF CELL LINES DERIVED FROM ADULT FEMALE MOUSE HYPOTHALAMUS

Introduction

The hypothalamus regulates reproductive function, growth, metabolism, stress responses and food intake. The arcuate nucleus (ARC) is a site for leptin signaling, which regulates body weight and food intake (Coppari et al. 2005), and both ARC and the anteroventral periventricular nucleus (AVPV) have *Kiss1*-expressing neurons forming an essential part of reproductive functioning (Clarkson et al. 2009). 17β-estradiol (E2) signaling in the hypothalamus, both through classical estrogen receptors ERs (ERα and ERβ) and membrane-associated ERs, including G protein-coupled estrogen receptor 1 (GPER1), is involved in regulating the transcription and release of gonadotrophin releasing hormone (GnRH) and luteinizing hormone (LH) (Petersen et al. 2003). E2 also regulates various neural pathways, such as those involved in sexual receptivity (Micevych and Dominguez 2009) and energy homeostasis (Bless et al. 2014).

Advances in medicine and molecular techniques have led to the discovery of several natural and synthetic compounds that act as selective estrogen receptor modulators (SERMs). SERMs exhibit neuroprotective effects by inducing neurite outgrowth (Nilsen et al. 1998) and preventing neuronal loss after kainic acid injury (Azcoitia et al. 2006) in the rodent brain through both genomic and non-genomic mechanisms (Dhandapani 2002; DonCarlos et al. 2009). Synthetic SERMs, *e.g.*, tamoxifen and raloxifene, and the

selective ER downregulator (SERD) fulvestrant (ICI 182,780), exhibit both antagonistic and agonistic activity at ERs and GPER1 in the rodent hypothalamus (Alfinito et al. 2008; Littleton-Kearney et al. 2002; McKenna et al. 1992; Patisaul et al. 2004). In addition to synthetic SERMs, naturally occurring SERMs, including coumestrol, genistein, and daidzein, act through both ERα and ERβ and regulate sexual behaviors and gonadotropin secretion in the hypothalamus (Whitten et al. 2002). However, *in vivo* studies fail to answer whether the effects of SERMs on gene expression are through direct action at the neuron, specifically in the hypothalamus, or through some indirect, systemic effects.

To explore the molecular mechanisms for effects of estrogens and SERMs in the hypothalamus, we tested five cell lines (mHypoA-50, 51, 55, 59, and 63) representative of mature neurons from the adult female mouse hypothalamus. All five cell lines exhibit gene expression patterns that are characteristic of the ARC or AVPV hypothalamic regions (Dhillon et al. 2011; Friedman 2013; Kim 2010; Treen et al. 2016). For more information on the cell lines, see Belsham et al., 2009 (Belsham et al. 2009).

For screening experiments, genes were selected based on their known brain functions and their response to E2. From prior studies, each of the aforementioned hypothalamic cell lines expresses both *Esr1* and *Esr2* (Dhillon et al. 2011; Friedman 2013; Kim 2010). Further, mHypoA-50 and 55 cells express *Gper1* (Friedman 2013). In addition, we examined other E2-regulated hypothalamic genes: growth hormone (*Gh*), prolactin (*Prl*), and kisspeptin (*Kiss1*), which are expressed in various regions of the hypothalamus (Addison and Rissman 2012; Aramburo et al. 2014; Brown et al. 2010; Gottsch et al. 2004; Grattan and Kokay 2008; Smith et al. 2005). *Gh* is involved in cognition and

memory (Donahue et al. 2006; Harvey 2010), and exhibits a sexually dimorphic pattern of gene expression in the medial preoptic area (MPOA) and ARC regions of the hypothalamus (Addison and Rissman 2012). *Prl* plays a role in neurogenesis, maternal behavior, food intake and stress (Torner et al. 2004; Yip et al. 2012). *Kiss1* is involved in the stimulation and release of GnRH, LH, and follicle-stimulating hormone (Gottsch et al. 2004; Kim 2010). *Kiss1* expression was reported in all cell lines tested in this study (Friedman 2013; Kim 2010), whereas this is the first examination of *Gh* and *Prl* expression in these cell lines. In addition, we examined the expression of *Nr4a1*, an orphan nuclear receptor, which is expressed in the hypothalamus, is regulated by E2 (Lundholm et al. 2008; Saucedo-Cardenas and Conneely 1996) and plays a functional role in stress responses (Helbling et al. 2014), food intake (Chen et al. 2015), and cognition (McNulty et al. 2012). None of these cell lines had been tested for *Nr4a1* gene expression. Therefore, a series of qPCR experiments were performed to a) confirm the presence of *Esr1*, *Esr2*, *Kiss1* in each cell line and *Gper1* in mHypoA-50 & 55; b) screen for presence of *Gper1* in mHypoA-51, 59 & 63 and *Gh*, *Prl*, and *Nr4a1* in all cell lines; and c) select one or more cell lines that express the genes of interest and respond to E2 treatment for use in future experiments to test estrogens and SERMs.

Materials and Methods

Cell line maintenance and treatments

Five adult female hypothalamic mouse cell lines (mHypoA-50, 51, 55, 59 & 63) were obtained from Cedarlane Labs (Burlington, Ontario, Canada). All cell lines were grown in monolayers in high-glucose DMEM (Sigma Aldrich, D5796), supplemented with 10%

fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained at 37°C with 5% CO_2. For examining E2 responses, all five cell lines were 'serum starved' for 16 h using phenol red-free DMEM supplemented with 10% charcoal stripped FBS + 1% penicillin/streptomycin. Time of serum starvation was optimized to examine E2 responses without affecting cell viability (data not shown). Cell lines were then treated with either dimethyl sulfoxide (DMSO, vehicle control) or 10nM 17β-estradiol (E2) dissolved in DMSO for 24 h.

RNA extraction and cDNA synthesis

RNA was extracted from cell lines using the miRCURY RNA isolation kit – Cell and Plant (Exiqon, 300110) and 800ng/µl of each extracted RNA sample was used to make cDNA using the High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, 4368814) according to manufacturer's instructions. Due to technical issues with both the DMSO and E2 treated mHypoA-50 cells, this cell line was excluded from both the screening and subsequent qPCR experiments. Data for mHypoA-50 in Table 5.1 is from a qPCR experiment conducted independently. All cDNA samples were diluted 1:3 before further use.

Quantitative Real-Time PCR (qPCR) & Data Analysis

Taqman primers were selected, using the manufacturer's recommendations, to avoid the detection of non-target sequences (See Supplementary Table 5.1). All qPCR reactions were 10 µl reactions and were run on ABI ViiA7 in 384 well plates. Reaction conditions were 50°C for 2 mins, 95°C for 10 min followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. qPCR reactions for the four cell line treatments (mHypoA-51, 55, 59 & 63) were run in triplicate within each experiment for each of the genes of interest: *Esr1,*

Esr2, *Gper1*, *Nr4a1*, *Gh*, *Prl*, and *Kiss1*, relative to control gene *Hmg20b*. Relative expression for each gene was then calculated using the delta-delta CT method, normalized to the delta Ct (target Ct – reference gene Ct) for DMSO-treated cells (Livak and Schmittgen 2001). Three additional qPCR experiments were run under the same conditions as above but only for *Esr1*, *Esr2*, *Gper1* and *Nr4a1* targets in mHypoA-55 and mHypoA-63. The mHypoA-55 and mHypoA-63 cells used in these experiments were from different passages (mHypoA-55: P30, P19, P20 and mHypoA-63: P31, P19, P18). There was no significant passage effect observed in the expression of the indicated genes as analyzed by two-factor ANOVA with 'E2' and 'passage' as factors, thus allowing the comparison of results obtained from different cell line passages. Data from these qPCR runs were analyzed by performing a student's t-test (DMSO vs. E2 treatments) for each gene in each of the two cell lines.

Results

Baseline expression of target genes

We confirmed the presence of both *Esr1* and *Esr2* in all five hypothalamic cell lines (Table 5.1, Fig. 5.1A-B). Similarly, we confirmed the expression of *Gper1* in mHypoA-50 and mHypoA-55 and for the first time report *Gper1* expression in mHypoA-51, mHypoA-59 and mHypoA-63 (Table 5.1, Fig. 5.1C). The expression of *Kiss1* in mHypoA-55, mHypoA-59 and mHypoA-63 was confirmed; however, we did not detect *Kiss1* mRNA expression in mHypoA-50 or mHypoA-51 (Table 5.1, Fig. 5.1D). We report the first examination of genes *Nr4a1*, *Gh* and *Prl* in all five hypothalamic cell lines (Table 5.1, Fig. 5.1E-G). *Nr4a1* was expressed in all five cell lines, with a moderate basal

level (without any treatment) of mRNA abundance (Table 5.1, Fig. 5.1E). *Gh* and *Prl*, on the other hand, had extremely low to undetectable levels of basal expression (Table 5.1, Fig. 5.1F-G); *Prl* was not detected in mHypo-55 or -63.

E2 regulation of genes of interest in cell lines

E2 increased *Esr1* expression in mHypoA-51 and mHypoA-63 cells and *Esr2* transcript expression in mHypoA-63 cells (Fig. 5.1A-B). In contrast to the increase in *Esr1*, E2 treatment resulted in a decrease in *Esr2* expression in mHypoA-51 cells (Fig. 5.1A-B). Unlike mHypoA-51 and mHypoA-63 cells, *Esr1* expression was slightly decreased in E2-treated mHypoA-59 cells. Treatment with E2 increased the expression of *Gper1* in mHypoA-55, mHypoA-59 and mHypoA-63, but not mHypoA-51 cells (Fig. 5.1C).

Further, E2 increased *Gh* expression in both mHypoA-59 and mHypoA-63 cells but not mHypoA-55 cells, with no detectable levels of *Gh* expression in E2-treated mHypoA-51 cells (Fig. 5.1F). With E2 treatment, a modest increase in *Prl* expression was observed in mHypoA-51 cells and a decrease in *Prl* expression was seen in mHypoA-59 cells. *Prl* expression was undetectable in both mHypoA-55 and mHypoA-63 cells with or without E2 treatment (Fig. 5.1G). E2 downregulated *Kiss1* gene expression in mHypoA-55 and mHypoA-59 cells, but increased *Kiss1* transcript expression in mHypoA-63 cells (Fig. 5.1D). *Kiss1* expression was undetectable in mHypoA-51 cells irrespective of E2 treatment.

In summary, all four cell lines were E2 responsive, with each cell line exhibiting a slightly different pattern of gene expression after 24 h of E2 treatment.

Identification of the most E2-responsive cell lines and reproducibility of PCR results

The mHypoA-55 and mHypoA-63 cells exhibited the most E2 responsivity for our genes of interest. Cell line mHypoA-55 showed the most E2 response of the cell lines examined, *i.e.*, a 23% decrease in *Nr4a1* (Fold Change/FC = 0.77) and a large increase in *Gper1* (FC = 9.08) expression following E2 treatment (Fig. 5.1E, Fig. 5.1C). Also, although the expression of *Prl*, *Kiss1,* and *Gh* was very low in this cell line, mHypoA-55 cells had the most consistent detection of *Gh* compared to any of the other cell lines (Fig. 5.1D, Fig. 5.1F-G).

Cell line mHypoA-63 exhibited an increase in expression of all ERs with E2 treatment (*Esr1*, FC = 1.59; *Esr2*, FC = 1.76; *Gper1*, FC = 2.76) when compared with DMSO (Fig. 5.1A-C). Further, mHypoA-63 had a relatively reliable detection of both *Gh* and *Kiss1* with an increase in both *Gh* and *Kiss1* expression, following E2 treatment (*Gh*, FC = 1.34; *Kiss1*, FC = 1.62; Fig. 5.1F, Fig.5.1D).

Due to the aforementioned E2 treatment responses of mHypoA-55 and mHypoA-63, we selected these two cell lines to confirm the reliability of our screening results with three additional qPCR experiments. Because of the very low to undetectable expression of *Gh*, *Prl*, and *Kiss1*, we followed up on gene expression of the ERs and *Nr4a1* (Fig. 5.2A-D).

Both mHypoA-55 and mHypoA-63 cells showed similar trends in gene expression following E2 treatment when compared to the previous qPCR screening results (Fig. 5.2A-D). However, the *Gper1* increase and *Nr4a1* decrease post E2 treatment detected in our initial screening experiment in mHypoA-55 cells were not statistically significant (*Gper1* and *Nr4a1*, $P = 0.7$; Fig. 5.1C-D). In the case of mHypoA-63 cells, there was no significant difference in *Esr1* ($P = 0.145$) or *Gper1* ($P = 0.413$) mRNA expression with

E2 treatment (Fig. 5.2A and 5.2D). However, the increase in expression of *Esr2* (P = 0.036; Fig. 5.2B) and decrease in *Nr4a1* expression (P = 0.002) in mHypoA-63 cells, after 24 h of E2 treatment, were statistically significant.

Discussion

The aims of this study were a) to screen five immortalized cell lines derived from adult female mouse hypothalamus for the expression of established genes of interest due to their regulation by E2 in the hypothalamus *in vivo*, b) examine the E2-responsiveness of these cell lines by measuring *Esr1*, *Esr2*, *Gper1*, *Kiss1*, *Nr4a1*, *Gh*, and *Prl* gene expression after E2 treatment, and c) select one or more cell lines for use in future studies to evaluate the mechanism of action of estrogens and SERMs in the ARC and AVPV of the mouse hypothalamus.

Earlier characterization of these cell lines had identified mHypoA-51, mHypoA-55, mHypoA-59 and mHypoA-63 to have an ARC phenotype and mHypoA-50 to have an AVPV phenotype (Dhillon et al. 2011; Friedman 2013; Kim 2010; Treen et al. 2016). Prior studies indicate that both classical ERs (*Esr1* and *Esr2*) are expressed in the hypothalamus with a relatively higher expression of *Esr1* as compared to *Esr2*, especially in the ARC (Kelly and Ronnekleiv 2015; Merchenthaler et al. 2004; Mitra et al. 2003). Our experiments confirmed the presence of both *Esr1* and *Esr2* in all cell lines and also indicate a relatively higher abundance of *Esr1* in all cell lines (Table 5.1, Fig. 5.1A-B). *Gper1* expression is high in the hypothalamus of the adult mouse brain, especially in the ARC, PVN, AVPV, periventricular hypothalamic nucleus and supraoptic nucleus (Hazell et al. 2009). *Gper1* is also known to be widely distributed in the hypothalamic-pituitary

axis of the rat (Brailoiu et al. 2007). *Gper1* expression was observed in all cell lines tested, with overall expression being relatively higher than *Esr2* but lower than *Esr1* in each cell line (Table 5.1, Fig. 5.1A-D). In summary, both classical ERs and GPER exhibited moderate to low levels of mRNA expression in these adult female hypothalamic cell lines.

Another important aspect of the study was the selection of hypothalamic cell lines that not only express ERs but also show a response to E2 treatment. E2 generally downregulates the expression of its receptors, *Esr1* and *Esr2*, *in vivo* in the rodent brain, although these effects may vary by brain region, timing, sex, and species (Lauber et al. 1991; Nomura et al. 2003; Patisaul et al. 1999; Simerly and Young 1991). E2 decreased expression of *Esr1* in mHypoA-59 and decreased *Esr2* expression in mHypoA-51 in the initial screening experiments (Fig. 5.1A-B). In contrast to a previous report (Friedman 2013), no increase in *Esr1* or *Esr2* expression was detected in mHypoA-55 with 24 h of E2 treatment. The difference in results may be due to the fact that in our experiments the cell lines were 'serum starved' for 16 h prior to E2 treatment to minimize the impact of estrogens in FBS affecting gene expression. In contrast, the previous study used only 4 h serum starvation prior to E2 treatment (Friedman 2013). However, we did observe an increase in *Esr1* mRNA in mHypoA-51 and mHypoA-63 cells and an increase in *Esr2* expression in mHypoA-63 cells after E2 treatment. Importantly, these E2 responses were most noticeable in mHypoA-55 and mHypoA-63 cells, with mHypoA-63 cells increasing the expression of all ERs and mHypoA-55 cells showing a large increase in *Gper1* transcript abundance with E2 treatment in the screening experiment. This is especially relevant to future experiments with SERMs to evaluate if artificially produced or

naturally occurring estrogens, like E2, regulate the expression of ERs in the ARC of the hypothalamus.

In addition to ERs, of special interest was the finding that these mouse hypothalamic cell lines express the orphan nuclear receptor, *Nr4a1*. *Nr4a1* is induced in the hypothalamus in response to stress (Helbling et al. 2014), and it plays a role in leptin's central control of food intake (Chen et al. 2015) and in learning and memory (McNulty et al. 2012). This is the first report demonstrating *Nr4a1* expression in a murine hypothalamic cell line. We report here that all five mHypoA cell lines exhibit moderate expression of *Nr4a1*, similar to previously published *in vivo* studies for mouse hypothalamus (Saucedo-Cardenas and Conneely 1996). Thus, these cell lines will be a valuable tool for *in vitro* mechanistic studies to identify pathways regulating *Nr4a1* expression.

An earlier study found that E2 treatment downregulates the expression of *Nr4a1* in the mouse hypothalamus (Lundholm et al. 2008) which, therefore, was the anticipated result for our hypothalamic cell lines. E2 suppressed *Nr4a1* expression in both mHypoA-55 and mHypoA-63 cell lines (Fig. 5.2D), with a statistically significant suppression in mHypoA-63 ($P = 0.002$). Both mHypoA-55 and mHypoA-63 not only express *Nr4a1* but also show a response to E2 treatment, which provides reasonable confidence that these lines are appropriate for future studies investigating the role of synthetic and naturally occurring SERMs on *Nr4a1* gene expression in female mouse hypothalamic neurons.

Lastly, the presence of gene targets *Gh*, *Prl*, and *Kiss1* was also evaluated in all cell lines. *Gh* and *Prl* mRNAs were of interest as they are both synthesized locally in the rodent brain in several hypothalamic nuclei, such as the MPOA and ARC, and also co-

localize with *Esr1* in these neurons (Addison and Rissman 2012; Aramburo et al. 2014; Brown et al. 2010; Grattan and Kokay 2008; Marano and Ben-Jonathan 2014). *Kiss1* is highly expressed in mouse hypothalamic nuclei, especially the ARC, AVPV, and PVN, and regulates the stimulation and release of GnRH in these regions (Friedman 2013; Gottsch et al. 2004; Smith et al. 2005). Also, one study indicated a downregulation of *Kiss1* expression in mHypoA-51 and mHypoA-63 cells after a 4 h E2 treatment, with the decrease less pronounced or lost 24 h post E2 treatment (Kim 2010). A more recent study, however, found an increase in *Kiss1* expression in mHypoA-51 cells, after a 24 h E2 treatment (Mittelman-Smith et al. 2015). We detected low to no expression of *Kiss1*, *Gh*, and *Prl* in the five cell lines. Previous studies describing *Kiss1* expression in mHypoA-51 and mHypoA-63 used an input RNA concentration that was 2.5 times higher than that used in our experiments to prepare cDNA for qPCR experiments (Friedman 2013; Kim 2010; Mittelman-Smith et al. 2015), providing a possible explanation for discrepancy among studies regarding detection of *Kiss1*. Our results indicate an extremely low basal mRNA expression of *Kiss1*, *Gh*, and *Prl* in the lines tested.

In summary, five hypothalamic cell lines derived from adult female mouse hypothalamus were screened and evaluated for the relative abundance of the transcripts of five genes of interest and their response to E2 treatment. We report the first examination of *Nr4a1*, *Gh*, and *Prl* mRNA in these cell lines and our results suggest that mHypoA-55 and mHypoA-63 cells may be valuable for future experiments with E2 and SERMs. However, further studies comparing the gene expression profiles of these cell lines to specific hypothalamic neuronal populations using RNA sequencing techniques

may provide a deeper insight into the suitability of these cell lines for studying E2 and SERM responses.

Table 5.1

Average Ct values of target gene mRNA in each hypothalamic cell line after 16h cell starvation and 24 h DMSO.

Cell Line	*Esr1*	*Esr2*	*Gper1*	*Kiss1*	*Nr4a1*	*Gh*	*Prl*
mHypoA-50[a]	30.0	33.8	32.9	-	23.3	35.0	34.9
mHypoA-51	29.0	32.6	31.5	-	25.0	36.9[b]	39.1
mHypoA-55	28.4	32.5	31.0	34.6[b]	24.6	35.7	-
mHypoA-59	27.7	34.2	31.1	35.6[b]	25.1	36.9[b]	37.8
mHypoA-63	31.2	34.5	29.6	35.6[b]	25.3	37.3[b]	-

[a] data for mHypoA-50 from a separate experiment (see methods); [b] undetermined Ct value for some replicates; '-' = undetermined Ct value for all replicates

Fig. 5.1 Relative mRNA expression of target genes A) *Esr1*, B*) Esr2*, C) *Gper1*, D) *Kiss1*, E) *Nr4a1*, F) *Gh*, and G) *Prl* after 24 h 10nM E2 treatment (black bars) compared to control treatment (DMSO, white bars) in 4 hypothalamic cell lines, (L-R): 51 - mHypoA-51; 55 – mHypoA-55; 59 – mHypoA-59 and 63 – mHypoA-63. Missing bars indicate undetectable levels of mRNA, *i.e*., Ct > 39.5.

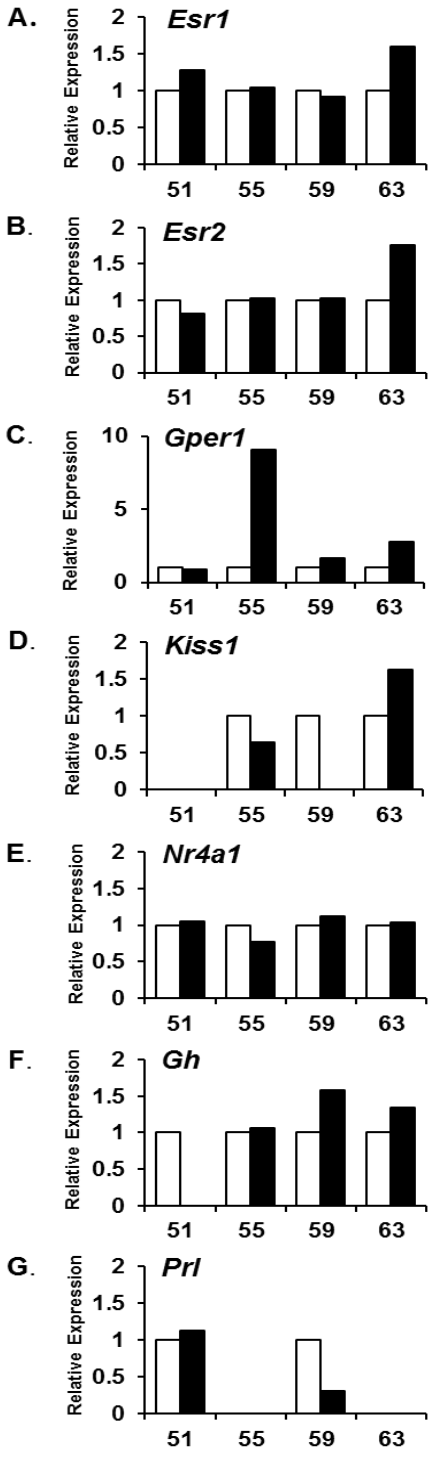
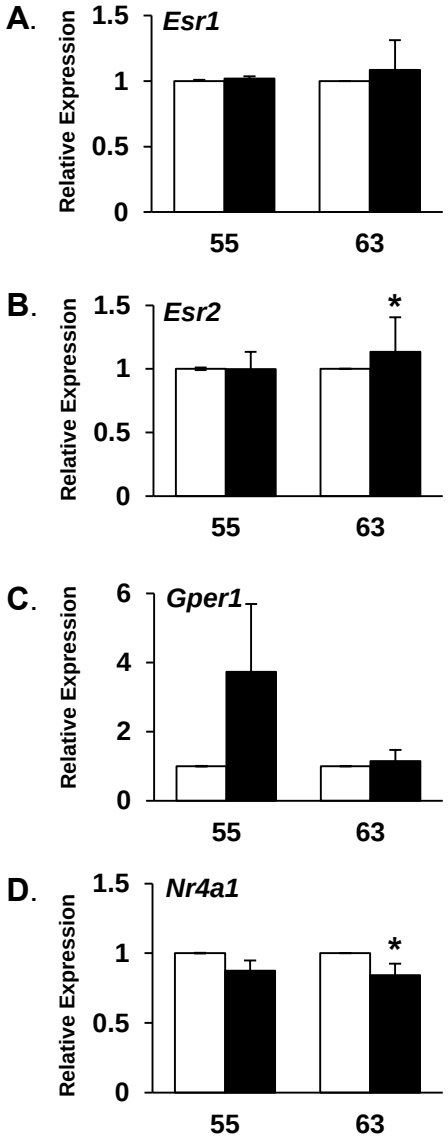


Fig. 5.2 Relative mRNA expression of target genes in cell lines mHypoA-55 & mHypoA-63. A) *Esr1,* B) *Esr2*, C) *Gper1*, D) *Nr4a1* after 24 h treatment with DMSO (white bars) or 10nM E2 (black bars) in 2 hypothalamic cell lines, (L-R): 55 – mHypoA-55; 63 – mHypoA-63. Three independent experiments represented (mean +/- S.E.M.). * represents a statistically significant difference between DMSO and E2 treatment groups (t-test, $P < 0.05$).



Supplementary Table 5.1 Genes measured with qPCR and assay IDs for TaqMan primers (Life Technologies)

Gene Code	Gene Name	Gene ID (NCBI)	Assay ID
Esr1	Estrogen receptor 1	13982	Mm00433149_m1
Esr2	Estrogen receptor 2	13983	Mm00599821_m1
Gper1	G-protein coupled estrogen receptor	76854	Mm02620446_s1
Kiss1	Kisspeptin	280287	Mm03058560_m1
Nr4a1	Nuclear receptor, subfamily 4, group A, member 1	15370	Mm01300401_m1
Gh	Growth hormone	14599	Mm00433590_g1
Prl	Prolactin	19109	Mm00599950_m1

CHAPTER 6

SUMMARY AND FUTURE CONSIDERATIONS

General Summary

The main goals of the experiments discussed in this dissertation were to answer the following questions:

1) Do glyceollins (Glys) oppose the 17-β estradiol (E2) effects on gene expression in the whole brain, *i.e.,* do Glys act anti-estrogenically in the female mouse brain?
2) Are there any brain-region specific effects of Gly on gene expression, specifically in the hypothalamus, hippocampus and cortex?
3) Are there any differences between acute vs. chronic Gly dosing on gene expression in the hypothalamus, hippocampus and cortex?
4) Can we use immortalized neuronal cell lines derived from adult female mice to evaluate if Gly affects gene expression through direct action at the neuron?

The experiments described in this dissertation are a first attempt at evaluating the effects of Gly in the female mouse brain. We focused our experiments on gene expression as a means of understanding Gly's overall effects, if any, on brain-related pathways and processes. As initial research on Gly was focused on its anti-estrogenic effects, suppressing estrogen-responsive tumors both *in vitro* in cancer cell lines and *in vivo* in xenograft mouse tumor models (Burow et al. 2001; Salvo et al. 2006), our initial

hypothesis was that Gly would oppose the E2 effect on gene expression in the brain. To test this hypothesis, we first performed a whole brain microarray analysis to determine the effects of E2 alone, Gly alone and in combination (E2+Gly) on gene expression in the female mouse brain (Chapter 2). The results from the microarray experiment provided some evidence for Gly opposition of the effects of E2 on expression of a few genes but interestingly also showed E2 opposition of Gly effects on gene expression, with Gly exhibiting a gene expression pattern distinct from both E2 groups (Bamji et al. 2015). Moreover, Gly by itself significantly upregulated the expression of genes involved in basic cell division, immune responses and neurogenesis while downregulating the expression of a gene that promotes neurodegeneration. Thus, this experiment provided the first indication of Gly's potential neuroprotective effects in the female mouse brain.

As estrogen receptors (ERs), growth hormone (*Gh*) and prolactin (*Prl*) have a moderate to low basal expression in the brain, our microarray experiment failed to detect differences between treatments on the expression of these genes of interest. Previous research showed that the strongest anti-estrogenic effect of Gly (which includes Gly isomers I, II and III), was exhibited by the Gly I isomer, through action at estrogen receptor (ER) alpha (ERα) (Zimmermann et al. 2010). We were interested in evaluating Gly's effects on expression of *Gh* and *Prl*, as they are expressed throughout the brain, regulated by E2 and are often found to be co-expressed with ERα, especially in hypothalamic neurons (Addison and Rissman 2012; Brown et al. 2010; Donahue et al. 2006; Furigo et al. 2014). Further, *Gh* and *Prl* regulate important physiological responses. *Gh* plays a role in food intake, memory formation, learning and mood (Abba et al. 2005; Bohlooly-Y et al. 2004; Donahue et al. 2006; Nyberg and Burman 1996; Quinnies et al.

2015; Sun et al.) and *Prl* regulates neurogenesis, maternal behavior, food intake and stress (Marano and Ben-Jonathan 2014; Torner et al. 2004). Therefore, our next experiment, described in Chapter 3, was an RNA-sequencing (RNA-Seq) analysis which not only gave us a broader picture of Gly's gene expression pattern in relation to E2, but also enabled the detection of differential expression of *Gh* and *Prl*. The RNA-Seq data confirmed some of the results of our microarray experiment, with Gly and E2 regulating similar genes/pathways in both experiments and no significant E2 or Gly effects on ER expression. As we examined a much larger set of transcripts in the RNA-Seq experiment, we also found evidence for Gly regulation of genes that was similar to E2 (for example, if E2 upregulated a gene, Gly also upregulated the gene, but the magnitude of the effect may differ), which we did not observe in the microarray experiment. Overall, the results from both Chapters 2 and 3 provided evidence that Gly may not act as a pure anti-estrogen in the female mouse brain, but may act as a selective estrogen-receptor modulator (SERM) and ER-independent effects on gene expression, as well.

Although the whole brain studies provided useful information, they did not take into account the heterogeneity of the brain. Distinct brain regions have highly specific functions and can differ greatly in terms of gene expression. Thus, in Chapter 4, using quantitative real-time polymerase chain reaction (qPCR), we evaluated brain region-specific effects of Gly on expression of select gene targets (*Esr1*, *Gper1* (hypothalamus only), *Nr4a1*, *Gh* and *Prl*) in three brain regions — the hypothalamus, hippocampus and cortex. In addition, as the whole brain experiments involved an 11-day chronic treatment with Gly, we tested the effects of acute (single dose of Glys followed by tissue harvest at 2, 24 and 48 hr) and chronic (multiple doses of Gly for 7 consecutive days) Glys

exposure on gene expression. Our results showed that neither an acute nor a chronic dose of Gly had any effect on the expression of ERs or *Nr4a1* in any of the brain regions tested. The lack of a Gly effect on ER expression was consistent in all experiments conducted in this dissertation. This is not totally surprising as ER expression varies between different brain regions, and within each brain region (Merchenthaler et al. 2004). Thus, our use of grossly dissected whole hypothalamic, hippocampal and cortical brain regions may be the reason for the lack of an observed Gly effect on ERs. *Nr4a1*, on the other hand was highly upregulated by Gly, but not E2, compared to control, whereas E2 suppressed that Gly upregulation in the E2+Gly treatment in the microarray study. However, no Gly upregulation of *Nr4a1* was observed in the RNA-Seq or in the qPCR experiments in Chapter 4. Moreover, we observed an effect of acute Gly dosing (2hr and 24hr) on the expression of *Gh* in all three brain regions and *Prl* in the hypothalamus and cortex. Both Gly treatments (Gly and E2+Gly) upregulated the expression of *Gh* and *Prl* compared to non-Gly treatments (control and E2). Therefore, the results from Chapter 4 indicate that a single, acute dose of Gly can potentially influence the regulation of feeding, neurogenesis, stress and cognition, through its action on *Gh* and *Prl* expression.

Lastly, all the experiments conducted in Chapters 2-4 involved a peripheral administration of Gly, through intraperitoneal (i.p.) injections. We were interested in determining an *in vitro* neuronal system to test if the observed Gly effects on gene expression in our previous experiments were through direct action at the neuron or through some indirect peripheral mechanism of action. To examine this question, we tested five immortalized adult hypothalamic cell lines (mHypoA-50, -51, -55, -59 and -63) for the expression of our genes of interest (ERs, *Nr4a1*, *Gh* and *Prl*) and also

evaluated their E2-responsiveness in order to select a line(s) that may be useful in studying the action of estrogens and SERMs like Gly in the female hypothalamus (Chapter 5). Based on relatively reliable expression of our genes of interest and E2-responsivity, we were able to select two lines (mHypoA-55 and -63) which may be useful in future experiments to evaluate direct Gly effects at the neuron on gene expression.

Biomedical significance

The brain-related health benefits of estrogens in women are well known, with decreasing levels of estrogen in postmenopausal women being associated with cognitive decline. Hormone replacement therapy (HRT, estrogen + progesterone) and estrogen replacement therapy (ERT), prior to the release of the WHI report in 2003 (Chlebowski et al. 2003), were increasingly popular means to alleviate cognitive decline and were shown to improve working memory (Duff and Hampson 2000) and alleviate symptoms of depression (Whooley et al. 2000) in postmenopausal women. However, HRT also increased the risk of estrogen-responsive breast, ovarian and uterine tumors, leading to an interest in soy plant products genistein and daidzein, estrogenic compounds potentially possessing the neuroprotective effects of estrogens while reducing the risk for estrogen-responsive tumors. Indeed, both genistein and daidzein exhibit several neuroprotective effects (Liu et al. 2008; Malinowska et al. 2010; Pan et al. 2012; Wang et al. 2014), but also induce neurotoxicity in primary neuronal cultures at high concentrations (Jin et al. 2007). Additionally, genistein and daidzein were found to possess some anti-tumor effects (Barnes 1995; Zhang et al. 2016), but also acted estrogenically in the periphery on reproductive tissues, *e.g.*, the uterus and mammary gland (Gaete et al. 2012; Santell et al. 1997) and increased uterine cell proliferation and breast tumor proliferation *in vitro*,

limiting their use as ERT alternatives. Thus, gaining knowledge of the effects of these compounds in the brain, if any, is vital to fully understand the health benefits of these soy compounds. Gly possesses multiple anti-tumor, anti-microbial, and antioxidant properties in addition to its ability to regulate glucose and lipid metabolism (Chapter 1). Research on Gly gained momentum due to the fact that, in addition to all the health-promoting benefits of soy, unlike genistein and daidzein, Gly acted as an anti-estrogen in the periphery (Salvo et al. 2006). To determine Gly's potential as not only an anticancer therapeutic but as a HRT dietary supplement, it is important to evaluate its effects in the brain. The experiments conducted in this dissertation are the first of their kind to determine Gly's actions in the female mouse brain, with hypothesis-generating microarray and RNA-Seq analyses that can serve as a foundation for future studies.

Future Considerations for Gly effects in the mouse brain

Our data indicate that Gly may act through both ER-mediated and potentially non ER-mediated mechanisms; but this finding needs to be empirically tested. Initial studies testing the effects of soy isoflavone supplements (containing genistein and daidzein) reported that the soy supplement acted anti-estrogenically by affecting both ERα and ERβ in the rat hypothalamus. The soy supplement suppressed the ERα-mediated, E2-dependent upregulation of oxytocin receptor expression in the ventromedial nucleus of the hypothalamus (Patisaul et al. 2001) and increased ERβ mRNA expression in the paraventricular hypothalamic nucleus, unlike E2 which decreased ERβ expression (Patisaul et al. 2001). However, in a subsequent study, the same group found that genistein alone had no effect on ERα- mediated oxytocin receptor expression, but unlike E2, increased the expression of ERβ mRNA in the paraventricular hypothalamic nucleus

(Patisaul et al. 2002). The results from these two studies highlight the need for evaluating the effects of individual components in soy isoflavonoid supplements. Given the known functions mediated by ERs in the brain, it is necessary to understand if Gly affects gene expression through ERs, if so, which ERs may be involved and if it translates to effects on cognitive and reproductive behaviors. Hence, future research on Gly should focus on using selective ER knockout models to determine whether the effects of Glys on gene expression are ER-mediated and if so, which ERs are involved in Gly's mechanism of action. Also, as mentioned in Chapter 1, most studies used a mixture of Gly isomers (Gly I, II and III) and different Gly isomers can have estrogenic or SERM-like effects at different ERs (van de Schans et al. 2016). Thus research focused on the independent effects of individual Gly isomers on gene expression in the brain, instead of using a Gly mixture, may be useful in the development of disease-specific therapeutic interventions. Two immortalized adult hypothalamic cell lines selected in Chapter 5 (mHypoA-55 and mHypoA-63) can be used to evaluate the direct vs. indirect effects of Gly on gene expression in the brain. Other neuronal cell lines including immortalized adult hippocampal cell lines have also recently become commercially available and can be used for similar studies. Importantly, as mentioned earlier, compounds like genistein and daidzein eventually become neurotoxic at higher concentrations, so evaluating a dose-response curve for Gly on neuronal action would be useful in determining an appropriate dose of Gly in food supplements. Lastly, when we initially started working with Gly, we noticed that animals that were injected with Gly for just a few days were difficult to handle, jumpy and seemingly anxious. Our open-field anxiety trials (not included in this dissertation) did not detect any differences between treatments, but there was an

indication of possible defensive aggression towards the mouse handler. In order to develop Gly as a nutritional supplement, it will be key to evaluate its effects on various reproductive, cognitive as well as mood-related behaviors such as anxiety and aggression. Researchers must test various Gly doses on gene expression and behavior in animals, determine the underlying molecular mechanisms of Gly action through *in vivo* and *in vitro* studies, and perform appropriate studies in other animal models and ultimately in human subjects to realize the potential of Gly as a therapeutic agent for metabolic and adjuvant chemotherapeutic use.

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APPENDIX

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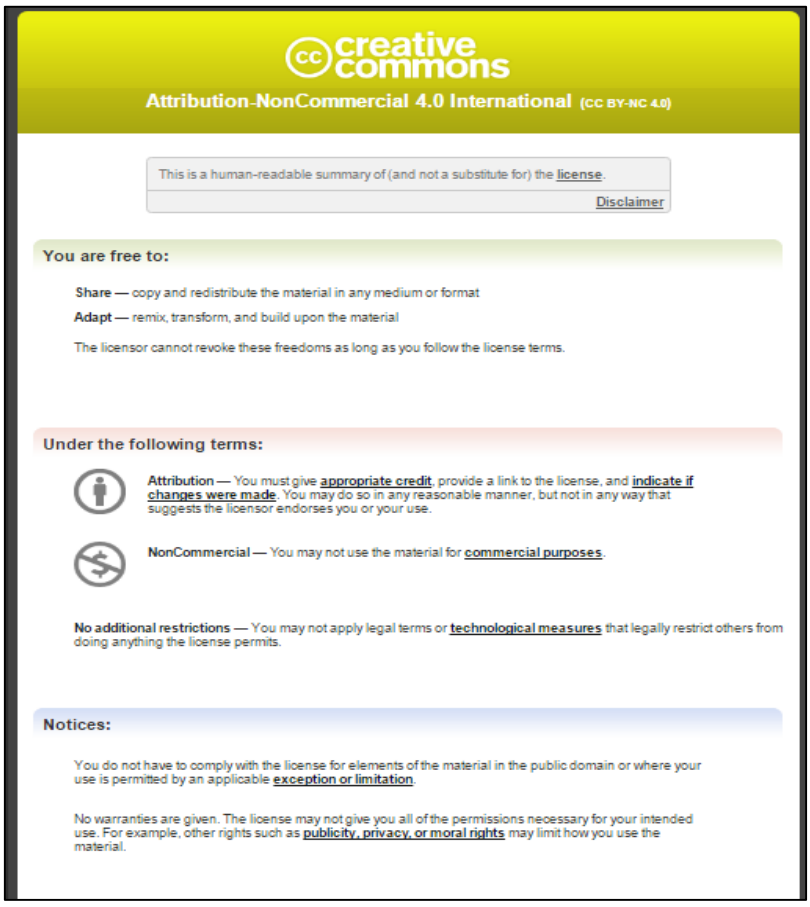

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Education

Ph.D.	University of Louisville, Department of Biology (expected 8/2016)	August 2010 – Present
M.Sc.	University of Pune (India), Department of Zoology Graduated with Distinction	July 2007 – May 2009
B.Sc.	University of Pune (India), Nowrosjee Wadia College, Department of Zoology Graduated with Distinction	May 2007

Employment

Graduate Teaching Assistant, Biology 331 (Genetics & Molecular Biology Laboratory), Department of Biology, University of Louisville, KY	August 2010 - Present

Research Experience

University of Louisville, Department of Biology, Louisville, KY November 2011 - Present
Ph.D. Student
Advisor: Dr. Cynthia Corbitt
Project Title: Effects of soy glyceollins on gene expression *in vivo* in the female mouse brain and *in vitro* in adult female mouse hypothalamic neurons.

University of Louisville, Department of Biology, Louisville, KY August 2010 – October 2011
Ph.D. Student
Advisor: Dr. David Reed (deceased)
Project Title: Founder contributions, heterozygosity, growth rate and heritability in an inbred population of alligator snapping turtles (*Macrochelys temmincki*).

University of Pune, Department of Zoology, Pune, India June 2008 – May 2009
Master's Student
Advisor: Dr.Kalpana Pai
Project Title: Ecological study of physicochemical properties and their effects on Rotifera populations at the Botanical Garden Tank, Zonal Agricultural Research Station in Pune.

Peer-Reviewed Publications

Bamji SF, Page RB, Patel D, Sanders A, Alvarez, AR, Gambrell C, Naik K, Raghavan AM, Burow ME, Boue SM, Klinge CM, Ivanova M, Corbitt C (2015) Soy glyceollins regulate transcript abundance in the female mouse brain, *Functional and Integrative Genomics*: 15(5): 549-561. PubMed PMID: 25953511; doi: 10.1007/s10142-015-0442-3.

Awards and Honors

Graduate Student Publication Award, University of Louisville, Department of Biology	April 2016
Bill Furnish Teaching Award, University of Louisville, Department of Biology	April 2015
Second Place – Graduate Oral Presentation, Kentucky Academy of Science 99th Annual Meeting, Morehead State University, Morehead, KY	November 2013

Presentations

Poster Presentation	"Neuroendocrine characterization of cell lines derived from adult female mouse hypothalamus" Society for Neuroscience Annual Meeting, Chicago, IL	October 2015

Poster Presentation	"Neuroendocrine characterization of cell lines derived from adult female mouse hypothalamus" Neuroscience Day, University of Louisville, KY	April 2015
Oral Presentation	"Effect of soy glyceollins on transcription in the female mouse brain" Bioinformatics Journal Club, University of Louisville, KY	December 2013
Oral Presentation	"Effect of soy glyceollins on transcription in the female mouse brain" Kentucky Academy of Science 99th Annual Meeting, Morehead State University, Morehead, KY	November 2013
Poster Presentation	"Effects of Glyceollins on Whole Transcript Expression in the Mouse Brain" Society for Neuroscience Annual Meeting, New Orleans	October 2012
Poster Presentation	"Effects of Glyceollins on Whole Transcript Expression in the Mouse Brain" Neuroscience Day, University of Louisville, KY	April 2012

Other Conferences Attended

Animal Behavior Conference, Indiana University, Bloomington, Indiana	April 2016
14th Annual UT-KBRIN Bioinformatics Summit, Paris Landing State Park, Buchanan, TN	March 2015
Anatomical and Functional Modularity of the Cerebral Cortex, Neuroscience Symposium, Louisville, KY	August 2013
Neuroscience Day, University of Louisville, Louisville, KY	April 2013
Animal Behavior Conference, Indiana University, Bloomington, Indiana	March 2013
UT- ORNL – KBRIN Bioinformatics Summit, Louisville, KY	March 2012
National Symposium on "Recent Trends in Modern Biology", Department of Zoology, University of Pune	March 2009
1st International Stem Cell Summit, Indian Institute of Technology, Madras, India	November 2008
National Symposium on "Recent Trends in Modern Biology", Department of Zoology, University of Pune	March 2008

Memberships

Society for Neuroscience member	April 2012- Present

Kentucky Academy of Science member	August 2010 – Present

Professional and Community Service

Brain Awareness Week, Louisville Chapter of Society for Neuroscience	March 11, 2014
Brain Awareness Week for Bullitt County High School, Louisville Chapter of Society for Neuroscience	May 24, 2012
Brain Awareness Week, Louisville Chapter of Society for Neuroscience	March 19, 2012