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Soy Glyceollins Regulate Transcript Abundance in the Female Mouse Brain

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Abstract

Glyceollins (Gly), 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, Gly exhibits mechanisms of action not involving estrogen receptor (ER) signaling. To date, effects of Gly on gene expression in the brain are unknown. For this study, we implanted 17- β estradiol (E2) or placebo slow-release pellets into ovariectomized CFW mice followed by 11 days of exposure to Gly or vehicle i.p. injections. We then performed a microarray on total RNA extracted from whole brain hemispheres and identified differentially expressed genes (DEGs) by a 2×2 factorial ANOVA with an FDR = 0.20. In total, we identified 33 DEGs with a significant E2 main effect, 5 DEGs with a significant Gly main effect, 74 DEGs with significant Gly and E2 main effects (but no significant interaction term), and 167 DEGs with significant interaction terms. Clustering across all DEGs revealed that transcript abundances were similar between the E2+Gly and E2-only treatments. However, gene expression after Gly-only treatment was distinct from both of these treatments and was generally characterized by higher transcript abundance. Collectively, our results suggest that whether Gly acts in the brain through ER-dependent or ER-independent mechanisms depends on the target gene.

Keywords

anti-cancer therapeutic; estrogen; functional genomics; glyceollins; phytoalexins

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Conflicts of Interest

The authors have no conflicts of interest to report.

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. 1). Among the most exciting findings to date are results which indicate that Gly opposes the activity of 17 β -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 ER α (Zimmermann et al. 2010). However, other studies concluded that Gly can also exhibit weak estrogenic activity via ER β , 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 myotubes *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 ER α (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 & 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 RNAlater (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 2×2 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 vs. A plots of replicate GeneChips, and pseudo-images of the $\log_2(\text{intensity})$ 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 ($\log_2(\text{RMA}) = 5.953$). 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 2×2 factorial ANOVA of the form $\log_2(\text{RMA})_{ijk} = \beta_0 + G_i + E_j + (GE)_{ij} + \varepsilon_{ijk}$ 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, Online Resource 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 50°C for 2min, 95°C for 10min, then 40 cycles of 95°C for 15sec and 60°C 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 (R_n) 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 2×2 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. 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. 2b; raw values, mean (pg/ml) $\pm$ SEM: Con, 4.13 ± 1.13 ; Gly, 3.17 ± 0.003 ; E2, 265.65 ± 55.65 ; E2+Gly, 76.05 ± 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. 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 Online Resource 2: [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 to 4 (see Online Resources 3–6 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 (Online Resource 3), but no interaction effect, 21 genes were upregulated in the E2 groups (*i.e.*, E2 and E2+Gly) (Online Resource 2a) when compared to the non-E2 groups (*i.e.*, Con and Gly). Those with known brain-related functions are described in Table 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 (Online Resource 2b), 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 (Online Resource 2c–d, Online Resource 4), 4 have known brain-related functions (Table 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*, Online Resource 2d) 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 3). These 74 genes fell into 4 different patterns of expression (Online Resource 2e–h, Online Resource 5) 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, Online Resource 2e). 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, Online Resource 2f). 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 (Online Resource 4–5).
- c. Upregulated in Gly groups compared to non-Gly groups and also upregulated in E2 groups compared to non-E2 groups (10 DEGs, Online Resource 2g). 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, Online Resource 2h). 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 (Online Resource 6). Twenty-one of these have known brain-related functions (Table 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 (Online Resource 2i).

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’ (*Ifna*, *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 (Online Resource 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 (Online Resource 2i). 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. 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 *Apa2*) 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 intragroup variability prevented the identification of *Prl* as a DEG.

Average plasma E2 levels in the E2+Gly group (76.05 ± 21.15 pg/ml) were only about 30% that of the E2 treatment group (265.65 ± 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 4, Online Resource 6), 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. 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 *Tnfrsf13b*) 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). Interestingly, *Ppia*, which is downregulated in Gly groups (Tables 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 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. Interaction plots showing **a)** body mass (mean $\pm$ SEM, ANOVA $p = 0.36$) **b)** natural log transformed plasma E2 levels (mean $\pm$ SEM, ANOVA $p < 0.0001$, E2 $p < 0.0001$, Gly $p = 0.0315$, E2 $\times$ 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. 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. 4.

Bar plots of the microarray (grey bars) and RT-qPCR (black bars) data for the eight genes (*Cdh12*, *Fcrls*, *Gh*, *Prl*, *Nr4a1*, *Ngp*, *Tph2*, *Slc6a4*) whose expression levels were technically replicated. Log₂(RMA) values from the microarray experiment and log₂(R) values from the RT-qPCR experiment were centered and scaled to facilitate side-by-side comparisons.

Table 1

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

Gene Symbol	Gene Name	Function	P-value	FC ^a	Ref
<i>ApoA2</i>	apolipoprotein A2	neurotransmitter release	0.0002	1.14	Kirov et al, 2008
<i>Calm</i>	Calmodulin	development & maintenance of neuronal function	0.0003	1.27	Takaishi et al, 2003
<i>Dgcr8</i>	Dicer homolog 8	maintenance of short & long term synaptic plasticity	0.0002	1.13	Fenelon et al, 2013
<i>Spon1</i>	Spondin-1	dendritic & axonal growth - hippocampus	0.0002	1.11	Feinstein et al, 1999; Ho et al, 2004

^aFC = fold change (E2 + E2+Gly)/(Con + Gly)

Table 2

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

Gene Symbol	Gene Name	Function	P-value	FC ^a	References
<i>Hmgn1</i>	hemicentin 1	cell division & development of tissue architecture	0.0007	1.24	Xu et al, 2013
<i>miR-495</i>	microRNA 495	targets BDNF involved in synaptic plasticity & neurogenesis	0.0006	1.75	Wu et al, 2010
<i>Ppia</i>	peptidylprolyl isomerase A	apoptosis; promotes neurodegeneration	0.0003	0.91	Nigro et al, 2013
<i>Tnfrsf13b</i>	tumor necrosis factor (ligand) superfamily, member 13b	innate & adaptive immune response in the CNS	0.0006	1.12	Farina et al, 2007

^aFC = fold change (Gly + E2+Gly)/(Con + E2)

Table 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							
<i>Atp12a</i>	ATPase, H ⁺ /K ⁺ transporting, nongastric, alpha polypeptide	mitochondrial dysfunction	0.0204	1.16	0.0004	0.76	Zhang et al, 2010
<i>Dgkz</i>	diacylglycerol kinase zeta	dendritic spine maintenance; neurite outgrowth	0.0247	1.11	0.0011	0.84	Seo et al, 2012; Yakubchuk et al, 2005
<i>Ifitm5</i>	interferon induced transmembrane protein 5	enriched in GABAergic neurons	0.0030	1.15	0.0031	0.88	Hardt et al, 2008
<i>mir-211</i>	microRNA 211	antiproliferative effects on glioma cells	0.0048	1.12	0.0005	0.83	Asuthkar et al, 2012
<i>mir-30d</i>	microRNA 30d	expressed in aging mouse brains	0.0044	1.09	0.0002	0.86	Li et al, 2011; Eda et al, 2011
<i>mir-503</i>	microRNA 503	promotes apoptosis of dendritic cells	0.0022	1.19	0.0004	0.79	Min et al, 2013
Gly down, E2 up^a							
<i>Ppap2b</i>	phosphatidic acid phosphatase type 2B	neuronal differentiation precursor	8.78E ⁻⁰⁶	0.85	0.0056	1.05	Sanchez-Sanchez, 2012
<i>Ppia</i>	peptidylprolyl isomerase A	apoptosis; promotes neurodegeneration	1.63E ⁻⁰⁵	0.92	0.0103	1.02	Nigro et al, 2013
<i>Vdac3</i>	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							
<i>Lrx1</i>	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							
<i>Prokl</i>	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 4

Brain-related DEGs with significant 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
<i>Alkbh3</i>	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
<i>Alkbh5</i>	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
<i>Bcs1l</i>	BCSI-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
<i>Coro7</i>	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
<i>Derl2</i>	Derl-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
<i>Grfa3</i>	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
<i>Igsf1</i>	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
<i>Kif6</i>	kinesin family member 6	neuronal migration	0.0012	1.18	1.06	0.98	0.83	0.93	0.89	Falnikar et al, 2013
<i>Manf</i>	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
<i>miR-210</i>	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
<i>miR-23a</i>	microRNA 23a	oligodendrocyte differentiation & myelin synthesis	0.0035	1.16	0.93	0.96	0.82	1.03	0.80	Lin et al, 2013; Smimova et al, 2005
<i>Nlr5</i>	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
<i>Nme1</i>	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
<i>Nr4a1</i>	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
<i>Ryr1</i>	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
<i>Smarc1l</i>	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
<i>Spg1l</i>	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
<i>Svop</i>	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

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Gene Symbol	Gene Name	Function	Int P-value ^a	FC GvC	FC EvC	FC EGvC	FC EGvG	FC EGvE	FC EvG	References
<i>Usp22</i>	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
<i>Wnt7b</i>	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
<i>Zbtb7a</i>	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

^aInt P-value = E2 x Gly interaction term P-value; FC = fold change