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Aging alters the expression of genes for neuroprotection and synaptic function following acute estradiol treatment

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Abstract

This study used microarray analysis to examine age-related changes in gene expression 6 and 12 hr following a single estradiol injection in ovariectomized mice. Estradiol-responsive gene expression at the 6 hr time point was reduced in aged (18 mo) animals compared to young (4 mo) and middle-aged (MA, 12 mo) mice. Examination of gene clustering within biological and functional pathways indicated that young and MA mice exhibited increased expression of genes for cellular components of the synapse and decreased expression of genes related to oxidative phosphorylation and mitochondrial dysfunction. At the 12 hr time point, estradiol-responsive gene expression increased in aged animals and decreased in young and MA mice compared to the 6 hr time point. Gene clustering analysis indicated that aged mice exhibited increased expression of genes for signaling pathways that are rapidly influenced by estradiol. The age differences in gene expression for rapid signaling pathways may relate to disparity in basal pathway activity and estradiol mediated activation of rapid signaling cascades.

Keywords

Estrogen; Hippocampus; Microarray; Signaling

Introduction

In humans, age-related impairments in hippocampal-dependent memory begin in middle-age and cognitive weakening continues with advancing age (Foster, 2006; Small et al., 1999). Estrogen treatment in women (Sherwin, 2006), nonhuman primates (Lacreuse et al., 2002; Rapp et al., 2003), and rodents (Aenlle et al., 2009; Foster et al., 2003; Markham et al., 2002) has been shown to protect against cognitive decline. However, it is becoming apparent that estradiol treatment initiated late in life is less effective (Adams et al., 2001; Daniel et al., 2006; Foster et al., 2003; Sherwin and Henry, 2008).

The mechanism for differential estradiol effects across the lifespan is unclear. In younger animals, estradiol has numerous effects on the hippocampus that could provide a mechanism for improved cognition. For example, estradiol can rapidly activate signaling pathways for neuroprotection (Guerra et al., 2004; Jover-Mengual et al., 2007; Kuroki et al., 2001; Sarkar et al., 2008; Wu et al., 2005) and synaptogenesis (Akama and McEwen, 2003; Mukai et al., 2007) and estradiol effects on neuroprotection and synaptogenesis may be impaired in aged animals (Adams et al., 2001; Brinton, 2008; Miranda et al., 1999; Yildirim et al., 2008) suggesting a possible breakdown in estrogen signaling.

To determine whether the age differences in synaptogenesis and neuroprotection result from a weakening of the signaling pathways, we investigated differences in gene expression following an acute estradiol treatment. *In vitro* (Carroll et al., 2006; Schnoes et al., 2008) and *in vivo* studies (Fertuck et al., 2003; Naciff et al., 2007; Pechenino and Frick, 2009) have provided evidence for distinct temporal patterns of estrogen-mediated gene expression. In general, genes related to the regulation of transcription are altered within the first 2 hrs of treatment. Protein changes associated with this early transcription contribute to the amplification in the number of altered genes occurring between 4-12 hr. Furthermore, this second wave of altered genes expression, between 4-12 hr, includes genes related to the functional effects of estrogen treatment for specific cell systems. Therefore, 17 β -estradiol was injected in ovariectomized mice and estradiol-responsive genes were identified by transcript profiling at 6 and 12 hr after treatment. Pathway analysis of estradiol-responsive genes identified age-related differences in functional pathways related to oxidative phosphorylation, synaptic plasticity, and estrogen responsive signaling cascades.

Materials and Methods

Subjects

Procedures involving animal subjects have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida and were in accordance with guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Initially 85 female C57/BL6 mice were obtained from National Institute of Aging for gene array analysis, with one gene chip per animal. However, quality controls for gene arrays indicate that 5 chips were outliers and the data for these animals was removed from further analysis. Therefore, a total of 80 female mice (young: n = 26, 4 months; middle-aged: n = 26, 12 months; aged: n = 28, 18 months) were employed in this study. Animals were housed 3-5 per cage and maintained on 12:12 light:dark cycle (lights on at 6 am). Following one-week habituation, mice were anesthetized (2 mg ketamine and 0.2 mg xylazine per 20 gm of body weight) and ovaries were removed through a small midline incision on the abdomen. All mice received ad lib access to food (Purina mouse chow, St Louis, MO) and water, until the surgery when they were placed on Casein based chow (Cincinnati Lab Supply, Cincinnati, OH), which is low in phytoestrogens found in soy based chow.

Hormone administration

Briefly, a single injection of 17 β -estradiol (Sigma Chemical Co, St Louis, MO) or mineral oil was initiated 10 days after ovariectomy (OVX) at 10 pm or 4 am. To control for time of day effects, all animal were sacrificed between 10 - 11 am, ~4 hr after lights on and either 6 hrs (injection at 4 am) or 12 hrs (injection at 10 pm) following the injection of estradiol or oil. Estradiol was dissolved in light mineral oil (Fisher Scientific, Pittsburgh, PA) to concentration of 0.1 mg/ml. Oil or estradiol (5 μ g) in oil was injected subcutaneously at the nape of the neck in volumes of 0.05 ml. The groups included: young receiving oil and sacrificed 6 hr (n = 5) and 12 hr (n = 3) later; young receiving estradiol and sacrificed 6 hr (n = 10) and 12 hr (n = 8) later; middle-aged receiving oil and sacrificed 6 hr (n = 5) and 12 hr (n = 6) later; middle-aged receiving estradiol and sacrificed 6 hr (n = 9) and 12 hr (n = 6) later; aged receiving oil and sacrificed 6 hr (n = 5) and 12 hr (n = 7) later; aged receiving estradiol and sacrificed 6 hr (n = 9) and 12 hr (n = 7) later. To determine effectiveness of estradiol treatment, uteri were excised at the time of sacrifice and weighed immediately. An analysis of variance (ANOVA) was used to compare main effects on uterine weight.

At the time of sacrifice, each animal was anesthetized with CO₂ and decapitated. The brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid. Both

hippocampus were removed, frozen in liquid nitrogen, and stored at -80°C . RNA was isolated from each sample using Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, MD). RNA concentration was determined using spectrophotometer and a subset of samples was examined using Agilent 2100 Bioanalyzer (Santa Clara, CA). Microarray analysis was performed for individual animals (one chip per animal).

Microarray Hybridization and signal detection

An amount of $5\mu\text{g}$ of total RNA was synthesized to cRNA using Affymetrix amplification kit following the manufacture's protocol. Hybridization of cRNA was carried out by the Interdisciplinary Center for Biotechnology Research Microarray Core, University of Florida. Hybridization of Affymetrix Mouse 430 2.0 Arrays occurred for 17 hours at 60°C in accordance with manufacture's instructions and arrays were scanned using an Affymetrix Microarray scanner. Images were analyzed using Affymetrix Gene Chip Operating System software (GCOS version 1.1) and scaled to 500. Hybridization signal intensities between GeneChips were normalized using dChip's (Li and Wong, 2001) model-based expression index with the PM-only model. The model was used to set thresholds for identify outlier probe sets. Arrays with a large number of outlier probe sets ($> 5\%$ of total) were removed from further analysis. Data was then transferred into Microsoft excel for further analysis. Probe sets were annotated using Affymetrix® NetAffx™ (12/2007).

Real Time-PCR

Real time PCR (RT-PCR) was performed to verify microarray results. RNA from each group was treated with Turbo DNase (Ambion, Austin TX) to remove any remaining genomic DNA. RNA was then converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Primers and probes for WDFY1, GABRA2, NNT, PPARGC1A, AFT4, ENTPD4 and GAPDH were purchased from Applied Biosystems. Briefly, $3\mu\text{g}$ of total RNA from a single animal was incubated with appropriate reagents at 25°C for 10 min and then heated to 37°C for 120 min using 7300 Fast Real-Time PCR System (Applied Biosystems). For relative quantification of RNA, 100ng in $2.5\mu\text{l}$ of cDNA was added to $12.5\mu\text{l}$ of Taqman® Universal PCR Master Mix (2X), $1.25\mu\text{l}$ of 20X Gene Expression Assay Mix, a probe specific primer mixture (Table 1), and $8.75\mu\text{l}$ of nuclease-free water for a total volume of $25\mu\text{l}$. Thermal cycler conditions were set at 2 min at 50°C , 10 min at 95°C and cycles 15s at 95°C and 1 min at 60°C for 40 cycles. The point at which the fluorescence crosses the threshold (C_t) was determined using 7300 Real-Time PCR System and SDS Software 1.3.1 analysis software (Applied Biosystems). Each sample was in triplicate and normalized to corresponding GAPDH values ($\Delta C_{t\text{sample}}$) and then compared to normalized young oil ($\Delta C_{t\text{reference}}$). The mean normalized values were compared using $\Delta\Delta C_t$ method as described by Applied Biosystems to derive fold change (Aenlle et al., 2009), where $\Delta\Delta C_t(\Delta C_{t\text{sample}})(\Delta C_{t\text{reference}})$.

Statistical analysis

Probe set filtering and initial statistical analysis was performed according to our previously published work (Aenlle et al., 2009; Blalock et al., 2003). Briefly, the number of present calls for each probe was determined across all chips and the probed was removed if fewer than 80% of the chips exhibited a present call for the probe. For all studies, differential expression was determined using two-tailed t -tests with the alpha level set at 0.025 in accordance with our previous studies (Aenlle et al., 2009; Blalock et al., 2003). The probes sets that exhibited an increase or decrease in expression following treatment were submitted to Ingenuity Pathway Analysis's (IPA; Ingenuity Systems). With alpha set at $p<0.025$ we were able to obtain >800 molecules for generating networks, in accordance with IPA best practices for pathway analysis. The IPA program uses a right-tailed Fisher's Exact Test to compute the likelihood that the relationship between the list of submitted genes and a set of

genes representing a given pathway is due to chance. A similar procedure was employed for determining overrepresentation of genes related to synaptic structure using the Expression Analysis Systematic Explorer (EASE) through the NIHDAVID Bioinformatics Resources (Hosack et al., 2003).

Results

For each age group, all animals treated with oil (young = 8, MA = 11, aged = 12) were used as controls to determine effects of treatment for age matched animals sacrificed 6 hr (young = 10, MA = 9, aged = 9) or 12 hr (young = 8, MA = 6, aged = 7) after a single estradiol injection. To determine the effectiveness of the estradiol treatment uterine weight was compared across groups (young oil 20 ± 5 mg; young estradiol 6 hr 54 ± 3 mg; young estradiol 12 hr 50 ± 3 mg; middle-aged oil 30 ± 6 mg; middle-aged estradiol 6 hr 52 ± 3 mg; middle-aged estradiol 12 hr 43 ± 10 mg; aged oil 34 ± 4 mg; aged estradiol 6 hr 56 ± 3 mg; aged estradiol 12 hr 56 ± 4 mg). An ANOVA indicated an overall treatment effect ($p < 0.0001$) in the absence of an age differences and post hoc FLSD tests indicated a significant increase in uterine weight at 6 hr ($p < 0.0001$) and 12 hr ($p < 0.0001$) following treatment relative to oil treated controls.

Age differences in estradiol-responsive genes for synaptogenesis, and neuroprotection 6 hr after treatment

Figure 1 illustrates the number of estradiol-responsive probes for the 6 and 12 hr time points. At the 6 hr time point the MA mice exhibited the greatest shift in gene expression with approximately twice as many probes exhibiting altered expression relative to young animals and approximately a ten fold increase in the number of altered probes relative to aged mice. Age-related differences in the pattern of estradiol-responsive gene expression were also apparent. For probes that were observed to change expression at 6 hrs, young and MA mice exhibited increased expression for ~60% of the probes, while the majority (64%) of estradiol-responsive probes were decreased in aged animals. A few probes were altered in the same direction across the different age groups; however, in some cases estradiol effects were in the opposite direction (Fig 1B).

To examine markers of synaptic components, estradiol-responsive genes were grouped according to age and whether the genes increased or decreased expression. The gene groups were submitted to DAVID Bioinformatics Resources to determine overrepresentation of genes related to the gene ontology classification for synapse cellular components (GO: 0045202). The results indicate that estradiol treatment was associated with increased expression of synaptic genes only for young (17 genes, $p < 0.0005$) and MA animals (26 genes, $p < 0.00005$) at the 6 hr time point. Four of the genes (ENAH, GRIA4, PJA2, GRIP1, GRIA1) were increased in both age groups (Table 2). A significant clustering was not observed for synaptic component genes that decreased expression (young: 4 genes; MA: 6 genes). Furthermore, aged animals did not exhibit altered expression, increasing or decreasing, for genes related to synaptic components.

Estrogen responsive genes were submitted to IPA to determine whether expression changes were associated with gene-enrichment for signaling pathways. Table 3 shows the pathways that exhibited significant ($p < 0.01$) overrepresentation. For genes that increased expression at the 6 hr time point, only young animals exhibited overrepresentation in specific signaling pathways including PPAR/RAR signaling, which has been linked to neuroprotection (Martin et al., 2006; Rosa et al., 2008; Sanguino et al., 2006; Santos et al., 2005). Interestingly, while significant gene enrichment was not observed for the PPAR/RAR pathway in MA mice, three of the six genes that increased in MA mice were common for the young group (CLOCK, GNAQ, NCOR1). For genes that decreased expression at the 6 hr time point,

young and MA animals exhibited clustering of genes for oxidative phosphorylation and mitochondrial dysfunction (Table 4), with two genes NDUFV1 and NDUFV2 decreased in both age groups.

Age differences in estradiol-responsive genes 12 hr after treatment

The number of probes influenced by treatment decreased from the 6 to 12 hr time points for young and MA mice. In contrast, aged animals exhibited approximately a five fold increase in the number of estradiol-responsive probes at 12 hr relative to the 6 hr time point (Fig 1A). Most of the probes (67%) for aged animals exhibited decreased expression at 12 hr. When estradiol-responsive genes were compared across age groups, common probes were usually altered in the opposite direction in aged animals compared to the other two groups (Fig 1C) and include a number of genes involved in the regulation of transcription (Table 5).

To examine overrepresentation in functional categories, the list of significantly altered genes was submitted to DAVID Bioinformatics Resources for examination of synaptic components. For all age groups, overrepresentation of synaptic component genes was not observed. Data were then submitted to IPA for determination of over representation in functional pathways ($p < 0.01$). No significant clustering was observed for genes that exhibited decreased expression, regardless of age group (Table 6). In the case of increased expression, only aged animals exhibited gene enrichment which was largely focused on signaling pathways that are rapidly influenced by estrogen including α -adrenergic signaling (Aydin et al., 2008; Bowman et al., 2002; Favitt et al., 1991; Heikkinen et al., 2002), Ca^{2+} signaling (Brewer et al., 2006; Foster, 2005; Zhao and Brinton, 2007), synaptic plasticity (Cordoba Montoya and Carrer, 1997; Smith and McMahon, 2005; Warren et al., 1995), and IGF-1 signaling (Azcoitia et al., 1999; Donahue et al., 2006; Perez-Martin et al., 2003). Several of the genes interact with multiple signaling pathways (Table 7). Finally, the PPAR pathway was increased at 6 hr in young and 12 hr in aged mice; however, only one gene, CHUK, was common for young 6 hr and aged 12 hr groups.

The increase in the number of altered genes at 12 hr for aged animals suggests that gene changes observed in younger mice may have been delayed in older animals. To examine this possibility we employed the gene expression data for 6 hr in young and MA mice and compared it to gene expression in aged mice at 6 and 12 hr to determine the number of genes that changed in the same direction. As illustrated in Fig 1B for age mice, the number of genes that changes in the same direction at 6 hr was 13 compared to young and 18 compared to MA. When we used the gene expression from age mice at 12 hr, we expected to observe an increase of ~4 fold, since the number of genes for the aged group increase from 198 to 940. However, relative to the young 6 hr group we saw a small increase from 13 to 19 and relative to MA animals the number of genes decreased from 18 to 4.

Four genes that increased (WDFY1, GABRA2, NNT, PPARGC1) and two that decreased (ATF4, ENTPD4) in young mice treated with estradiol were selected for validation of microarray results using RT-PCR. These genes were selected because they exhibited altered expression in the same direction at 6 hr and 12 hr after treatment at $p < 0.025$, except for PPARGC1 which was increased at 12 hr for $p < 0.05$. RNA isolated from oil treated mice ($n = 3$) was used as the control to calculate the fold change for mice ($n = 3$) treated 6 hr earlier with estradiol. Similarly, the fold change was calculated for values on the microarray for young oil and estradiol (6 hr) treated mice. Figure 2 illustrates that the direction and extent of altered transcription was similar for the microarray and RT-PCR.

Discussion

Age differences in estradiol-responsive gene signatures 6 hr after treatment

The current study examined altered gene expression in the hippocampus following estradiol treatment over the course of aging. The results reveal that aged animals were less responsive to estradiol treatment examined 6 hr after an acute treatment. In young and MA animals, estradiol treatment reduced expression for genes involved in oxidative phosphorylation and mitochondrial dysfunction. The decreased expression may represent feedback regulation due estradiol effects on oxidative phosphorylation. Altered oxidative phosphorylation is a major outcome of estradiol treatment in young animals and recent work indicates that in the brain, estradiol can enhance mitochondrial efficiency and decrease oxidative stress (Irwin et al., 2008; Massart et al., 2002; Nilsen et al., 2007; Stirone et al., 2005; Zheng and Ramirez, 1999). It is unclear whether estradiol influences oxidative phosphorylation to the same extent in aged animals. This point is important since previous research indicates that regulation of mitochondrial function and oxidative phosphorylation may constitute a cornerstone for estrogen's neuroprotective effects (Simpkins and Dykens, 2008). Thus it will be important for future studies to determine whether acute estradiol effects on oxidative phosphorylation are reduced with advanced age.

Age differences in genes that increased expression were also apparent. Estradiol treatment is associated with an increase in dendritic spines in the hippocampus of young adult rats (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992; Woolley and McEwen, 1993; Woolley et al., 1996), and this process is impaired in older rats (Adams et al., 2001; Miranda et al., 1999; Yildirim et al., 2008). We observed that young and MA, but not aged mice, exhibited an increase in expression of genes related to the synapse 6 hr after acute estradiol treatment. Young mice exhibited an increase in genes related to PPAR signaling. Although, MA did not exhibit a significant number of genes in this pathway, for the 6 genes that increased, 3 were common to young and MA animals. Aged animals exhibited increased expression of genes related to the PPAR pathway at 12 hr post treatment suggesting that the interaction of estrogen and PPAR signaling is maintained in advanced age. The ability of estrogen to increase gene expression of this pathway may be important for hippocampal aging since PPAR signaling has been implicated in the progression of Alzheimer's disease (Dupuy et al., 2001) and neuroprotection from inflammation (Kapadia et al., 2008; Vegeto et al., 2008). Furthermore, age and sex specific changes have been noted for hippocampal PPAR signaling (Sanguino et al., 2006), suggesting that older females may be at greater risk.

Age differences in estradiol-responsive gene signatures 12 hr after treatment

In contrast to young and MA mice, which exhibited a decline in the number of altered gene between 6 and 12 hr, the number of genes with altered expression increased during this time in aged animals. For aged mice at the 12 hr time point after an acute injection, gene expression increased in signaling pathways that are rapidly influenced by estradiol including; Ca^{2+} signaling (Brewer et al., 2006; Foster, 2005; Zhao and Brinton, 2007), cAMP signaling (Gu and Moss, 1996), IGF-1 signaling (Azcoitia et al., 1999), and synaptic plasticity (Foy et al., 2008; Sharrow et al., 2002). The rapid activation of these pathways by estrogen is due to membrane interactions and not the result of classic transcriptional regulation. However, there is some indication for a reciprocal interaction between rapid membrane effects of estrogen on Ca^{2+} , G-protein coupled receptor, and trophic factor signaling and estrogenic modulation of genes in these pathways (Foster, 2005).

Mechanisms for age-related differences in estradiol-responsive gene signatures

In the case of increased expression of genes for rapid signaling cascades, it may be important that these same signaling cascades decline during aging (Foster, 2005). Thus, aged cells may be differentially sensitive to estradiol influences due to age-related changes in baseline function and differential activation of rapid of signaling cascades by estradiol. Estradiol effects on Ca^{2+} signaling provides a prime example. Estradiol rapidly influences Ca^{2+} signaling (Sarkar et al., 2008; Wu et al., 2005). In turn, Ca^{2+} signaling can regulate gene expression through “non-classical” transcriptional regulation, independent of estrogen nuclear receptor mechanisms (Bading et al., 1993; Foster, 2005). Aged hippocampal neurons exhibit altered Ca^{2+} homeostasis and estrogen has effects on Ca^{2+} -dependent processes, which are opposite that observed during aging (Foster, 2007). For example, the Ca^{2+} -dependent afterhyperpolarization is increased with age and estradiol reduces the afterhyperpolarization (Kumar and Foster, 2002). Furthermore, estradiol pretreatment may have a greater effect on Ca^{2+} regulation in aged cells (Brewer et al., 2006; Brewer et al., 2009). Together the results indicate that age differences in gene expression for rapid signaling pathways may relate to disparity in basal pathway activity and estrogen mediated activation of rapid signaling cascades.

In addition, to age-related changes in rapid signaling cascades, it is likely that changes in estrogen receptors contribute to differences in gene expression. In brain regions like the hippocampus that express both estrogen receptor alpha ($\text{ER}\alpha$) and beta ($\text{ER}\beta$), the magnitude and direction of gene regulation will depend on the relative expression of each receptor and the interaction of receptors (Gonzales et al., 2008; Gottfried-Blackmore et al., 2007). While it is unclear how estrogen receptor expression changes in the hippocampus of mice, aging female mice exhibit a decrease in the transcription and expression of $\text{ER}\beta$ in the cortex (Sharma and Thakur, 2006; Thakur and Sharma, 2007). In contrast, an age-related shift in the hippocampal expression of $\text{ER}\alpha$ splice variants may reduce the sensitivity to estrogen treatment in women (Ishunina et al., 2007). In the hippocampus of rats, expression of both $\text{ER}\alpha$ and $\text{ER}\beta$ declines during aging (Mehra et al., 2005) and the loss of $\text{ER}\alpha$ is associated with the decreased responsiveness of hippocampal synapses to estradiol (Adams et al., 2002). Indeed, previous work indicates an important role for $\text{ER}\alpha$ in the estrogen-mediated increase in synaptic markers (Jelks et al., 2007; Morissette et al., 2008; Mukai et al., 2007). However, several of these studies report a similar, though usually blunted effect of $\text{ER}\beta$ activation (Jelks et al., 2007; Morissette et al., 2008; Patrone et al., 2000), suggesting that $\text{ER}\beta$ may be less active but have similar effects on transcription (Lindberg et al., 2003).

An age-related change in $\text{ER}\alpha$ and $\text{ER}\beta$ or a decline in rapid signaling pathways could have reduced or delayed estradiol induced signaling and gene regulation. In the current study there appears to be a delay in the expression of PPAR genes. However, only one gene was common for young 6 hr and aged 12 hr groups. Although, gene enrichment was observed for the PPAR pathway in young and aged mice, IPA analysis indicated that many more pathways were differentially influenced between the two age groups. Similarly, synaptic component genes increased for young, but not for aged mice. Finally, examination of all genes indicated little correspondence in the gene changes between young 6 hr and aged 12 hr groups. Together, the results indicate that delayed activation is not responsible for most of the age differences in altered gene expression between 6 and 12 hr. The longer-term effects of estradiol on gene transcription in aged animals remain to be determined.

It is possible that gene changes observed in aged animals could act as a priming response for successive estradiol induced changes beyond the 12 hr time point. For example, estradiol application to hippocampal slices rapidly increases ERK/MAPK activation and NMDA receptor function (Bi et al., 2003) and the magnitude of LTP (Foy et al., 2008) in young, but not aged animals. In contrast, *in vivo* priming with estradiol 48 hr prior to sacrifice can

enhance LTP in slices from young and aged animals (Smith and McMahon, 2005; Yun et al., 2007). Previous works suggests that both estrogen receptors and rapid signaling cascades are involved in the estradiol-mediated spine growth and synaptogenesis (Akama and McEwen, 2003; Lee et al., 2004; Mukai et al., 2007; Murphy and Segal, 1996; Murphy and Segal, 1997; Yildirim et al., 2008; Znamensky et al., 2003). Thus, while young mice exhibit a rapid increase in the expression of the synaptic marker synaptophysin, an increase in synaptophysin can also be observed in aged mice following treatment with estradiol over several days (Frick et al., 2002; Spencer et al., 2008). Similarly, behavioral studies suggest that a single estradiol injection delivered after training can improve memory in young and MA animals, but not aged animals (Frick, 2009), consistent with the idea that aged animals are less responsive to a single injection. Estradiol treatment for several days prior to training reliably improves memory in middle-aged animals (Foster, 2005; Frick, 2009); however, the effects in aged animals can vary across species. Treatment prior to training improves memory in mice (Frick et al., 2002; Heikkinen et al., 2004; Vaucher et al., 2002), and is less effective in aged rats (Foster et al., 2003; Savonenko and Markowska, 2003; Talboom et al., 2008). Similar differences in responsiveness are noted for estradiol effects on synaptic markers, which can be increased in aged mice (Frick et al., 2002; Spencer et al., 2008), but not in aged rats (Adams et al., 2001; Miranda et al., 1999; Yildirim et al., 2008). The difference in rats and mice may be due to differences in the expression of estrogen receptors during aging as noted above. Regardless, the results indicate that aged animals are less responsive to a single injection of estradiol, however; depending on the species, estradiol priming may rescue estrogen responsiveness. It would be enlightening to determine whether an increase in the expression of estrogen receptors or an enhancement of rapid signaling would ameliorate age-related differences in gene changes, synaptic plasticity and memory following estradiol treatment.

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Figure 1.

Estradiol-responsive gene expression is altered over the course of aging. **A**) Illustration of the number of probes that were increased or decreased by estradiol treatment in young (Young), middle-aged (MA) and aged (Aged) animals at 6 hr (filled bars) or 12 hr (open bars) after a single estradiol injection. MA animals exhibited over two times the number of altered probes at 6 hr relative to the other two age groups. The number of altered probes decreased at 12 hr relative to 6 hr for young and MA animals. In contrast, aged animals exhibited approximately a five fold increase in the number of estradiol-responsive probes during this time period. **B&C**) Venn diagrams of the number of differentially expressed probes in response to estradiol treatment at **B**) 6 hr and **C**) 12 hr. The numbers in parentheses represent probes that changes in opposite directions.

Figure 2. Validation of estradiol treatment effects in young animals at 6 hr for six genes using RT-PCR. The bars represent the mean fold change in gene expression for young mice 6 hr following estradiol treatment compared to the mean of age-matched oil treated animals using RT-PCR (open bars) and for microarray measures (filled bars).

Table 1
Context Sequence of Genes for RT-PCR Analysis

Gene Symbol	Assay ID	Context Sequence
WDFY1	Mm00840455_m1	GGGGTGTGATGGAATTCACGTTT
GABRA2	Mm01211683_m1	CGGGAAGAGTGTAGTCAATGACAAG
NNT	Mm01298455_m1	GCCAACATCTCTGGTTATAAGGCTG
PPARGC1A	Mm00447183_m1	CGCAACATGCTCAAGCCAAACCAAC
ATF4	Mm00515324_m1	GCCATGGCGCTCTTCACGAAATCCA
ENTPD4	Mm00491888_m1	TTCCTGCCCTTGAGAGACATCCGGC
GAPDH	Mm99999915_g1	GAACGGATTGGCCGTATTGGGCGC

Table 2
Synaptic Component Genes Increased at 6 hr in Young and MA Mice

The Affymetrix probe identifier, gene symbol, gene description, *t*-test p-value and fold change are provided for genes of synaptic components that increase 6 hr following treatment in young and MA mice.

Affymetrix	Symbol	Description	p-value	Fold
Young				
1458298_at	CADPS	Ca ²⁺ dependent activator protein for secretion	1.58E-02	1.20
1443876_at	CAMK2A	Calcium/calmodulin-dependent protein kinase II alpha	1.00E-02	1.19
1423286_at	CBLN1	Cerebellin 1 precursor protein	1.33E-02	1.53
1433607_at	CBLN4	Cerebellin 4 precursor protein	9.86E-03	1.38
1433451_at	CDK5R1	Cyclin-dependent kinase 5, regulatory subunit (p35) 1	1.49E-03	1.26
1422887_a_at	CTBP2	c-terminal binding protein 2	9.81E-03	1.20
1442223_at	ENAH	Enabled homolog (drosophila)	9.46E-03	1.20
1455444_at	GABRA2	Gamma-aminobutyric acid receptor, subunit alpha 2	1.06E-06	2.75
1434098_at	GLRA2	Glycine receptor, alpha 2 subunit	1.29E-02	1.34
1458285_at	GRIA1	Glutamate receptor, ionotropic, ampa1 (alpha 1)	5.61E-03	1.34
1440891_at	GRIA4	Glutamate receptor, ionotropic, ampa4 (alpha 4)	1.55E-02	1.48
1436575_at	GRIN3A	Glutamate receptor ionotropic, nmda3a	1.35E-02	1.20
1435951_at	GRIP1	Glutamate receptor interacting protein 1	4.60E-03	1.20
1437363_at	HOMER1	Homer homolog 1 (drosophila)	1.94E-02	1.19
1417376_a_at	IGSF4A	Immunoglobulin superfamily, member 4a	1.19E-02	1.22
1450435_at	L1CAM	L1 cell adhesion molecule	1.18E-02	1.15
1452328_s_at	PJA2	Praja 2, ring-h2 motif containing	1.78E-02	1.41
Middle-age				
1439220_at	ANK3	Ankyrin 3, epithelial	1.93E-02	2.01
1458525_at	APP	Amyloid beta (a4) precursor protein	2.94E-03	2.03
1445798_at	DLGH1	Discs, large homolog 1 (drosophila)	1.95E-02	1.74
1446585_at	DLGH2	Discs, large homolog 2 (drosophila)	1.32E-02	1.37
1429768_at	DTNA	Dystrobrevin alpha	5.52E-03	1.27
1445329_at	DTNB	Dystrobrevin, beta	5.79E-03	1.66
1446426_at	ENAH	Enabled homolog (drosophila)	4.17E-03	1.85
1454022_at	EPHB2	Eph receptor b2	9.43E-03	1.58
1458285_at	GRIA1	Glutamate receptor, ionotropic, ampa1 (alpha 1)	1.36E-02	1.37
1453098_at	GRIA2	Glutamate receptor, ionotropic, ampa2 (alpha 2)	1.27E-02	1.75
1443285_at	GRIA4	Glutamate receptor, ionotropic, ampa4 (alpha 4)	4.53E-03	2.08
1440602_at	GRIK2	Glutamate receptor, ionotropic, kainate 2 (beta 2)	6.52E-03	2.57
1421350_a_at	GRIP1	Glutamate receptor interacting protein 1	1.69E-02	1.57
1458861_at	GRM7	Glutamate receptor, metabotropic 7	2.12E-02	1.42
1440637_at	ITSN1	Intersectin 1 (sh3 domain protein 1a)	1.58E-02	1.75
1424848_at	KCNMA1	Potassium large conductance calcium-activated channel	9.27E-03	2.99

Affymetrix	Symbol	Description	p-value	Fold
1440807_at	MAGI2	Membrane associated guanylate kinase	1.97E-03	2.90
1420171_s_at	MYH9	Myosin, heavy polypeptide 9, non-muscle	1.25E-02	1.34
1422520_at	NEF3	Neurofilament 3, medium	2.28E-02	1.13
1447216_at	NRXN1	Neurexin I	1.37E-02	2.10
1457212_at	NRXN3	Neurexin III	9.69E-04	2.11
1444126_at	PJA2	Praja 2, ring-h2 motif containing	1.52E-04	2.07
1442620_at	PSD3	Pleckstrin and sec7 domain containing 3	3.20E-04	2.12
1438282_at	SYT1	Synaptotagmin I	2.99E-03	2.02
1429729_at	SYT11	Synaptotagmin 11	1.22E-02	1.88
1459009_at	UTRN	Utrophin	2.27E-02	1.44

Estradiol-Responsive Pathways 6 hr Post Treatment

Table 3

Pathways with overrepresentation of genes that were observed to increase or decrease expression 6 hr following treatment. The p-value is calculated from a right-tailed Fisher's Exact Test. The number of altered genes is also provided.

Increasing	p-value	Genes	Decreasing	p-value	Genes
Young					
PPAR/RARa activation	5.E-03	15	Oxidative phosphorylation	5.E-05	17
Glutamate receptor signaling	5.E-03	8	Mitochondrial dysfunction	1.E-03	14
Circadian rhythm signaling	1.E-02	4			
Middle-age					
None			Oxidative phosphorylation	1.E-05	24
			Mitochondrial dysfunction	5.E-03	18
			Protein ubiquitination pathway	1.E-02	25
Aged					
None			None		

Table 4
Oxidative Phosphorylation and Mitochondrial Dysfunction Genes Altered at 6 hr in Young and Middle-Aged Mice

The Affymetrix probe identifier, gene symbol, gene description, *t*-test p-value and fold change are provided for genes of in the oxidative phosphorylation and mitochondrial dysfunction pathways that were decrease 6 hr following treatment in young and MA mice.

Affymetrix	Symbol	Description	p-value	Fold
Young				
1417607_at	COX6A2	cytochrome c oxidase subunit VIa polypeptide 2	4.53E-03	-1.37
1424364_a_at	UCRC	ubiquinol-cytochrome c reductase complex (7.2 kD)	1.86E-02	-1.31
1416057_at	NDUFB11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3kDa	1.98E-02	-1.28
1417286_at	NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	9.06E-03	-1.27
1454716_x_at	COX5B	cytochrome c oxidase subunit Vb	2.13E-02	-1.25
1437680_x_at	GLRX2	glutaredoxin 2	7.65E-03	-1.22
1423676_at	ATP5H (includes EG:10476)	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	2.48E-02	-1.17
1453229_s_at	HCG 25371	hCG25371	7.61E-03	-1.16
1428360_x_at	NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa	1.42E-02	-1.16
1416526_a_at	PARK7	Parkinson disease (autosomal recessive, early onset) 7	3.80E-03	-1.16
1416495_s_at	NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)	2.01E-03	-1.14
1428322_a_at	NDUFB10 (includes EG:4716)	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	3.85E-03	-1.14
1455283_x_at	NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	1.12E-02	-1.14
1415980_at	ATP5G2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)	1.45E-02	-1.13
1428075_at	NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	3.76E-03	-1.13
1426689_s_at	SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	1.11E-02	-1.11
1428179_at	NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	9.55E-03	-1.10
1415966_a_at	NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	1.02E-02	-1.07
1449622_s_at	ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1	1.66E-02	-1.07
Middle-age				
1429329_at	COX10	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase (yeast)	7.36E-04	-1.38
1419544_at	ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit c1	1.98E-02	-1.32
1426742_at	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1	9.93E-03	-1.30
1415967_at	NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	3.37E-03	-1.30
1428782_a_at	UQCRC1	ubiquinol-cytochrome c reductase core protein I	3.35E-03	-1.29
1455640_a_at	TXN2	thioredoxin 2	7.96E-03	-1.29
1423711_at	NDUFAF1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	1.42E-03	-1.27
1417799_at	ATP6V1G2	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2	1.13E-02	-1.25
1423737_at	NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	5.67E-04	-1.24

Affymetrix	Symbol	Description	p-value	Fold
1451312_at	NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)	4.13E-03	-1.23
1424488_a_at	PPA2	pyrophosphatase (inorganic) 2	4.49E-04	-1.23
1448331_at	NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	8.45E-03	-1.22
1450968_at	UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	1.08E-02	-1.22
1437013_x_at	ATP6V0B	ATPase, H ⁺ transporting, lysosomal 21kDa, V0 subunit b	4.57E-03	-1.21
1432264_x_at	COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like	1.44E-02	-1.21
1448153_at	COX5A	cytochrome c oxidase subunit Va	2.60E-05	-1.20
1448292_at	UQCR	ubiquinol-cytochrome c reductase 6.4kDa subunit	2.33E-02	-1.20
1448286_at	HSD17B10	hydroxysteroid (17-beta) dehydrogenase 10	4.13E-03	-1.19
1448589_at	NDUFB5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	1.29E-03	-1.18
1451096_at	NDUFS2	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	4.95E-03	-1.18
1428631_a_at		ubiquinol-cytochrome c reductase core protein II	2.34E-02	-1.17
1428179_at	NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	1.60E-03	-1.16
1416663_at	NDUFA9 (includes EG:4704)	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	2.01E-03	-1.16
1416952_at	ATP6V1D	ATPase, H ⁺ transporting, lysosomal 34kDa, V1 subunit D	1.41E-02	-1.16
1448203_at	ATP5L	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit G	7.34E-03	-1.15
1415671_at	ATP6V0D1	ATPase, H ⁺ transporting, lysosomal 38kDa, V0 subunit d1	1.19E-03	-1.13
1428075_at	NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	1.56E-02	-1.12

Table 5
Genes from aged mice which exhibited expression opposite young or MA mice at 12 hr

Genes from aged mice which exhibited expression opposite young or MA mice at 12 hr. The Affymetrix probe identifier, gene symbol, gene description, *t*-test *p*-value and fold change are provided for genes in young (Y), MA, and aged (A) mice 12 hr following treatment. The *p*-values are all < 0.025 for aged mice and for either young or MA mice.

Affy ID	Gene	Protein	p-value			Fold Change		
			Y	MA	A	Y	MA	A
1452369_at	MAG11	MEMBRANE ASSOCIATED GUANYLATE KINASE, WW AND PDZ DOMAIN CONTAINING 1	6.3E-04	4.4E-01	3.8E-03	-1.29	-1.10	1.51
1434008_at	SCN4B	SODIUM CHANNEL, TYPE IV, BETA	2.2E-02	3.9E-01	3.9E-03	-1.26	-1.16	1.47
1419008_at	NPY5R	NEUROPEPTIDE Y RECEPTOR Y5	9.2E-01	2.1E-02	5.3E-05	1.01	-1.52	1.45
1426495_at	2410042D21RIK	RIKEN CDNA 2410042D21 GENE	5.2E-01	1.2E-02	1.9E-03	1.08	-1.51	1.41
1448795_a_at	TBRG4	TRANSFORMING GROWTH FACTOR BETA REGULATED GENE 4	6.8E-01	1.6E-02	5.3E-03	-1.05	-1.34	1.33
1427329_a_at	IGH-6	IMMUNOGLOBULIN HEAVY CHAIN 6 (HEAVY CHAIN OF IGM)	5.0E-02	2.3E-02	1.3E-02	-1.41	-1.27	1.32
1455277_at	HHIP	HEDGEHOG-INTERACTING PROTEIN	8.4E-05	7.1E-01	1.1E-02	-1.45	1.06	1.29
1426582_at	ATF2	ACTIVATING TRANSCRIPTION FACTOR 2	1.4E-01	2.1E-02	2.4E-02	1.27	-1.45	1.28
1429249_at	4833424O15RIK	RIKEN CDNA 4833424O15 GENE	7.1E-01	1.9E-02	5.9E-03	1.04	-1.58	1.27
1456904_at	EST		8.0E-01	1.0E-02	2.2E-02	1.02	-1.18	1.26
1426806_at	OBFC2A	OLIGONUCLEOTIDE BINDING FOLD CONTAINING 2A	2.6E-01	1.9E-02	1.9E-02	1.15	-1.51	1.25
1428429_at	RGMB	RGM DOMAIN FAMILY, MEMBER B	2.9E-01	2.2E-02	1.3E-02	1.13	-1.43	1.25
1435165_at	CNTN2	CONTACTIN 2	2.1E-02	4.4E-01	1.1E-02	-1.23	-1.09	1.25
1416286_at	RGS4	REGULATOR OF G-PROTEIN SIGNALING 4	4.9E-01	2.3E-02	5.9E-04	1.05	-1.13	1.23
1433719_at	SLC9A9	SOLUTE CARRIER FAMILY 9 (SODIUM/HYDROGEN EXCHANGER), ISOFORM 9	2.3E-02	4.4E-01	3.5E-03	-1.18	-1.07	1.21
1455734_at	CRBN	CEREBLON	2.1E-01	2.4E-02	2.3E-03	1.09	-1.15	1.20
1436056_at	KIF13B	KINESIN FAMILY MEMBER 13B	7.6E-03	6.6E-01	2.4E-02	-1.23	1.04	1.20
1419184_a_at	FHL2	FOUR AND A HALF LIM DOMAINS 2	4.7E-03	5.6E-01	1.7E-02	-1.17	-1.03	1.20
1456967_at	TRIM66	KIAA0298 HYPOTHETICAL PROTEIN (HUMAN)	1.2E-02	7.4E-01	1.8E-02	-1.24	1.04	1.19
1448752_at	CAR2	CARBONIC ANHYDRASE 2	7.9E-01	2.4E-02	9.7E-03	1.02	-1.25	1.16
1449164_at	CD68	CD68 ANTIGEN	9.2E-01	1.3E-02	7.7E-03	-1.00	-1.33	1.15
1428903_at	3110037116RIK	RIKEN CDNA 3110037116 GENE	9.8E-03	9.8E-01	1.2E-02	-1.13	-1.00	1.14
1423332_at	SDCBP	SYNDECAN BINDING PROTEIN	7.4E-01	1.8E-02	1.4E-02	1.03	-1.13	1.13

Affy ID	Gene	Protein	p-value			Fold Change		
			Y	MA	A	Y	MA	A
1429087_at	1110054005RIK	RIKEN CDNA 1110054005 GENE	2.3E-02	4.7E-02	2.0E-02	-1.20	-1.18	1.13
1429227_x_at	NAPIL1	NUCLEOSOME ASSEMBLY PROTEIN-1	2.1E-01	1.5E-02	2.4E-03	1.09	-1.25	1.13
1424801_at	ENAH	ENABLED HOMOLOG (DROSOPHILA)	3.8E-01	1.9E-02	2.5E-02	1.05	-1.20	1.12
1416458_at	ARF2	ADP-RIBOSYLATION FACTOR 2	9.6E-01	1.4E-02	2.0E-02	-1.00	-1.26	1.12
1434440_at	GNAI1	GUANINE NUCLEOTIDE BINDING PROTEIN, ALPHA INHIBITING 1	6.2E-01	1.4E-02	2.3E-02	1.05	-1.10	1.12
1424594_at	LGALS7	LECTIN, GALACTOSE BINDING, SOLUBLE 7	1.0E-02	3.1E-01	6.3E-03	-1.12	-1.05	1.12
1455403_at	MANEA	MANNOSIDASE, ENDO-ALPHA	1.8E-02	9.3E-01	2.3E-02	-1.12	1.01	1.11
1434612_s_at	SBNO1	SNO, STRAWBERRY NOTCH HOMOLOG 1 (DROSOPHILA)	8.1E-01	2.2E-02	2.4E-02	1.01	-1.17	1.11
1448963_at	NFYC	NUCLEAR TRANSCRIPTION FACTOR-Y GAMMA	1.8E-02	4.5E-01	1.9E-03	-1.10	-1.05	1.11
145011_at	STARD4	RIKEN CDNA 4632419C16 GENE	9.2E-03	4.4E-03	2.1E-02	-1.23	-1.17	1.10
1417364_at	EEFIG	EUKARYOTIC TRANSLATION ELONGATION FACTOR 1 GAMMA	8.8E-01	2.4E-02	1.2E-02	-1.01	-1.13	1.09
1452159_at	2310001A20RIK	RIKEN CDNA 2310001A20 GENE	2.1E-04	2.4E-01	2.2E-02	1.17	-1.10	-1.10
1417252_at	NT5C	5',3'-NUCLEOTIDASE, CYTOSOLIC	1.4E-03	1.2E-01	2.9E-03	1.32	-1.17	-1.12
1429048_at	BLOC1S2	BIOGENESIS OF LYSOSOME-RELATED ORGANELLES COMPLEX-1, SUBUNIT 2	2.1E-02	2.4E-02	1.5E-02	1.24	-1.28	-1.14
1434521_at	RFXDC2	REGULATORY FACTOR X DOMAIN CONTAINING 2 HOMOLOG (HUMAN)	7.8E-01	9.3E-03	7.9E-03	-1.01	1.20	-1.15
1459874_s_at	MTMR4	MYOTUBULARIN RELATED PROTEIN 4	4.9E-03	1.9E-01	1.3E-02	1.22	1.14	-1.16
1434745_at	CCND2	CYCLIN D2	1.1E-03	2.3E-01	1.5E-02	1.23	-1.09	-1.16
1456748_a_at	NIPSNAP1	4-NITROPHENYLPHOSPHATASE DOMAIN AND NON-NEURONAL SNAP25-LIKE PROTEIN H...	1.3E-02	6.0E-01	2.2E-02	1.21	1.04	-1.17
1426858_at	INHBB	INHIBIN BETA-B	1.5E-02	5.6E-01	2.2E-02	1.20	-1.05	-1.17
1441003_at	ERCC4	EXCISION REPAIR CROSS-COMPLEMENTING RODENT REPAIR DEFICIENCY, COMPLEME...	2.8E-01	2.9E-03	2.3E-02	-1.14	1.48	-1.19
1431890_a_at	MLLT3	DNA SEGMENT, CHR 4, ERATO DOI 321, EXPRESSED	6.5E-03	8.2E-01	2.5E-04	1.14	1.02	-1.20
1455940_x_at	WDR6	WD REPEAT DOMAIN 6	2.4E-02	3.6E-01	2.0E-02	1.16	1.14	-1.20
1447320_x_at	RPO1-3	RNA POLYMERASE 1-3	7.7E-03	1.4E-01	1.3E-02	1.29	-1.20	-1.20
1436443_a_at	KDELC1	KDEL (LYS-ASP-GLU-LEU) CONTAINING 1	7.4E-03	9.5E-01	1.6E-02	1.31	-1.01	-1.21
1448694_at	JUN	JUN ONCOGENE	3.4E-01	1.4E-02	2.8E-03	-1.05	1.13	-1.22
1436114_at	Rnf165	Ring finger protein 165	2.3E-02	2.4E-01	1.3E-03	1.19	1.11	-1.25
1455039_a_at	SIN3B	TRANSCRIPTIONAL REGULATOR, SIN3B (YEAST)	1.8E-02	6.7E-01	4.5E-03	1.23	1.04	-1.30

Affy ID	Gene	Protein	p-value			Fold Change		
			Y	MA	A	Y	MA	A
1441727_s_at	ZFP467	HYPOTHETICAL PROTEIN, MNCB-3350	1.6E-02	3.1E-01	5.6E-03	1.34	1.10	-1.33
1456573_x_at	NNT	NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE	2.0E-03	5.8E-01	1.7E-02	1.77	1.09	-1.33
1434210_s_at	LRIG1	LEUCINE-RICH REPEATS AND IMMUNOGLOBULIN-LIKE DOMAINS 1	2.1E-03	9.9E-01	2.1E-02	1.42	-1.00	-1.34
1438157_s_at	NFKBIA	NUCLEAR FACTOR OF KAPPA LIGHT CHAIN GENE ENHANCER IN B-CELLS INHIBITOR	1.4E-02	3.0E-01	2.4E-03	1.29	1.14	-1.35
1439422_a_at	CIQDC2	CIQ DOMAIN CONTAINING 2	2.4E-02	4.1E-01	3.2E-03	1.25	-1.06	-1.37
1429372_at	SOX11	SRY-BOX CONTAINING GENE 11	3.5E-03	6.7E-01	1.3E-02	1.51	1.06	-1.39
1446464_at	PSME4	PROTEASOME (PROSOME, MACROPAIN) ACTIVATOR SUBUNIT 4	9.3E-01	5.4E-03	1.5E-02	-1.02	2.11	-1.58
1454869_at	WDR40B	WD REPEAT DOMAIN 40B	8.2E-01	2.1E-02	1.3E-03	-1.07	1.59	-1.74

Table 6

Estradiol-Responsive Pathways 12 hr Post Treatment

Pathways with overrepresentation of genes with altered expression 12 hr following treatment. The p-value is calculated from a right-tailed Fisher's Exact Test. The number of altered genes is also provided.

Increasing		p-value	Genes	Decreasing	p-value	Genes
Young						
	None			None		
MA						
	None			None		
Aged						
	α -Adrenergic signaling	5.E-05	13	None		
	Calcium signaling	1.E-04	16			
	Long-term depression signaling	5.E-04	13			
	Long-term potentiation signaling	1.E-03	12			
	G-protein coupled receptor signaling	5.E-03	13			
	cAMP-mediated signaling	1.E-02	11			
	IGF-1 signaling	1.E-02	10			
	PPAR signaling	1.E-02	7			
	Neuregulin signaling	1.E-02	8			

Table 7

Signaling Genes Altered at 12 hr in Aged Mice

The Affymetrix probe identifier, gene symbol, gene description, *t*-test *p*-value and fold increase are provided for genes increased 12 hr following treatment in signaling pathways in aged mice. An x indicates that the gene is a member of the pathway.

						Signaling Pathways								
Affymetrix	Symbol	Description	p-value	Fold	α -Adrenergic	Calcium	LTD	LTP	G-Protein	cAMP	IGF	PPAR	Neuregulin	
1426585_s_at	MAPK1	mitogen-activated protein kinase 1	1.47E-02	1.14	x	x	x	x	x	x	x	x	x	
1416351_at	MAP2K1	mitogen-activated protein kinase 1	1.08E-02	1.11	x		x	x	x	x	x	x	x	
1453419_at	MRAS	muscle RAS oncogene homolog	1.27E-02	1.25	x		x	x	x	x	x	x	x	
1452032_at	PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	4.08E-05	1.12	x	x		x	x	x				
1440132_s_at	PRKAR1B	protein kinase, cAMP-dependent, regulatory, type I, beta	5.23E-03	1.14	x	x		x	x	x	x			
1460419_a_at	PRKCB	protein kinase C, beta	2.03E-02	1.16	x		x	x	x				x	
1418754_at	ADCY8	adenylate cyclase 8 (brain)	1.26E-02	1.24	x		x	x	x	x				
1426582_at	ATF2	activating transcription factor 2	2.39E-02	1.28		x		x	x	x				
1433592_at	CALM1	calmodulin 1 (phosphorylase kinase, delta)	3.37E-03	1.18	x	x		x		x				
1434440_at	GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	2.30E-02	1.12	x		x		x	x				
1450186_s_at	GNAS	GNAS complex locus	1.96E-02	1.14	x		x		x	x				
1417279_at	ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	1.33E-02	1.28	x	x	x	x						
1426645_at	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	5.62E-03	1.36								x	x	
1422103_a_at	STAT5B	signal transducer and activator of transcription 5B	2.07E-02	1.16								x	x	
1417091_at	CHUK	conserved helix-loop-helix ubiquitous kinase	1.42E-03	1.26					x			x		
1421622_a_at	RAPGEF4	Rap guanine nucleotide exchange factor (GEF) 4	2.09E-02	1.49					x	x				
1416286_at	RGS4	regulator of G-protein signaling 4	5.89E-04	1.23					x	x				

Affymetrix	Symbol	Description	p-value	Fold	Signaling Pathways									
					α -Adrenergic	Calcium	LTD	LTP	G-Protein	cAMP	IGF	PPAR	Neuregulin	
1450202_at	GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1	4.12E-03	1.35		x		x						
1452533_at	RYR3	ryanodine receptor 3	1.02E-03	1.36		x	x							
1440962_at	SLC8A3	solute carrier family 8 (sodium/calcium exchanger), member 3	3.51E-03	1.26	x	x								
1450655_at	PTEN	phosphatase and tensin homolog	2.15E-02	1.28									x	
1419073_at	TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	1.08E-02	1.16										x
1418099_at	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	5.29E-03	1.26								x		
1419036_at	CSNK2A1	casein kinase 2, alpha 1 polypeptide	4.59E-03	1.11							x			
1421992_a_at	IGFBP4	insulin-like growth factor binding protein 4	1.76E-02	1.32							x			
1422313_a_at	IGFBP5	insulin-like growth factor binding protein 5	1.65E-03	1.57							x			
1417933_at	IGFBP6	insulin-like growth factor binding protein 6	8.90E-03	1.35							x			
1450431_a_at	NEDD4	neural precursor cell expressed, developmentally down-regulated 4	1.33E-02	1.10							x			
1452046_a_at	PPP1CC	protein phosphatase 1, catalytic subunit, gamma isoform	1.95E-02	1.15					x					
1420534_at	GUCY1A3	guanylate cyclase 1, soluble, alpha 3	8.46E-03	1.56			x							
1420871_at	GUCY1B3	guanylate cyclase 1, soluble, beta 3	3.69E-03	1.43			x							
1453260_a_at	PPP2R2A	protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	2.23E-02	1.23			x							
1452788_at	PPP2R5E	protein phosphatase 2, regulatory subunit B', epsilon isoform	1.44E-02	1.87			x							
1417943_at	GNB4	guanine nucleotide binding protein (G protein), gamma 4	4.75E-04	1.30	x									
1452363_a_at	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	1.76E-03	1.15		x								
1417606_a_at	CALR	calreticulin	2.45E-02	1.16		x								
1434572_at	HDAC9	histone deacetylase 9	7.05E-04	1.35		x								
1424852_at	MEF2C	myocyte enhancer factor 2C	6.59E-03	1.27		x								

						Signaling Pathways							
Affymetrix	Symbol	Description	p-value	Fold	α -Adrenergic	Calcium	LTD	LTP	G-Protein	cAMP	IGF	PPAR	Neuregulin
1450243_at	RCAN2	regulator of calcineurin 2	1.57E-02	1.50		x							
1423721_at	TPM1	tropomyosin 1 (alpha)	1.41E-02	1.13		x							