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ORIGINAL ARTICLE

Activity of estradiol and selective estrogen receptor modulators in the mouse N20.1 oligodendrocyte/ astrocytes cell line

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Abstract

OBJECTIVE: The mechanism through which estrogen exerts its neuroprotective and anti-neurodegenerative effects in the central nervous system is poorly understood. Human glial cells are implicated in the pathogenesis of Alzheimer's disease and have both α and β estrogen receptors (ER).

We developed a glial cell model for ER function using the N20.1 mouse oligodendroglial cell line to evaluate the response of ERα and ERβ to estradiol (E2), a raloxifene analog LY117018 (LY) and 4-hydroxytamoxifen (4OHT).

DESIGN: We tested the ability of exogenous ER to activate transcription in response to ligands (100 nM) using the glial cell line N20.1 in a transient cotransfection assay with an ERα or ERβ expression vector, an ERE-driven reporter and a Renilla luciferase transfection control.

RESULTS: Endogenous ER was not detected in the N20.1 cells by Western immunoblotting. E_2 stimulated both ERα and ERβ on both ERE- and AP-1 driven promoters. The transcription stimulation by E_2 in the ERα and ERβ through the AP-1driven promoter, though significant, was not of the same magnitude as the stimulation of the ERα through the ERE-driven promoter. 4OHT and LY did not show significant transcriptional activation of either the ERα or ERβ, through either the ERE or AP-1 driven promoters. LY, at a 10-fold higher concentration than E_2, showed a difference in its antagonist activity on the ERβ through the AP-1 pathway when compared with the ERE- driven promoter, demonstrating not only promoter specificity, but also receptor specificity.

CONCLUSIONS: This is the first description of the activity of 4OHT and LY on estrogen receptors in glia.

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Introduction

In the brain, 17 β-estradiol (E_2) has been shown to influence the development, plasticity, and survival of neurons [2,11,19]. These multiple actions in the central nervous system (CNS) may have a beneficial impact on the development of age-related neuronal degenerative diseases. In particular, a beneficial role of E_2 in Alzheimer's disease and Parkinson's disease has been suggested based on results of both human and animal studies [7,18,26,28]. The ER is expressed in all known neural cells including microglia. The glia in the CNS includes the microglia, astrocytes and oligodendrocyte. ERs are known to be expressed in all three glial types [10,41], although details of their distribution and signaling pathway have not been characterized, with the exception of a recent paper characterizing the localization of ERα and its activation of the non-classical signaling pathway in astroglia [23].

E_2 has recently been shown to have anti-inflammatory activity in glial cells [34]. Of the glial cells, the astrocytes have perhaps the greatest potential for involvement in the mediation of E_2 neuroprotective effects. They have been implicated in the process of synaptic remodeling and also appear to have a critical role in the protection or survival of neurons in the brain, since ablation of astrocytes in vivo results in a significant decrease in neuronal survival [5]. We selected the N20.1 mouse cell line for our studies because it has several features of immature oligodendrocytes and expresses markers of both oligodendrocytes and astrocytes. N20.1 glial cells not only express high levels of glial fibrillary acidic protein (GFAP), a classical astroglial marker [31,33], but they provide a reproducible source of glial cells and are easy to grow and transfect.

A variety of selective estrogen receptor modulators (SERMs) have been developed to retain the favorable effects and minimize the adverse effects of E_2. Tamoxifen (4OHT) is an antiestrogen in breast tissue [12] and appears to function as an E_2-like ligand in uterine tissue [14]. Raloxifene (RAL) has been reported to retain the antiestrogenic properties of 4OHT in breast tissue and to show minimal E_2-effects in the uterus; in addition, it has potentially beneficial E_2-like effects in non-reproductive tissues such as bone and cardiovascular tissue [3,39,40].

The ER activates transcription both from classical response elements (EREs), to which ER binds directly, and from various alternative response elements, to which ER does not bind directly. While the relative significance of ER activation at heterologous or alternative response elements is not yet clear, there are indications that such activation is as important as activation at EREs [37]. 4OHT activates AP-1 target genes in uterine cells but not in breast tumor cells [36]. This cell specific effect parallels the effect of 4OHT upon the growth of these cell types. Moreover, RAL lacks E_2- like effects on AP-1 targets in uterine cells [22] and does not exert E_2-like effects on cell proliferation. Extending the parallel, E_2-liganded ER enhances AP-1 target genes in some breast cell lines but represses AP-1 target genes in others [24]. Where ER stimulates transcription of AP-1 targets it stimulates proliferation, and where it represses AP-1 transcription it inhibits proliferation. Thus, ER action at AP-1 sites may reflect some of the mechanisms that ER uses to regulate genes that are involved in the cellular growth response.

The mechanism of action by which 4OHT and RAL may exert their agonist or antagonist activity in the glial cell has not been previously described. Therefore, any potential effect of these SERMs on neurodegeneration remains to be elucidated. Furthermore, these ligands are used in large populations of women with osteoporosis and/or breast cancer, without knowing their possible impact in preventing or protecting against neuronal degeneration. We addressed the role of ER subtypes and the responses to E_2 and two SERMs, raloxifene analog LY117018 (LY) and 4OHT, in the N20.1 glial cell line.

Methods

Plasmids: hERα: HEGO in pSG5, was obtained from Drs. Pierre Chambon and Hinrich Gronemeyer. hERβ (aa1-530) in pSG5 as described by Ogawa et al, (21), was obtained from Dr. J.A Gustafsson.

Culture Conditions: The N20.1 cell line, originally provided by Dr. Tony Campagnoni, was derived from glial precursors immortalized with SV40 T antigen [33]. It was passaged and maintained at a permissive temperature of 34 °C or at 37 °C for comparison, in Dulbecco's modified Eagle's medium/ F-12/ supplemented with 10% fetal bovine serum (FBS) 4.0g/L glucose, and 2.4g/L sodium bicarbonate, supplemented with 10% NuSerum (Collaborative Biomedical Products) 100 μg/ml G418 and 20μg/ml gentamicin were added after filtration. For experiments, cells were grown almost to confluence at 37 °C in an atmosphere of 5% CO_2. The cells were then seeded and cultured for an additional 2 days prior to transfection. One day before transfection charcoal-stripped FBS (Hyclone) was substituted for NuSerum FBS at 10% for estradiol-free media; all other components remained constant.

N20.1 Cell Transfection: N20.1 cells were seeded at a density of 2.5 x 10^5 cells per 35-mm well (six-well dishes; Costar, Cambridge, MA, U.S.A.) 48 hours prior to transfection in 2 ml of growth medium. At 20 hours prior to transfection, the cells were washed with 1X PBS and incubated with media containing charcoal-stripped FBS. Cells were transfected with 2 μg of total plasmid DNA and 2.5 μl/μg of SuperFect (Qiagen) per 1 μg of DNA according to the manufacturer's specifications. Cells were transfected with 0.66 μg of either ERα wild type or ERβ wild type vector DNA, 1.32 μg of 2ERE-luciferase reporter DNA and 0.013 μg of Renilla Luciferase. pRL-SV40 was used to control for differences in transfection efficiency. Each construct was transfected at least in quadruplicates per experiment. Cells were incubated with the DNA/SuperFect cocktail for 4 hours. After that, E_2, 4OHT, and LY were added (10^{-7} M). E_2 (10^{-8} M) and LY (10^{-6} M) were used alone and in combination to evaluate inhibition of E_2 by LY. The ligands added to the cells were dissolved in ethanol and vehicle

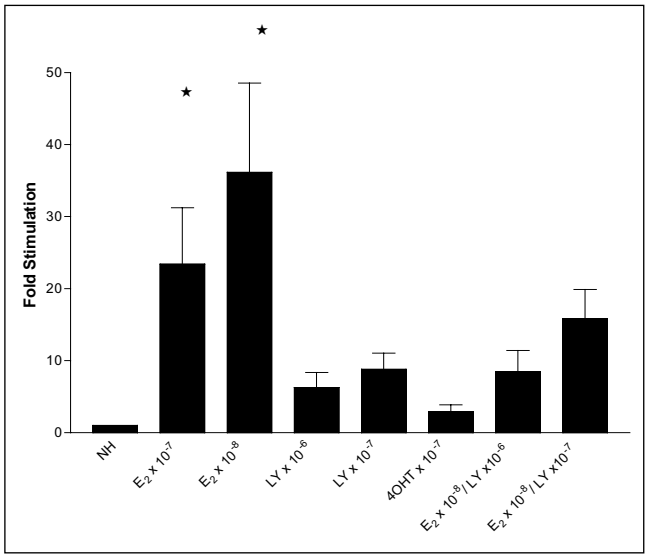

Figure 1. E2, Raloxifene Analog, Tamoxifen Stimulation and Raloxifene Analog antagonism of E2 of ERα Through the ERE Pathway in N20.1 Glial Cells.
Transcription activation *(top)* was measured using a transient transfection assay with wt ERα (HEGO), an ERE-driven reporter (p2ERE-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in N20.1 Glial cells described under "Methods". The activity of ER is measured by the relative luciferase activity, RLU, which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The RLU was measured in the absence of hormone (vehicle control) and in response to E_2 (10^{-7} M and 10^{-8}M); 4OHT (10^{-7} M), LY (10^{-6} M and 10^{-7} M); and the combination of LY (10^{-6} M and 10^{-7} M) and E_2 (10^{-8} M). The values are the mean ± S.E of at least four independent experiments, each carried out in triplicate. *P=<0.05 compared to nh (no hormone).

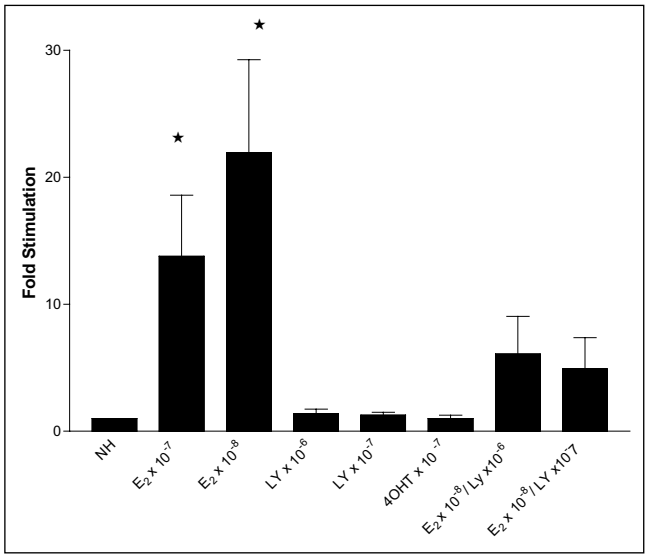

Figure 2. E_2, Raloxifene Analog, and Tamoxifen Stimulation and Raloxifene Analog antagonism of E_2 of ERβ Through the ERE Pathway in N20.1 Glial Cells.
Transcription activation *(top)* was measured using a transient transfection assay with wt ERβ (HEGO), an ERE-driven reporter (p2ERE-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in N20.1 Glial cells described under "Methods". The activity of ER is measured by the relative luciferase activity, RLU, which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The RLU was measured in the absence of hormone (vehicle control) and in response to E_2 (10^{-7} M and 10^{-8}M); 4OHT (10^{-7} M), LY (10^{-6} M and 10^{-7} M); and the combination of LY (10^{-6} M and 10^{-7} M) and E_2 (10^{-8} M). The values are the mean ± S.E of at least four independent experiments, each carried out in triplicate. *P=<0.01 compared to nh (no hormone).

control was used. Lysates were prepared approximately 40 hours post DNA addition. Aliquots of lysate were evaluated for reporter gene expression using the Dual Luciferase Assay Kit (Promega) for determination of luciferase activities. Luminescence was measured as relative light units (RLU) using a TD 20/20 luminometer (Turner Designs).

Western blotting: We prepared whole N20.1 cell extract in Laemmli buffer (16) and SDS-gel electrophoresis (50μg protein/lane) was carried out. We determined protein concentration using the Bradford method. Proteins were transferred to nitrocellulose. The membrane was immunoblotted with ERα: ER Ab-15 (Clone AER611); ERβ (Ab–24), both purchased from NeoMarkers. Bands were visualized using the ECL™ system.

Data analysis: The results (mean ± SEM) are expressed relative to the activity of the ER in the absence of ligand (fold-stimulation). Comparisons were analyzed with ANOVA (Dunnett's).

Results

We first wanted to determine whether the mouse N20.1 cell line expresses endogenous ER. N20.1 cells were maintained and propagated in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum (FBS), 4.0g/L glucose, and 2.4g/L sodium bicarbonate. There was no endogenous ERα or ERβ detected by Western blot either at 34 °C or at 37 °C; although a delay in cell growth was observed at 37 °C. Therefore, all subsequent experiments were carried out using ERα and ERβ that were introduced into the cells by transient transfection.

Activity through ER*α* and ER*β* at an ERE-driven promoter: We tested the activity of E_2, LY, and 4OHT through the ERα and ERβ in an ERE-driven promoter.

In the ERα transfected cells, E_2 at 10^{-7} M and 10^{-8} M showed significant activity through the ERE driven promoter ($p<0.05$ compared to no ligand, ANOVA (Dunnett's)). The increase in fold stimulation by E_2 in the ERα expressing cells was 23.37 ± 7.8 at 10^{-7} M, and 36.12 ± 12.3 at 10^{-8} M. LY did not show any significant stimulation at either 10^{-6} M or at 10^{-7} M in the ERα cells through the ERE-driven promoter ($p>0.05$). The transcription stimulation by 4OHT via ERα was not statistically significant ($p>0.05$) (*Figure 1*).

We then tested the ability of LY, at 10^{-6} M and 10^{-7} M, to block the activity of E_2 through the ERα. LY, 10^{-6} M and 10^{-7} M, in combination with E_2, 10^{-8} M, decreased the activity of E_2 from 36.12 ± 12.3 fold to 5.26 ± 1.4 and 15.87 ± 1.4 fold, respectively (*Figure 1*).

In the ERβ transfected cells, E_2 at 10^{-7} M and 10^{-8} M, also showed significant stimulatory activity through the ERE-driven promoter ($p<0.01$ compared to no ligand, ANOVA (Dunnett's)). The increase in fold stimulation by E_2, 10^{-7} M, in the ERβ expressing cells was 13.77 ± 4.8.

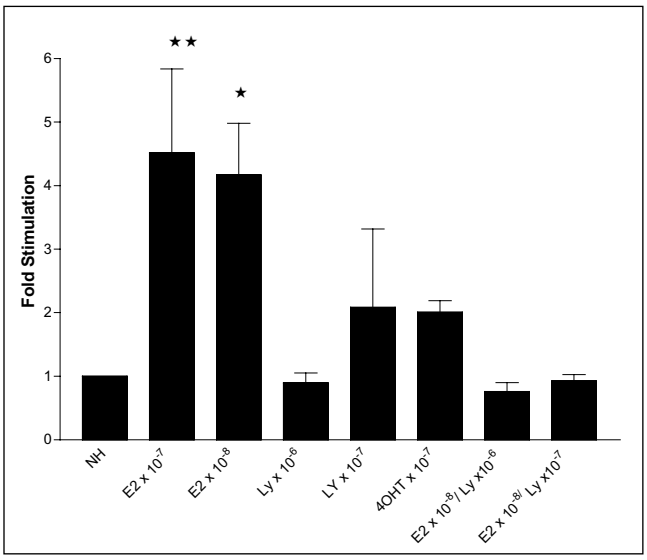

Figure 3. E_2, Raloxifene Analog, and Tamoxifen Stimulation and Raloxifene Analog antagonism of E_2 of ERα Through the AP-1 Pathway in N20.1 Glial Cells.
Transcription activation *(top)* was measured using a transient transfection assay with wt ERα (HEGO), an ERE-driven reporter (p2ERE-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in N20.1 Glial cells described under "Methods". The activity of ER is measured by the relative luciferase activity, RLU, which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The RLU was measured in the absence of hormone (vehicle control) and in response to E_2 (10^{-7} M and 10^{-8}M); 4OHT (10^{-7} M), LY (10^{-6} M and 10^{-7} M); and the combination of LY (10^{-6} M and 10^{-7} M) and E_2 (10^{-8} M). The values are the mean ± S.E of at least four independent experiments, each carried out in triplicate. *P=<0.05, **P=<0.01 compared to nh (no hormone).

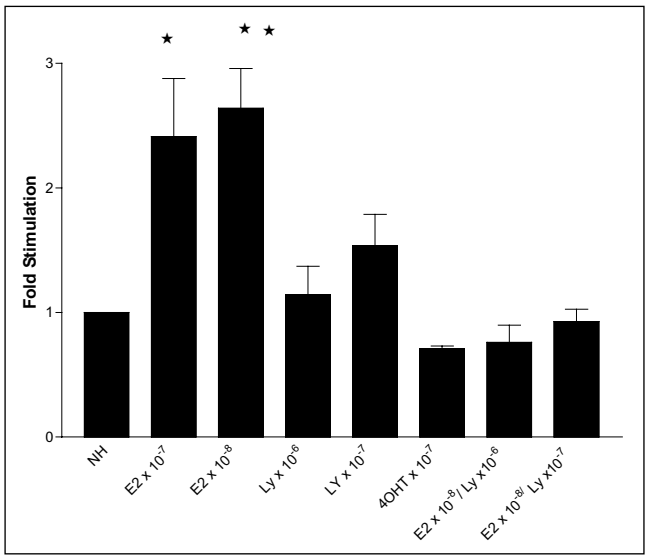

Figure 4. E_2, Raloxifene Analog, and Tamoxifen Stimulation and Raloxifene Analog antagonism of E_2 of ERβ Through the AP-1 Pathway in N20.1 Glial Cells.
Transcription activation *(top)* was measured using a transient transfection assay with wt ERβ (HEGO), an ERE-driven reporter (p2ERE-luciferase), and a *Renilla* luciferase transfection control (pRL-SV40) in N20.1 Glial cells described under "Methods". The activity of ER is measured by the relative luciferase activity, RLU, which is the ratio of firefly luciferase activity to *Renilla* luciferase activity. The RLU was measured in the absence of hormone (vehicle control) and in response to E_2 (10^{-7} M and 10^{-8}M); 4OHT (10^{-7} M), LY (10^{-6} M and 10^{-7} M); and the combination of LY (10^{-6} M and 10^{-7} M) and E_2 (10^{-8} M). The values are the mean ± S.E of at least four independent experiments, each carried out in triplicate. *P=<0.05 **P=<0.01 compared to nh (no hormone).

The stimulation by E_2, at 10^{-8} M, in the ERβ cells was 21.93 ± 7.3. LY did not show any significant stimulation at either 10^{-6} M or at 10^{-7} M. (p>0.05) (*Figure 2*).

The transcription stimulation by 4OHT (10^{-7} M) via the ERβ receptor was not statistically significant (p>0.05). There was no significant activity when using the empty pSG5 vector (not shown). We then tested the ability of LY, at 10^{-6} M and 10^{-7} M, to block the activity of E_2 through ERβ. LY, at 10^{-6} M and 10^{-7} M, in combination with E_2, 10^{-8} M, decreased the activity of E_2, in the ERβ transfected cells, from 21.93 ± 7.3 fold to 6.07 ± 2.9 and 4.91 ± 2.4 fold, respectively (*Figure 2*).

These results show that E_2, but not LY and 4OHT, exerts agonist activity through the ERα and ERβ receptors in the mouse N20.1 cell line (*Figure 1 and 2*). A 10 and 100 –fold molar excess of LY substantially reduced the E_2-stimulated activity of both ERα and ERβ, to levels only 2 fold greater than in the absence of E_2 (*Figure 1 and 2*). Therefore, LY is an effective antagonist of E_2 through both ERα and ERβ on an ERE-driven promoter in the N20.1 cell line.

Activity through ER*α* and ER*β* at an AP-1 driven promoter: We tested the activity of E_2, LY, and 4OHT through the ERα and ERβ in an AP-1-driven promoter.

In the cells expressing ERα, E_2 showed a significant increase in the fold stimulation at a concentration of 10^{-7} M (4.5 1± 1.08 SEM; p<0.01) and at 10^{-8} M (4.16 ± 1.18 SEM, p<0.05). LY (10^{-6} M and 10^{-7} M) and 4OHT (10^{-7} M) did not exhibit significant stimulation through the ERα on the AP-1 driven promoter (p>0.05) (*Figure 3*). We then tested the ability of LY, 10^{-6} M and 10^{-7} M, to block the activity of E_2 through the ERα . LY, 10^{-6} M and 10^{-7} M, in combination with E_2, 10^{-8} M, decreased the activity of E_2 from 36.12 ± 12.3 fold to 5.26 ± 1.4 and 15.87 ± 1.4 fold, respectively (*Figure 3*).

In the ERβ expressing cells, there was also significant stimulation by E_2 when used at the two concentrations tested. The increase in activity of the ERβ by E_2, 10^{-7} M, was 2.46 ± 0.46 SEM (p<0.05) and E_2, 10^{-8} M, 2.64 ± 0.32 SEM (p<0.01) (*Figure 3*).

The transcription stimulation by E_2 in the ERα and ERβ through the AP-1 driven promoter, though significant, was not of the same magnitude as the stimulation of the ERα through the ERE-driven promoter. Neither LY (10^{-6} M and 10^{-7} M) nor 4OHT (10^{-7} M) exhibited significant stimulation through the ERβ on the AP-1 driven promoter (p>0.05) (*Figure 4*).

We then tested the ability of LY, 10^{-6} M and 10^{-7} M, to block the activity of E_2 through the ERβ. LY, 10^{-6} M and 10^{-7} M, in combination with E_2, 10^{-8} M, decreased the activity of E_2 from 4.16 ± 0.8 fold to 0.76 ± 0.1 and 0.92 ± 0.09 fold, respectively (*Figure 4*).

A 10- and 100-fold molar excess of LY substantially reduced the E_2-stimulated activity of both ERα and ERβ, to levels similar to those observed in the absence of E_2 **(Figure 3 and 4)**. However, a 10 –fold

molar excess of LY, was only effective in antagonizing the transcription activity of the ERα, but not of the ERβ ($p<0.05$). Therefore, LY is also an effective antagonist of E_2 through ERα and ERβ, at 10^{-6} M but not at10^{-7} M, on an AP-1-driven promoter in the N20.1 cell line. The difference observed in the lack of antagonist activity of LY in the ERβ through the AP-1, when compared with the ERE- driven promoter at 10 –fold higher concentration than E_2, demonstrates not only promoter specificity, but also, receptor specificity.

Discussion

Our results show that E_2 10^{-7} M and E_2 10^{-8} M stimulated both ERα and ERβ on both ERE and AP-1 driven promoters. The transcription stimulation by LY and 4OHT via the ERα and ERβ was not statistically significant, in either the ERE- driven or in the AP-1 driven promoters.

When we tested the ability of LY to block the activity of E_2 on the ERα and ERβ through the ERE-driven and AP-1 driven promoters, a 100-fold higher concentration of LY was an effective antagonist of E_2 through the ERα and ERβ in the ERE- and AP-1 driven promoters. However, when LY was used at only 10-fold higher concentration than E_2, it did not prove to be an effective antagonist of the ERβ on an AP-1 driven promoter. This observation shows that the mechanisms by which LY modulates transcription activity, depends not only in the type of the promoter but also on the type of receptor. This finding is supported by the observation that RAL reduces the activation caused by E_2 in a dose dependent manner to the amount observed with RAL alone [22] demonstrating that RAL induction is weaker than the induction by E_2 and that RAL-induced (LY in our study) results from binding to ERα. The lack of stimulation observed using the empty vector pSG5 as a control supports the observation that the transcription stimulation by hormone was ER-mediated.

Even though 4OHT has been reported to activate AP-1 target genes in uterine cells [36], we did not observe significant transcriptional activation with 4OHT through an AP-1 driven promoter in the N20.1 glial cells.

Recent data from the Women's Health Initiative Memory study, demonstrating that E_2 plus progestin therapy increases the risk for probable dementia in postmenopausal women aged 65 years or older, has complicated even further the unresolved debate about the usefulness of E_2 in protection against neurodegenerative disease [29]. However, there is compelling epidemiological evidence that postmenopausal E_2 therapy offers protection against neurodegeneration. Multiple case-control studies of previous exposure to E_2 support the reduction of developing Alzheimer's disease [38]. The biological plausibility of this observation is supported by evidence for the interaction between sex steroids and brain function through the two ER subtypes ERα and ERβ in the brain. It has been shown that there are neuronal populations that express both receptors and some that express only one of the two receptors [4]. Neurons of the supraoptic nucleus express ERβ but not ERα, whereas in the anteroventral periventricular nucleus of the preoptic area there is ERα but not ERβ. Although ERα and ERβ are predominantly expressed in neurons, their presence in glial cells of the spinal cord in vivo has been confirmed [25].

An interesting hypothesis has recently been raised suggesting that E_2-induced neuroprotection achieved with physiological doses of E_2 involves, at least in part, mediation by astrocytes [6]. Through an indirect protective mechanism, physiological levels of estrogen stimulate the release of astrocyte-derived neuroprotective factors influencing protection of neurons from cell death. This possible parallel pathway of indirect and direct protection could explain how E_2 could achieve widespread protection of the cerebral cortex, striatum and hippocampus despite the fact that ER is not globally expressed in all neurons in these areas [6].

As additional support of astrocytes being a candidate for a mediator of E_2 action in the brain, E_2 has been demonstrated to increase glial cell proliferation and enhance expression of the astrocyte specific marker, GFAP [30]. Only ERβ colocalizes with the astrocyte marker GFAP [1]. If ERβ is the predominant ER in astrocytes in the brain, this receptor could be important in brain development, neuronal migration, and sexual differentiation of the brain. Furthermore, colocalization of estrogen receptors in astrocytes in a variety of brain regions has been confirmed immunocytochemically in brain sections derived from guinea pig, rat, and human [1,8,13,17,20,27]. Striking morphological abnormalities have been described in the brains of ERβ knockout (BERKO) mice demonstrating that the ERβ is necessary for neuronal survival [35]. The development of the ERβ knockout mice provided a model to study the function of the ERβ in the brain demonstrating that the ERβ is necessary for neuronal survival and that morphological abnormalities occur in the absence of the ERβ in the brain [35]. Recently, direct evidence has been provided of the expression of both ERα and ERβ in oligodendrocytes *in vitro* and, most importantly, the expression of ERβ in oligodendrocytes *in vivo* [41]. Although previous classical studies examining effects of hormones on myelin differentiation and structure could easily be dismissed as secondary phenomena, the data provided by recent studies suggest E_2 may directly affect oligodendrocyte function *in vivo* [41]. Based on this observation, our innovative glial cell model becomes an important and valid tool for further functional studies.

Another variable to consider is that different ligands may elicit different responses when the receptor binds to different effector sites [22]. Through receptor interactions with different response elements, the same ligand can cause activation or repression of different sets of genes. Interestingly, antiestrogens can induce transcriptional activation through the ERs at these non-classical sites.

The response to both E_2 and antiestrogens at an AP-1 site depends on the subtype of the receptor [22]; E_2 elicits transcriptional activation with ERα, but transcriptional repression with ERβ. Also, the two ER

subtypes can respond differently to RAL at an AP-1 site. The stimulation of AP-1 targets by SERMs is especially evident with ERβ. For example with RAL, ERβ is 10-fold more efficient in activating AP-1 targets than ERα with E_2 [15]. We anticipated a higher response of LY in the ERβ through the AP-1 site than the one described in the present study. One possible explanation for the lack of response of the ERβ through the AP-1 site to LY in our study, apart from not having been previously described in this type of cells, is the observation that SERMs only activate ERβ through the AP-1 sites at very high concentrations, even higher concentrations than required for the binding of the SERM to the receptor [37]. Micromolar concentrations are required for RAL to activate ERβ, and these are unlikely to be found in patients because of poor bioavailability.

To our knowledge there is no previous description of the activity and mechanisms of action of 4OHT and LY in a glial cell model, despite the fact that certain women can be treated for 5 years with 4OHT to reduce the risk of breast cancer [9,32]. Testing the activity of other SERMs in this model remains crucial.

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