Impact of the selective estrogen receptor modulator, raloxifene, on neuronal survival and outgrowth following toxic insults associated with aging and Alzheimer’s disease

Kathleen O’Neill, a Shuhua Chen, a and Roberta Diaz Brinton a,b,*

Abstract

The current study investigated the estrogen agonist–antagonist properties of the selective estrogen receptor modulator, raloxifene (Ral), on neuroprotection and neuronal markers of memory function. Low concentrations of raloxifene significantly reduced basal markers of membrane damage and had no deleterious effect on neuronal survival. However, high concentrations of raloxifene (1000–5000 ng/ml) induced a significant increase in markers of membrane damage and a significant decrease in neuronal survival. At subtoxic concentrations, raloxifene induced significant neuroprotection against beta amyloid $\beta_{25–35}$-, hydrogen peroxide- and glutamate-induced toxicity. Results of analyses to determine whether raloxifene acted competitively or synergistically with $17\beta$-estradiol revealed that a postmenopausal level of $17\beta$-estradiol exerted a significantly greater increase in neuronal survival against beta-amyloid- and glutamate-induced toxicity compared to 50 ng/ml raloxifene. The combined presence of raloxifene and $17\beta$-estradiol was significantly neuroprotective against beta amyloid $\beta_{25–35}$- and glutamate-induced excitotoxicity but was significantly lower than $17\beta$-estradiol alone while not significantly different than raloxifene alone. Morphologic analyses demonstrated that raloxifene significantly increased neuronal outgrowth of hippocampal neurons within a narrow dose range that was blocked by a glutamate NMDA receptor antagonist. Raloxifene did not promote the outgrowth of basal forebrain or cortical neurons. Results of this study indicate that raloxifene exerted partial estrogen agonist action in the absence of $17\beta$-estradiol whereas in the presence of $17\beta$-estradiol, raloxifene exerted a mixed estrogen agonist–antagonist effect.

Introduction

Selective estrogen receptor modulators, or SERMs, also known as tissue selective estrogens, are a class of drugs with mixed estrogen agonist–antagonist effects (Wijayaratne et al., 1999) and have been proposed to be the next generation of alternatives to traditional estrogen replacement therapy (Cosman and Lindsay, 1999; Katzenellenbogen et al., 2001). Ultimately, the ideal SERM or tissue-selective action estrogen would exert estrogen agonist in the bone and in brain while simultaneously acting as an estrogen receptor antagonist in tissues such as the breast and uterus (Katzenellenbogen and Korach, 1997; Mitlak and Cohen, 1997; Purdie and Beardsworth, 1999). The therapeutic goal of SERMs is to prevent diseases associated with estrogen deficiency, such as osteoporosis and potentially Alzheimer’s disease without promoting estrogen-associated tumor genesis. Recent analyses that showed that a common hormone replacement component, medroxyprogesterone acetate, blocks the neuroprotective and neurotrophic mechanisms of $17\beta$-estradiol (Nilsen and Brinton, 2002a,b, 2003) coupled with the most recent results of the Women’s Health Initiative, which found a twofold increase in the risk of Alzheimer’s disease in women on combined estrogen and medroxyprogesterone acetate replacement therapy (Rapp et al., 2003; Shumaker et al., 2003), highlight the importance of designing optimal estrogenic molecules for the brain.
versely affected in Alzheimer’s disease. Specifically, these studies investigated whether Ral induced neuronal markers of memory function and neuroprotection. Results of these studies indicate that Ral can act as a partial estrogen agonist in the absence of E$_2$ and acts as a partial antagonist in the presence of E$_2$.

Materials and methods

Primary neuron culture

Primary cultures of dissociated hippocampal, cortical and basal forebrain neurons (derived from septal area) were performed as described by Brinton et al. (2000). Briefly, brain regions were dissected from the brains of embryonic day 18 (E18-d) rat fetuses (IAUC Animal Protocol Approval number 9052), treated with 0.05% trypsin in Hank’s balanced salt solution (5.4 mM KCl, 0.4 mM KH$_2$PO$_4$, 137 mM NaCl, 0.34 mM Na$_2$HPO$_4$·7H$_2$O, 10 mM glucose, 10 mM HEPES) for 5 min at 37°C and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. For morphological analyses, between 20,000 and 40,000 cells were seeded onto polylysine-coated (10 µg/ml) 22-mm coverslips whereas cultures used for biochemical analyses were plated onto 0.1% polyethyleneamine-coated 35-mm Petri dishes at a density of 10$^5$ neurons/ml. Nerve cells were grown in Neurobasal medium (NB; Life Sciences) supplemented with B27, 5 U/ml penicillin, 5 mg/ml streptomycin, 0.5 mM glutamine and 25 µM glutamate (Gibco, Rockville, MD), at 37°C in 10% CO$_2$. Phenol red was included in the medium as a pH indicator and any estrogenic activity of the phenol red additive was present in both control and experimental cultures. Cultures grown in serum-free NBM yielded approximately 99.5% neurons and 0.5% glia. However, glial cells were not apparent in cultures following just 24 h in culture.

Morphological analysis

Nerve cells attached to polylysine-coated coverslips were removed from the culture dish and rapidly mounted into a recording chamber. Volume of the chamber was 200 µl and pH was maintained at 7.4. Videomicroscopic recording of nerve cells was accomplished using a Dage-MTI camera equipped with a Newvicon tube linked to an Olympus BH-2 microscope and a Panasonic time-lapse video recorder (Model AG-6050). Recordings were made using phase-contrast optics with a 40 × objective and a 1.50 multiplier with 100 W tungsten source passed through a green filter. Nerve cell recordings were conducted following 24-h exposure to raloxifene (gift of Wyeth-Ayerst Laboratories). Selection of nerve cells for analysis was random, and all recording and morphological analyses were conducted blind to the experimental condition. Morphological analysis was achieved using a BioQuant Image analysis system.

The tissue-selective agonist and antagonist actions of two SERMs, tamoxifen (TMX) and raloxifene (Ral), have led to intensive preclinical and clinical investigations to explore the potential application of such chemical entities as an ideal hormone replacement therapy (Goldstein et al., 2000; Grandbois et al., 2000; Purdie and Beardsworth, 1999; Yang et al., 1996). The most recently FDA approved molecule for the treatment of osteoporosis is Ral (Evista), a nonsteroidal benzothiophen derivative that binds with high affinity to the nuclear estrogen receptor (Baker et al., 1998; Brzozowski et al., 1997; Delmas et al., 1997; Yang et al., 1996). In breast, Ral acts as a classical antiestrogen to inhibit the growth of mammary or endometrial carcinoma (Purdie and Beardsworth, 1999) whereas in nonreproductive tissues, Ral acts as a partial estrogen agonist to prevent bone loss and lower serum cholesterol. In addition, Ral demonstrates a pharmacological profile similar to that of 17β-estradiol (E$_2$) in both ovariectomized rats and postmenopausal women (Delmas et al., 1997; Purdie and Beardsworth, 1999; Sato et al., 1996).

Studies conducted in neural preparations show that Ral can exert mixed agonist–antagonist effects. In rat hypothalamus, Ral was found to increase dopamine content twofold, acting as a partial agonist, but was able to block the fivefold induction of dopamine levels by E$_2$, acting as a full antagonist (Grandbois et al., 2000). In studies using a rat pheochromocytoma cell line (PC12), Ral showed estrogen agonist properties to increase process outgrowth in cells pretreated with NGF (Nilsen et al., 1998).

Genazzani et al. (1999) found that Ral exerted estrogen-like action on neuroendocrine opiatergic pathways when administered alone in ovariectomized rats, which was reversed in fertile or in ovariectomized rats treated with E$_2$ where it exerted an antiestrogen effect. Wu et al. (1999), investigating the impact of Ral alone or in combination with estradiol on choline acetyltransferase activity, found that ovariectomized female rats treated with either estradiol benzoate or Ral showed an increase in choline acetyltransferase activity.

Clinically, Ral significantly increased hot flushes, suggesting that it acts as an estrogen receptor antagonist on sites regulating vasomotor function in human females (Cohen and Lu, 2000), and hot flushes have been postulated to be associated with poorer cognitive performance (Birge, 1998). On the other hand, results from the MORE trial (Multiple Outcomes of Raloxifene Evaluation) concluded that Ral treatment for 3 years did not affect overall cognitive scores in postmenopausal women with osteoporosis and did not reverse menopause-associated memory deficits, which are reversed with estrogen replacement therapy (Sherwin, 2002; Yaffe et al., 2001). Together, results of these studies indicate that Ral can exert estrogen agonist and estrogen antagonist effects depending on the cell population and the environmental context.

The current investigation sought to determine whether the SERM raloxifene exerts estrogen agonist or antagonist effects in neurons involved in memory function and ad-
Analysis system designed for quantitative analysis of cellular morphological features. Cell size was controlled by selecting an equal number of cells from each coverslip that fell within three size categories: small, medium and large. Cell size was determined by the area of the field encompassed by the length of extensions. If a cell encompassed 1/4 of the monitor field, it was categorized as small; 1/2 the field was medium; cells encompassing the entire monitor field or required multiple fields for analysis were categorized as large. Neurons intermediate to these dimensions were graded by the analyst to the closest size category. Number of neurites was defined as the number of extensions greater than 50-\(\mu\)m long emanating directly from the cell body. Neurite length represents the summation of the length of all neurites/neuron. Branches were operationally defined as any extension that exceeded 10-\(\mu\)m length and occurred along the shaft of the neurite. Branches that occurred as second- or third-order processes were not included in this measure. Branch length represents the summation of the length of all branches present on an individual neuron. The number of bifurcation points represents the total number of points at which branches extend from the neuritic shafts plus those points at which branches extend from other branches for an entire neuron. Microspikes were defined as processes emanating from either neurites or branches that measured less than 10 \(\mu\)m. Statistically significant differences were determined by a one-way ANOVA followed by a Newman–Keuls post hoc analysis for individual differences.

**Neuronal survival**

For analyses of neuronal survival, between 40,000 and 60,000 cells were seeded onto poly-d-lysine-coated (10 \(\mu\)g/ml) 25-mm\(^2\) coverslips with grid size of approximately 600 \(\mu\)m\(^2\) composed of 520 alphanumeric grids. Hippocampal neurons were treated with varying concentrations of raloxifene (Ral) beginning at 3 days in vitro and exposed for a total of 4 days. Neurons were counted at 7 days in vitro and grids with viable neurons were selected for analysis over the entire period of the experiment. One hundred neurons per coverslip were selected for study and there were three coverslips per condition for a total of 300 neurons analyzed per condition per experiment. Following the time 0 neuron count, cultures were rinsed with 37\(^\circ\)C HEPES-buffered saline solution (HBS). Viable neurons in

Fig. 1. Effect of raloxifene on basal LDH release from neurons in culture. Primary hippocampal (A), cortical (B) and basal forebrain (C) neurons (7–9 days in vitro) were grown in the absence or presence of raloxifene for 4 days. At the end of the fourth day, media was harvested from all cultures and assayed for LDH content. Results are expressed as percent of control LDH release. Data are expressed as mean ± SEM and were combined across three separate experiments, \(n = 12\) per condition. *\(P < 0.05\), **\(P < 0.01\) and ***\(P < 0.001\) compared to control cultures. Low concentrations of raloxifene reduced basal release of LDH whereas high concentrations increased LDH release.
the same grid were counted before and 24 h following toxin exposure. Neuronal viability was determined by three morphological criteria: the presence of smooth, round neuronal phase bright soma, possession of at least one or more neurites longer than the diameter of the cell body, and granulation free neurites as described by Mattson (1992) and Mattson and Kater (1989). Statistically significant differences were determined by a one-way ANOVA followed by a Newman–Keuls post hoc analysis for individual comparisons.

**Efflux assay of lactate dehydrogenase (LDH)**

LDH release into the culture media following a neurotoxic insult was measured using the Cytotoxicity Detection Kit from Boehringer Mannheim Biochemicals and a spectrophotometer at 490 nm. Neurotoxins utilized included 0.2 mM glutamate (Sigma), 20 μM hydrogen peroxide (VWR) and 8 μg/ml β-amyloid peptide25–35 (Aβ25–35; Lot Number: B01200, Bachem). Seven-day-old neuronal cultures were pretreated with Ral at varying concentrations for 4 days before exposure to one of three toxic agents, at room temperature in HEPES buffer containing 100 mM NaCl, 2.0 mM KCl, 2.5 mM CaCl₂, 1.0 mM MgSO₄, 1.0 mM Na₂HPO₄, 4.2 mM NaHCO₃, 12.5 mM HEPES and 10.0 mM glucose. Glutamate was dissolved in the same buffer as hydrogen peroxide whereas Aβ25–35 was dissolved in sterile distilled water at a concentration of 1 mg/ml as a stock solution. This stock was aliquoted and stored at −20°C. Following exposure to the neurotoxins, cultures were washed two times with 37°C HEPES buffer and replaced with fresh NBM media. Cultures were then returned to the incubator and assessment of LDH release conducted 24 h following exposure to the neurotoxins. Statistically significant differences were determined by a one-way ANOVA followed by a Newman–Keuls post hoc analysis for individual comparisons.

**Hydrogen peroxide exposure**

One-week-old neuronal cultures were pretreated with Ral for 4 days. On the 11th day, cultures were rinsed with HEPES-buffered saline solution (HBS) and exposed to 20 μM hydrogen peroxide (H₂O₂) as determined by Brinton et al. (1997a,b). Dilution of H₂O₂ was made fresh from a 30% stock solution into HBS just before each experiment. Cultures were exposed to 20 μM H₂O₂ in HBS for 15 min at 37°C. Ral was present in the medium during H₂O₂ exposure. Following 15 min, the cultures were rinsed two times with HBS, and fresh media with and without Ral was replaced. Cultures were returned to the incubator for 24 h before harvesting media for LDH measurements. Statistically significant differences were determined by a one-way ANOVA followed by a Newman–Keuls post hoc analysis for individual comparisons.

![Fig. 2. Effect of raloxifene on hippocampal neuron survival.](image-url) Hippocampal neurons were treated 24 h after seeding with indicated concentration of raloxifene for 4 days. Viable neurons were counted just before raloxifene exposure and at each subsequent 24 h for 4 days following raloxifene exposure. Data are presented as percent of neurons surviving relative to Day 0 (mean ± SEM from three separate experiments; 100 hundred neurons were counted per neuronal culture; seven to eight cultures were analyzed per condition per experiment (*P < 0.05, **P < 0.01, ***P < 0.001).
ANOVA followed by a Newman–Keuls post hoc analysis for individual comparisons.

**Glutamate exposure**

One-week-old neuron cultures were pretreated with Ral for 4 days. On the 11th day, cultures were exposed to 0.2 mM glutamate for 5 min at room temperature in HEPES buffer containing 100 mM NaCl, 2.0 mM KCl, 2.5 mM CaCl₂, 1.0 mM MgSO₄, 1.0 mM NaH₂PO₄, 4.2 mM NaHCO₃, 12.5 mM HEPES and 10.0 mM glucose as previously described (Brinton et al., 1997a,b). Immediately following glutamate exposure, cultures were washed two times with HEPES buffer and replaced with fresh NBM with and without Ral. Cultures were returned to the incubator for 24 h before harvesting media for LDH measurements. Statistically significant differences were determined by a one-way ANOVA followed by a Newman–Keuls post hoc analysis for individual comparisons.

**Beta amyloid₂⁵–₃₅ (Aβ₂⁵–₃₅) exposure**

Aβ₂⁵–₃₅ (Lot Number: B01200), purchased from Bachem, was dissolved in sterile distilled water at a concentration of 1 µg/ml as a stock solution. This stock was aliquoted and stored at −20°C. Seven-day-old cultures were pretreated with varying concentrations of Ral for 4 days in NBM. Exposure to Aβ₂⁵–₃₅ followed the procedure previously described (Behl et al., 1997). On the seventh day of culture, NBM containing 8 µg/ml Aβ₂⁵–₃₅ alone or 8 µg/ml Aβ₂⁵–₃₅ plus varying concentrations of Ral were prepared, and cultures were incubated in test substances for 24 h at 37°C. Following 24 h of exposure to Aβ₂⁵–₃₅, culture medium was exchanged for Aβ₂⁵–₃₅-free medium with Ral. Cultures were returned to

Fig. 3. Effect of raloxifene on Aβ₂⁵–₃₅-induced toxicity in cultured hippocampal neurons. Photomicrograph images show fields of hippocampal neurons grown for 8 days in vitro under the indicated conditions. (A) Hippocampal neurons under control conditions appear healthy with a dark cell body and a phase bright halo and abundant clearly defined neuronal processes. (B) Hippocampal neurons exposed to 8 µg/ml Aβ₂⁵–₃₅ for 24 h display shrunken cell bodies and degeneration of neuronal processes. (C) Hippocampal neurons grown in the presence of 50 ng/ml raloxifene for 4 days before exposure to 8 µg/ml Aβ₂⁵–₃₅ display a clear reduction in features of degeneration. (D) Hippocampal neurons grown in the presence of 500 ng/ml raloxifene for 4 days before exposure to 8 µg/ml Aβ₂⁵–₃₅ exhibit a modest reduction in features of degeneration. Scale bar = 50 µm.
the incubator and allowed to incubate for 24 h before LDH measurements the following day.

Results

Impact of raloxifene on neuronal viability and survival

The neuroprotective effects of raloxifene (Ral) were investigated using three different neurotoxic insults—exposure to β-amyloid peptide25–35, H2O2 and excitotoxic glutamate. These were selected because of their role in degeneration associated with Alzheimer’s disease (Behl et al., 1994; Mattson et al., 1993) and were used at concentrations previously determined to induce a 40–50% decrement in viability (Brinton et al., 1997a,b). In addition, we investigated the cellular specificity of Ral action using hippocampal, cortical and basal forebrain neurons. Two measures of neuronal responses to toxic insults were assessed—LDH release and neuronal survival.

Initially we investigated the impact of Ral directly on measures of neurotoxicity. Analysis of LDH levels revealed that following 4 days of Ral exposure, low concentrations—50 ng/ml in hippocampus, 5–500 ng/ml in cortex and 5 ng/ml in basal forebrain—induced modest but significant reductions in basal levels of LDH release. In contrast, higher concentrations of Ral—1000–5000 ng/ml in hippocampal and cortical neurons and 5000 ng/ml in basal forebrain neurons—induced significant increases in basal LDH release (Fig. 1).

Using this initial data, an analysis of the impact of up to 4 days of exposure to 500–5000 ng/ml Ral on hippocampal neuron survival was conducted revealing that no adverse impact on neuronal survival occurred at 500 ng/ml Ral whereas 2500–5000 ng/ml induced a significant reduction in neuron survival within 48 h of continuous exposure (Fig. 2). This toxicity induced by Ral required concentrations that greatly exceed that achieved in plasma following a 60-mg dose and therefore must be considered a pharmacological and not a physiological effect.

The first series of neuroprotection experiments investigated the impact of Ral on the toxicity induced by a 24-h exposure to the toxic peptide beta amyloid25–35 (Aβ25–35). Neurons derived from the hippocampus were treated for 4 days with either 50 or 500 ng/ml Ral. Following this exposure, media was exchanged for that containing Aβ25–35 for 24 h. The following day, media was harvested from all cultures and assayed for LDH content. Results are expressed as percent of Aβ25–35-induced LDH release. Data are expressed as mean ± SEM and were combined across three separate experiments, n = 12 per condition. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with Aβ25–35-treated cultures.
in the absence or presence of Ral and after 24 h, degeneration of cultured hippocampal neuron processes and toxicity was apparent (Figs. 3A and B). Hippocampal neurons treated with a subtoxic concentration of Ral (50 ng/ml) exhibited a lesser degree of Aβ25-35-induced degeneration of neuronal processes than those treated with the higher Ral concentration of 500 ng/ml (Figs. 3C and D) and this was consistent with the magnitude of reduction observed in LDH release (Fig. 4A). Analysis of β-amyloid-induced LDH release demonstrated that exposure of hippocampal, cortical and basal forebrain neurons to nanogram quantities of Ral (5–500 ng/ml) significantly reduced the LDH released.

Fig. 5. Neuroprotective effect of raloxifene on H2O2-induced toxicity in hippocampal neurons. Photomicrograph images show fields of hippocampal neurons grown for 7 days in vitro under the indicated conditions. Left panel displays images of hippocampal neurons 24 h before exposure to hydrogen peroxide (left side) whereas the right panel shows the same field of neurons 24 h later with the middle and lower right panel showing neurons after a 15-min exposure to 20 μM H2O2 (right side). Control neurons appear healthy with dark cell bodies and long neuronal processes. Hippocampal neurons before H2O2 exposure exhibit the same features of viability as control neurons, whereas following exposure to H2O2 these neurons exhibited shrunken cell bodies and a loss of process integrity. Many neurons failed to survive. Hippocampal neurons exposed to 50 ng/ml raloxifene 4 days before exposure to H2O2 exhibited diminished toxicity and increased survival relative to neurons exposed only to H2O2. Scale bar = 50 μm.
following exposure to 8 μg/ml Aβ25–35 (Fig. 4). Thus, the significant neuroprotective effect of Ral was apparent in all three neuronal populations investigated. Also apparent in all neuronal populations was a U-shaped dose response showing the greatest neuroprotective efficacy and potency in hippocampal neurons in response to 50 ng/ml Ral [a 40% reduction in LDH release (Fig. 4A); $F = 7.78; df 4,55; P < 0.0001$]. This neuroprotective effect diminished at higher concentrations, and at the highest concentration, 5000 ng/ml, no neuroprotection was achieved. In cortex, Ral had a similar potency but a lesser efficacy with significant neuroprotection occurring at 5 ng/ml; however, the magnitude of neuroprotection is less than that observed in the hippocampus (see Fig. 4B; $F = 6.26; df 4,55; P < 0.0003$). In basal forebrain neurons, Ral exhibited the least potency and least efficacy of neuroprotection and only induced a significant

Fig. 6. Effect of raloxifene on survival of H$_2$O$_2$-treated hippocampal neurons. Hippocampal neurons were treated with indicated concentration of raloxifene for 4 days before exposure to 20 μM H$_2$O$_2$. Viable neurons were counted just before H$_2$O$_2$ exposure and 24 h following H$_2$O$_2$ exposure. Data are presented as percent of control neuron survival (mean ± SEM from three separate experiments; 100 hundred neurons were counted per neuronal culture; seven to eight cultures were analyzed per condition per experiment). **$P < 0.01$ compared with H$_2$O$_2$-treated cultures.

Fig. 7. Effect of raloxifene on H$_2$O$_2$-induced LDH release. Primary hippocampal (A), cortical (B) and basal forebrain (C) neurons (7–9 days in vitro) were grown in the absence or presence of raloxifene for 4 days followed by exposure to 20 μM H$_2$O$_2$ for 15 min at 37°C. Media was exchanged following the 15-min exposure to H$_2$O$_2$ for media with or without raloxifene. Following 24 h, media was harvested from all cultures and assayed for LDH content. Results are expressed as percent of H$_2$O$_2$-induced LDH release. Data are expressed as mean ± SEM and are combined across three separate experiments, $n = 18$ per condition, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with H$_2$O$_2$-treated cultures.
neuroprotective effect at 500 ng/ml [a 35% reduction in LDH release (see Fig. 4C) \( F = 2.97; df 4,55; P < 0.0272 \)]. The rank order of potency and magnitude of raloxifene-induced neuroprotection against \( A_{[25-35]} \) in the brain regions investigated was hippocampal neurons > cortical neurons > basal forebrain.

We next investigated the ability of Ral to act as a neuroprotective agent against \( H_2O_2 \). Hippocampal neurons exposed to 20 \( \mu M \) \( H_2O_2 \) exhibited pronounced morphological degeneration and death (Fig. 5). In contrast, hippocampal neurons exposed to 50 ng/ml Ral 4 days before exposure to \( H_2O_2 \) exhibited diminished toxicity and increased survival relative to neurons exposed only to \( H_2O_2 \) (Fig. 5). Quantitative analysis of the impact of Ral on \( H_2O_2 \)-induced hippocampal neuron death demonstrated that \( H_2O_2 \) alone induced death in 80% of the neurons, whereas neurons pretreated with 5 and 50 ng/ml of Ral had a 40% death rate such that 60% of neurons survived (Fig. 6); higher concentrations did not significantly promote neuron survival (Fig. 6). Biochemical analysis of the impact of Ral on LDH release following \( H_2O_2 \) exposure to hippocampal, cortical and basal forebrain neurons demonstrated that pretreatment induced significant reductions in \( H_2O_2 \)-induced LDH release (Fig. 7). Hippocampal neurons exhibited a maximum 25% reduction in \( H_2O_2 \)-induced LDH release, which occurred at 500 ng/ml (Fig. 7A; \( F = 25.57; df 4,55; P < 0.0001 \)) but neuroprotection was but still apparent at 1000 ng/ml. Cortical neurons also exhibited significant reduction in \( H_2O_2 \)-induced LDH release in the presence of 5 ng/ml Ral, which was maximal at 50 ng/ml and diminished in response to higher concentrations (Fig. 7B; \( F = 20.22; df 4,55; P < 0.0001 \)). The response in basal forebrain neurons was greater than in either hippocampus or cortex. At 5 ng/ml, Ral significantly reduced \( H_2O_2 \)-induced LDH release, and maximal neuroprotection was achieved at 50 ng/ml (Fig. 7C) with a 30–56% reduction in LDH release. The

![Fig. 8. Neuroprotective effect of raloxifene against glutamate-induced toxicity in cultured hippocampal neurons. Photomicrograph images show fields of hippocampal neurons grown for 8 days in vitro under the indicated conditions. (A) Hippocampal neurons under control conditions appear healthy with a dark cell body and a phase bright halo and abundant clearly defined neuronal processes. (B) Hippocampal neurons exposed to 0.2 mM glutamate for 5 min and visualized 24 h later display shrunken cell bodies and degeneration of neuronal processes. (C) Hippocampal neurons grown in the presence of 50 ng/ml raloxifene for 4 days before exposure to 0.2 mM glutamate for 5 min displayed a marked reduction in features of degeneration. (D) Hippocampal neurons grown in the presence of 500 ng/ml raloxifene for 4 days before exposure to 0.2 mM glutamate for 5 min display clear features of neuronal viability with a dark cell body and a phase bright halo and abundant clearly defined neuronal processes similar to those of control neurons not treated with 0.2 mM glutamate. Scale bar = 50 \( \mu m \).]
rank order of potency and magnitude of Ral neuroprotection from H₂O₂ toxicity in the brain regions investigated was basal forebrain > hippocampal neurons > cortical neurons.

Lastly, the impact of Ral on glutamate-induced toxicity in neuron cultures was determined. Results of these analyses demonstrated that 200 μM glutamate induced the visible and pronounced toxicity of hippocampal neurons (Figs. 8A and B) and the pretreatment of hippocampal neurons with 50 or 500 ng/ml Ral for 4 days exerted a neuroprotective effect against the neurotoxic effects of glutamate (Figs. 8C and D). Quantitative biochemical assessment demonstrated that Ral exerted a significant reduction in glutamate-induced LDH release (Fig. 9). Specifically, in hippocampal neurons, a significant reduction in LDH release was maximal at 5 ng/ml of Ral and sustained up to 500 ng/ml (F = 21.95; df, 5.66; P < 0.0001) but loss of neuroprotective efficacy occurred at 1000 ng/ml and a significant increase in LDH occurred at 5000 ng/ml (Fig. 9A). The magnitude of the Ral-induced reduction in glutamate-induced toxicity ranged from 7% to 20% across the dose-response range. Ral also induced a significant reduction in glutamate-induced LDH release in cortical neurons (F = 73.59; df, 4.60; P < 0.0001) with maximal neuroprotection at 500 ng/ml and a significant increase at 5000 ng/ml (see Fig. 9B). The magnitude of the Ral-induced reduction in glutamate-induced toxicity ranged from 15% to 43% across the dose-response range. In basal forebrain neurons, Ral was less effective, but still exhibiting a significant reduction in LDH release at 500 ng/ml (F = 9.84; df, 5.64; P < 0.0001) and a significant increase occurring at the 5000 ng/ml concentration (Fig. 9C). The magnitude of the raloxifene-induced reduction in LDH release from basal forebrain neurons ranged from 14% to 45% across the dose-response range. The rank order of potency and magnitude of Ral neuroprotection against glutamate toxicity in the brain regions investigated was cortical neurons > hippocampal neurons > basal forebrain neurons.

**Impact of raloxifene in the presence of 17 β-estradiol on neuronal survival following exposure to neurotoxins**

To determine whether Ral would act competitively or synergistically with 17 β-estradiol (E₂), a series of experiments was conducted to determine the impact of the combined presence of Ral and E₂ on neuronal survival following exposure to either Aβ[25–35] or glutamate. The concentration of 13 pg/ml E₂ used in this experiment Fig. 9. Effect of raloxifene on glutamate-induced LDH release. Primary hippocampal (A), cortical (B) and basal forebrain (C) neurons (7–9 days in vitro) grown in the absence or presence of raloxifene for 4 days were exposed to 0.2 mM glutamate for 5 min at room temperature. The following day media was harvested from all cultures and assayed for LDH content. Results are expressed as percent of glutamate-induced LDH release. Data are expressed as mean ± SEM and are combined across three separate experiments, n = 18 per condition, *P < 0.05, **P < 0.01 and ***P < 0.001 compared with glutamate-treated cultures.
corresponds to the average plasma level in postmenopausal women (Soules and Bremner, 1982; Trevoux et al., 1986). Results of these experiments demonstrated that hippocampal neurons pretreated with either Ral or E₂ followed by exposure to Aβ25–35 had a significantly greater increase in neuron survival compared to neurons exposed to Aβ25–35 alone \((F = 54.43; \text{df} 4,25; P < 0.001; \text{Fig. 10A})\). Not remarkably, neurons pretreated with 13 pg/ml E₂ showed a significantly greater increase in neuronal survival (25% increase in survival) against Aβ25–35-induced toxicity compared to neurons exposed to Aβ25–35 alone \((F = 54.43; \text{df} 4,25; P < 0.001; \text{Fig. 10A})\). Not remarkably, neurons pretreated with 13 pg/ml E₂ showed a significantly greater increase in neuronal survival (25% increase in survival) against Aβ25–35-induced toxicity compared to 50 ng/ml Ral (12% increase in neuronal survival) and the combined presence of Ral and E₂ was still neuroprotective but was significantly lower than E₂ alone while not significantly different than Ral alone (13% increase in neuronal survival). The same response profile occurred in response to glutamate-induced excitotoxicity. Ral alone exerted a modest but significant increase in neuronal survival following exposure to 200 μM glutamate (14% increase in neuronal survival; \(F = 59.99; \text{df} 4,39; P < 0.001\); \text{Fig. 10B}). As with Aβ25–35, 13 pg/ml E₂ showed a significantly greater increase (24% increase in neuronal survival) against glutamate-induced excitotoxicity compared to 50 ng/ml Ral. Finally, the combined presence of Ral and E₂ was still significantly neuroprotective against glutamate-induced excitotoxicity but was lower than E₂ alone while not significantly different than Ral alone (11% increase in neuronal survival). Together, these data indicate that Ral acted as a partial agonist or as a partial antagonist in the presence of E₂.

**Impact of raloxifene on neuronal outgrowth**

The goal of these analyses was to determine whether Ral would exert estrogen agonist action to promote cellular mechanisms associated with memory function. Our previous work investigating the neurotrophic action of individual estrogens, 17β-estradiol (E₂) and equilin indicated that a neurotrophic effect in one neuronal population did not predict a neurotrophic response in another nerve cell population (Brinton et al., 1997a,b, 2000). We therefore investigated the neurotrophic action of Ral in neurons derived from the hippocampus, cerebral cortex and basal forebrain. Each of these brain regions is involved in learning and memory (Posner et al., 1988) and vulnerable to the degenerative process of Alzheimer’s disease (Solodkin and Van Hoesen, 1997).

In neurons derived from the hippocampus, Ral significantly increased both macrofeatures of neuronal morphology, number of neurites \((F = 4.61; \text{df} 4,265; P < 0.0013)\) and neurite length \((F = 7.91; \text{df} 4,265; P < 0.0000)\) (Fig. 11). The microfeatures of neuronal morphology were also significantly increased with total number of branches \((F = 17.05; \text{df} 4,265; P < 0.0000)\), branch length \((F = 17.31; \text{df} 4,265; P < 0.0000)\), total number of branch bifurcations \((F = 6.26; \text{df} 4,265; P < 0.0001)\) and number of microspikes \((F = 8.06; \text{df} 4,265; P < 0.0000)\), all
showing greater abundance (Fig. 11). Analysis of the dose-response profile indicated that increased neuronal process outgrowth occurred at two concentrations—50 and 5000 ng/ml—and that the dose-response profile was biphasic with lower and intermediate concentrations exerting no neurotrophic effect while 50 and 5000 induced a
Neurotrophic effect. Such a profile suggests that the mechanism(s) underlying Ral-induced neuronal process outgrowth is strictly regulated by concentration and that either competing mechanisms are activated by Ral, one that is neurotrophic and another that is limiting, or that activation of the neurotrophic mechanism is differentially regulated directly by concentration.

Analysis of the effect of Ral upon either cortical or basal forebrain neuron morphology indicated that Ral was without significant effect (Tables 1 and 2). Results of the dose-response analysis in occipital cortex neurons demonstrated that Ral was ineffective in promoting neuronal process outgrowth (Table 1) in that neither of the microstructures—number of neurites ($F = 0.75$; $df = 4,362$; $P < 0.5594$) nor neurite length ($F = 1.94$; $df = 4,362$; $P < 0.1025$)—were significantly affected. No significant difference in microstructure occurred between control and Ral-treated neurons whereas statistically significant differences occurred between Ral concentrations ($F = 3.23$; $df = 4,362$; $P < 0.0127$; Table 1). Branch length was similarly affected ($F = 2.80$; $df = 4,362$; $P < 0.026$) with post hoc analysis of individual comparisons revealing no significant differences between control and the neurons treated with Ral. Also, the number of branch bifurcations was not significantly affected ($F = 3.30$; $df = 4,362$; $P < 0.0876$), but Ral did exert a significant suppressive effect on the number of microspikes ($F = 9.04$; $df = 4,362$; $P < 0.0000$) with concentrations at 50 ng/ml and above significantly decreasing microspike number (Table 1).

Results of analyses of basal forebrain neurons indicated no statistically significant effect of Ral on either the macrofeatures of basal forebrain morphology—number of neurites ($F = 0.95$; $df = 4,370$; $P < 0.4347$) and neurite length ($F = 0.90$; $df = 4,370$; $P < 0.4666$; Table 2). Similarly, macrofeatures were not affected by Ral exposure with number of branches ($F = 0.91$; $df = 4,370$; $P < 0.4583$), branch length ($F = 0.82$; $df = 4,370$; $P < 0.5136$), number of branch bifurcation points ($F = 1.00$; $df = 4,370$; $P < 0.4082$) and number of microspikes ($F = 0.85$; $df = 4,370$; $P < 0.4963$), all exhibiting no significant effect (Table 2).

Mechanism of raloxifene-induced outgrowth of hippocampal neurons

Previous work from several laboratories has demonstrated that the neurotrophic effect of 17 β-estradiol (E2) and other neurotrophic estrogens is dependent on the potentiation of the NMDA receptor (Brinton et al., 1997a,b; Foy et al., 1999; Woolley and McCauley, 1994). Our previous work has demonstrated that NMDA receptors are both expressed and functional in neurons derived from the E18 rat brain following 24 h in culture (Brinton et al., 1997a,b). Based on

Table 1
Neurotrophic efficacy of raloxifene in cultured cortical neurons

<table>
<thead>
<tr>
<th>Raloxifene concentration (ng/ml)</th>
<th>Length of neurites (μm)</th>
<th>Number of neurites</th>
<th>Length of branches (μm)</th>
<th>Number of branches</th>
<th>Number of bifurcations</th>
<th>Number of microspikes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>300.92 ± 23.90</td>
<td>4.50 ± 0.47</td>
<td>175.20 ± 20.82</td>
<td>6.13 ± 0.81</td>
<td>1.21 ± 0.47</td>
<td>13.76 ± 4.39</td>
</tr>
<tr>
<td>5</td>
<td>282.31 ± 19.43</td>
<td>4.52 ± 0.34</td>
<td>191.97 ± 23.69</td>
<td>6.43 ± 0.88</td>
<td>1.16 ± 0.70</td>
<td>13.76 ± 4.41</td>
</tr>
<tr>
<td>50</td>
<td>278.71 ± 19.06</td>
<td>4.32 ± 0.32</td>
<td>156.71 ± 24.31</td>
<td>5.70 ± 0.71</td>
<td>1.05 ± 0.57</td>
<td>11.41 ± 2.60</td>
</tr>
<tr>
<td>500</td>
<td>274.13 ± 23.82</td>
<td>4.55 ± 0.07</td>
<td>163.13 ± 0.05</td>
<td>5.93 ± 0.31</td>
<td>1.35 ± 0.34</td>
<td>10.23 ± 1.72</td>
</tr>
<tr>
<td>5000</td>
<td>270.58 ± 5.83</td>
<td>4.73 ± 0.04</td>
<td>160.77 ± 30.83</td>
<td>5.79 ± 1.33</td>
<td>1.17 ± 0.33</td>
<td>11.25 ± 1.73</td>
</tr>
</tbody>
</table>

Cortical neurons derived from E18-d rat fetuses were cultured in serum-free Neurobasal media in the absence and presence of raloxifene at concentrations of 5, 50, 500 and 5000 ng/ml for 24 h. Seventy-five cells per condition were assessed for neurotrophic parameters, including length of neurites, number of neurites, length of branches, number of branches, number of branch bifurcations, and number of microspikes. Data are derived from two separate experiments. No significant effect on six morphological indicators of neuronal process outgrowth was observed in raloxifene-treated cells.

Table 2
Neurotrophic efficacy of raloxifene in cultured basal forebrain neurons

<table>
<thead>
<tr>
<th>Raloxifene concentration (ng/ml)</th>
<th>Length of neurites (μm)</th>
<th>Number of neurites</th>
<th>Length of branches (μm)</th>
<th>Number of branches</th>
<th>Number of bifurcations</th>
<th>Number of microspikes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>198.70 ± 5.19</td>
<td>4.07 ± 0.11</td>
<td>125.11 ± 6.21</td>
<td>5.17 ± 0.29</td>
<td>1.01 ± 0.14</td>
<td>7.53 ± 0.79</td>
</tr>
<tr>
<td>5</td>
<td>198.65 ± 17.78</td>
<td>4.02 ± 0.02</td>
<td>125.31 ± 4.60</td>
<td>4.89 ± 0.26</td>
<td>1.28 ± 0.11</td>
<td>7.96 ± 1.70</td>
</tr>
<tr>
<td>50</td>
<td>187.94 ± 12.80</td>
<td>3.95 ± 0.22</td>
<td>122.15 ± 6.34</td>
<td>5.01 ± 0.25</td>
<td>1.02 ± 0.06</td>
<td>7.03 ± 0.87</td>
</tr>
<tr>
<td>500</td>
<td>189.19 ± 4.90</td>
<td>3.84 ± 0.08</td>
<td>118.88 ± 8.18</td>
<td>4.83 ± 0.34</td>
<td>0.98 ± 0.08</td>
<td>6.74 ± 0.46</td>
</tr>
<tr>
<td>5000</td>
<td>178.04 ± 5.09</td>
<td>3.88 ± 0.03</td>
<td>114.17 ± 6.05</td>
<td>4.45 ± 0.16</td>
<td>0.99 ± 0.23</td>
<td>7.35 ± 0.98</td>
</tr>
</tbody>
</table>

Basal forebrain neurons derived from E18-d rat fetuses were cultured in serum-free Neurobasal media in the absence and presence of raloxifene at concentrations of 5, 50, 500 and 5000 ng/ml for 24 h. Seventy-five cells per condition were assessed for neurotrophic parameters, including length of neurites, number of neurites, length of branches, number of branches, number of branch bifurcations and number of microspikes. Data are derived from three separate experiments. No significant effect on six morphological indicators of neuronal process outgrowth was observed in raloxifene-treated cells.
these data, we investigated whether Ral-induced neuronal outgrowth was mediated through the same mechanism as that found for E₂, that is, an NMDA-receptor-dependent mechanism by using an NMDA receptor inhibitor, AP-5. Again, videorecording and morphological analysis of hippocampal neurons were conducted 24-h post exposure and blind to the experimental condition. Results of these experiments indicated that neurons treated with 50 ng/ml Ral had significantly increased outgrowth of both macro- and micro-features of hippocampal neuron morphology (number of neurites, $F = 4.00; df = 3,294; P < 0.0082$; length of neurites, $F = 25.81; df = 3,294; P < 0.0000$; number of branches, $F = \ldots$)

Fig. 12. Raloxifene-induced neurotrophism is blocked by an NMDA glutamate receptor antagonist. Hippocampal neurons derived from embryonic day 18 rat fetuses were cultured in the presence or absence of 50 ng/ml raloxifene, 10 μM AP-5, or in the presence of the both raloxifene and AP-5. Seventy-five neurons per condition were morphologically analyzed blind to the experimental condition. Data represent mean ± SEM, $n = 75$ neurons per condition and are representative of three separate analyses. Results of these experiments indicate that the growth-promoting effects of raloxifene were abolished in the presence of the NMDA receptor antagonist AP-5 (*$P < 0.05$, ***$P < 0.001$).
inhibition of protein kinase C kinases vital to cell survival as is the case for tamoxifen. The mechanism for the neurotoxic effect of Ral remains unexplained, however, Ral exerted a neurotoxic effect at high concentrations that mimicked the SERMs tamoxifen agonists, however, Ral exerted a neurotoxic effect at high concentrations that mimicked the effect of estrogen agonists like 17 β-estradiol (E2) (Brinton et al., 1997a,b), conjugated equine estrogens (Brinton et al., 2000) and select phytoestrogens (Zhao et al., 2002). Unlike full estrogen agonists, however, Ral exerted a neurotoxic effect at high concentrations that mimicked the SERMs tamoxifen (O’Neill et al., 1999) and genistein inhibition of protein kinase C (Rowlands et al., 1995) and genistein inhibition of tyrosine kinase (Akiyama et al., 1987).

**Discussion**

Raloxifene (Ral) exerted a mixed effect on neuronal biological integrity that was dose-dependent. At low concentrations, Ral reduced the biochemical marker of plasma membrane damage that mimicked the effect of estrogen agonists like 17 β-estradiol (E2) (Brinton et al., 1997a,b), conjugated equine estrogens (Brinton et al., 2000) and select phytoestrogens (Zhao et al., 2002). Unlike full estrogen agonists, however, Ral exerted a neurotoxic effect at high concentrations that mimicked the SERMs tamoxifen (O’Neill et al., 1999) and genistein inhibition of protein kinase C (Rowlands et al., 1995) and genistein inhibition of tyrosine kinase (Akiyama et al., 1987).

**Neuroprotection by raloxifene**

Three different neurotoxic insults, β-amyloid peptide 25–35, H2O2 and glutamate, were investigated because of their proposed role in degeneration associated with Alzheimer’s disease (Ames et al., 1993; Behl, 1997; Behl et al., 1994; Mattson et al., 1993). Also investigated was the regional selectivity of Ral-induced neuroprotection against these three different neurotoxins. The results of these experiments revealed that Ral was neuroprotective against each of the neurotoxins investigated and that analysis of results from different brain regions revealed differences in the magnitude and potency of Ral-induced neuroprotection.

Hippocampal neurons exhibited the greatest Ral-induced neuroprotection followed by cortex and basal forebrain. Preexposure to < 5000 ng/ml Ral was significantly neuroprotective against β-amyloid 25–35 (Aβ 25–35)-induced toxicity. The concentration-response profile for Ral-induced neuroprotection against Aβ 25–35-induced toxicity was U-shaped, with lower concentrations exerting neuroprotection. On the other hand, higher concentrations exerted less neuroprotective efficacy. The 40% reduction in LDH release induced by 50 ng/ml of Ral in hippocampal neurons was associated with a 12% increase in hippocampal neuron survival compared to Aβ 25–35 alone. When compared to full estrogen agonists, which induce 70–80% survival (Brinton et al., 2000), Ral appears as a partial estrogen agonist against Aβ 25–35-induced neuron death.

Preexposure to Ral exerted a significant neuroprotective effect against free radical damage induced by hydrogen peroxide. Somewhat surprisingly, the magnitude of the effect of Ral on neuron survival was comparable to that observed previously for 17 β-estradiol and conjugated equine estrogens (Brinton et al., 1997a,b, 2000). Paradoxically, the rather modest effect of Ral on LDH release would not have predicted the magnitude of neuron survival, which was substantial. The reason for this disparity remains unsolved but may be due to the different days in vitro at which the neurons were assessed for the morphological analyses (7 DIV) vs. the LDH analyses, which were conducted at 12 DIV. Remarkably, Ral exerted the greatest neuroprotective effect in basal forebrain neurons followed by hippocampal neurons and then cortical neurons.

Preexposure of hippocampal, basal forebrain or cortical neurons to Ral resulted in a significant reduction in glutamate-induced excitotoxicity. The magnitude of Ral-induced neuroprotection was greatest in cortical neurons followed by basal forebrain then by hippocampal neurons. In cortical and basal forebrain neurons, 500 ng/ml Ral exerted maximal neuroprotection. In hippocampal neurons, the neuroprotective effect was modest, with maximal efficacy occurring at 5 ng/ml Ral. In each of the neuronal populations, the dose response was U-shaped, with higher concentrations exerting a diminished neuroprotective effect. The 20% reduction in LDH release induced by 50 ng/ml of Ral in hippocampal neurons was associated with a 14% increase in hippocampal neuron survival compared to glutamate alone. When compared to full estrogen agonists that induce 70–80% survival (Brinton et al., 2000), Ral appears to act as a partial estrogen agonist against glutamate-induced neuron death.

Experiments to determine whether Ral would act competitively or synergistically with E2 revealed that Ral acted as partial agonist and a partial antagonist. In the absence of E2, Ral exerted modest estrogen agonist activity. In the presence of E2, Ral still exerted modest estrogen agonist activity but antagonized the effect of E2. These data suggest that Ral acts competitively with E2 to activate mechanisms of neuroprotection. Together, these data indicate that Ral acted as a partial agonist and partial antagonist in the presence of E2.

**Proposed mechanism for raloxifene neuroprotection**

An antioxidant effect for estrogens has been demonstrated in a broad range of cell types (Behl et al., 1995; Brinton et al., 2000; Green et al., 1997; Klein and Berlin, 1996). Data from several laboratories indicate that estrogens could activate multiple mechanisms and that activation of these mechanisms can lead to protection against a wide range of neurotoxic agents. Several candidate mechanisms include regulation of glutathione action and activation of mitogen-activated protein kinases, ERK1 and ERK2 (Green et al., 1998; Singer et al., 1999; Singh et al., 2000). Given that estrogen-induced neuroprotection potentially involves mul-
multiple mechanism, the partial agonist profile of Ral might be explained by either partial activation of all estrogen inducible neuroprotective mechanisms or activation of a subset of these mechanisms. It is unlikely that the partial agonist–antagonist effects of Ral observed in the current study are due to the absence of conversion products because glucuronide conjugates of Ral exhibit little affinity for estrogen receptors and are more than two orders of magnitude less potent in other organ systems (Dodge et al., 1997). We are currently pursuing the mechanism of Ral-induced neuroprotection against each of the neurotoxic agents investigated in the current study.

The partial agonist or partial antagonist action of Ral could be further explained by two potential interactions, competitive inhibition of estradiol for the same site of action or partial induction of conformational requirements for full receptor function. The partial agonist or partial antagonist action observed in this study and that of Wu et al. (1999) suggests that both mechanisms are at work—competitive inhibition and partial activation. It is unclear whether the estrogen-induced neuroprotection requires a nuclear estrogen receptor and/or a nuclear site of action (Singer et al., 1999; Singh et al., 1999). However, the interaction of Ral with ER–α could provide a conceptual understanding of the partial agonist or partial antagonist actions of Ral (Wijayarathne et al., 1999) in the nervous system that expands on the model of competitive antagonism by a weak agonist. Using electron crystallography, Brzozowski et al. (1997) demonstrated that while Ral enters the binding pocket of the estrogen receptor, the activation process is inhibited due to the molecular structure of Ral. Ral has an alkylaminoethoxy side chain that physically protrudes from the binding pocket. Levenson and Jordan (1998) found that a nitrogen atom in the side chain binds to residue aspartate 351 in the ligand-binding domain of the ER and splints the key helix 12 of the ligand-binding domain. This then prevents the normal wild-type rotation to seal the pocket and configure the AF-2 domain for the adherence of coactivators (Levenson and Jordan, 1998) blocking transcription. Indeed, it is clear that mutation of aspartate 351 to a tyrosine residue will convert Ral from an estrogen antagonist to an agonist. Thus, in tissues such as breast and endometrium, where intact AF-2 Ral remains little affinity for estrogen receptors and in two orders of magnitude less potent in other organ systems (Dodge et al., 1997). We are currently pursuing the mechanism of Ral-induced neuroprotection against each of the neurotoxic agents investigated in the current study.

Neurotrophism by raloxifene

Three distinctive features of Ral-induced neuronal process outgrowth emerged. First, the neurotrophic effect of Ral was neuron population specific. Ral induced a neurotrophic effect in hippocampal neurons but was not neurotrophic for cortical or basal forebrain neurons. The mechanism underlying the regional differences that would account for estrogen agonist action in the hippocampus and not in cortex and basal forebrain remains unknown, but is a topic of current investigation in our laboratory.

Second, in hippocampal neurons, the dose response was not linear but biphasic. This biphasic type of dose-response profile is reminiscent of that induced by E2 (Brinton et al., 1997a,b). Nonlinear dose–response curves for full estrogen agonists, E2 (Brinton et al., 1997a,b) and conjugated equine estrogens (Brinton et al., 2000), appear to be a consistent feature of this response. Similarly, a nonlinear dose-response profile was also observed for Ral-induced process outgrowth in NGF-treated PC 12 cells (Nilsen et al., 1998).

An obvious question that emerges from the dose-response analysis is why 5000 ng/ml Ral induced hippocampal neuron process outgrowth whereas this same concentration led to a significant decrease in neuronal survival. This paradox could be a function of the sampling paradigm for the different experiments. For the neurotrophic experiments, only surviving neurons were recorded for analysis whereas for the survival experiments viable and nonviable neurons were analyzed in all conditions. Alternatively, since Ral is promoting neuron process outgrowth in hippocampal neurons through an NMDA-receptor-dependent mechanism, Ral may be preferentially affecting healthy neurons and promoting the demise of neurons with compromised calcium buffering capacity.

Third, the neurotrophic effect of Ral in hippocampus was dependent on the glutamate NMDA receptor function comparable to that observed for full estrogen agonists. Blockade of the NMDA glutamate receptor abolished the neurotrophic effect of Ral as it had E2 and equilin-induced neurotrophism (Brinton et al., 1997a,b; Woolley and McEwen, 1994; Woolley et al., 1997). The mutual dependency of E2, equilin (a full estrogen agonist) and Ral on the glutamate NMDA receptor for expression of the neurotrophic response supports the postulate that in this population of neurons, on this response, Ral acts as an estrogen agonist.

The ability of Ral to promote hippocampal neuron process outgrowth suggests that Ral could act as an estrogen agonist to promote short-term memory function within the hippocampus. However, the dose–response curve for Ral-induced hippocampal neuron outgrowth would predict that any estrogenic effect would be highly dose dependent and that the range of efficacious concentrations would be very narrow. The lack of an effect in cortical and basal forebrain neurons indicates that not all regions of the brain involved in memory function are similarly affected by Ral. Data from human trials indicate that Ral does not reverse the memory deficits associated with estrogen loss in postmenopausal women (Nickelsen et al., 1999; Yaffe et al., 2001). These
data are consistent with the prediction that concentrations of Ral required to promote neurite outgrowth in vitro are not achieved in vivo and that the partial agonist properties of Ral are not sufficient to promote memory function in humans.

Potential functional significance of raloxifene-induced neuroprotection and neurotrophism

The current investigation sought to determine whether the SERM Ral exerted estrogen agonist or antagonist effects in neurons involved in memory function and adversely affected in Alzheimer’s disease. The neuroprotective effects of Ral would predict that some beneficial effect would be achieved from low doses of Ral but not from higher doses. The magnitude of the neuroprotection varied across the neuronal populations investigated and ranged from a 10% to 50% reduction in markers of neuronal damage depending on the neuronal population. It remains to be determined whether this magnitude of neuroprotection could lead to a decreased risk of developing Alzheimer’s disease, as has been found with estrogen replacement therapy (Yaffe et al., 1998; Zandi et al., 2002).

Results of these studies indicated that Ral acts as a partial estrogen agonist in the absence of 17β-estradiol (E2), while it exerts a partial antagonism effect in the presence of E2. While the concentration of Ral in brain achieved with the 60 mg therapeutic dose remains to be determined, it is evident that sufficient quantities can cross the blood–brain barrier to increase the frequency of hot flushes, a hypothalamic effect of estrogen receptor antagonists (Cohen and Lu, 2000). The toxicity induced by Ral requires concentrations that greatly exceed that achieved in plasma following a 60-mg dose and therefore must be considered a pharmacological and not a physiological effect.

The data within this report would predict that Ral could act as a partial estrogen agonist in brain to provide some neuroprotective benefit. The data would further predict that Ral could act as a partial estrogen agonist in hippocampus to promote memory function, but that the concentration required to promote the effect may likely exceed which that can cross the blood–brain barrier, and that range of effective concentrations would be very narrow.

Acknowledgments

This research was supported by grants from the National Institutes of Aging (PO1 AG1475:Project 2), Wyeth-Ayerst Laboratories, the Kenneth T. and Eileen L. Norris Foundation to RDB and a predoctoral fellowship from the American Foundation for Pharmaceutical Education to KO. The authors gratefully acknowledge the contributions of USC STAR Program students, Jasmin Minaya, George Oji, Jeffrey Kim and Alisa Sirinkingkaew.

References


Brinton, R.D., Proffitt, P., Tran, J., Luu, R., 1997a. Equilin, a principal component of the estrogen replacement therapy premarin, increases the growth of cortical neurons via an NMDA receptor-dependent mechanism. Exp. Neurol. 147, 211–220.


