Prevention of age-related dysregulation of calcium dynamics by estrogen in neurons

Gregory J. Brewer a,*, Joel D. Reichensperger a, Roberta D. Brinton b

a Departments of Neurology, Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School Medicine, P.O. Box 19626, Springfield, IL 62794-9626, USA
b Department of Molecular Pharmacology and Toxicology, University of Southern California School of Pharmacy, Los Angeles, CA 90089, USA

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Abstract

To determine the impact of aging and 17β-estradiol on neuronal Ca2+ homeostasis, intracellular Fura-2 Ca2+-imaging was conducted during 20-pulses of glutamate in hippocampal neurons cultured from embryonic (E18), middle-age (10 months) and old (24 months) rat brain. Marked age-related differences in intracellular Ca2+ ([Ca2+]i) homeostasis and striking regulation by 17β-estradiol were seen. Embryonic neurons exhibited the greatest capacity to regulate Ca2+ homeostasis followed by middle-age neurons. In old neurons, the first peak [Ca2+]i was substantially greater than at other ages and the return to baseline [Ca2+]i rapidly dysregulated with an inability to restore [Ca2+]i following the first glutamate pulse which persisted throughout the 20 pulses. 17β-Estradiol pretreatment of old neurons profoundly attenuated the peak [Ca2+]i rise and delayed the age-associated dysregulation of baseline [Ca2+]i, normalizing responses to those of middle-age neurons treated with estradiol. The efficacy of 17β-estradiol extended below 10 pg/ml with full protection against toxicity from glutamate and Aβ (1–40).

These results demonstrate age-associated dysregulation of [Ca2+]i homeostasis which was largely prevented by 17β-estradiol with implications for estrogen/hormone therapy.

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1. Introduction

Aging is associated with increased susceptibility to toxic insults [8] and dysregulation of intracellular Ca2+ homeostasis which are linked with or are causative for neurodegenerative disease [23–25]. The Ca2+ homeostasis dysregulation hypothesis of brain aging and neurodegeneration proposes that basal levels of [Ca2+]i increase in aging neurons and that return to baseline [Ca2+]i following stimulation is compromised [40,41]. Loss of [Ca2+]i homeostasis is implicated in several brain disorders including stroke, epileptic seizures and in the pathogenesis of Alzheimer’s disease (AD) [28,24].

Alterations in [Ca2+]i homeostasis could arise from multiple regulatory mechanisms, including [Ca2+]i buffering, extrusion and/or influx of Ca2+ [26,41,16]. A major instigator of the loss in Ca2+ homeostasis in these neurological disorders is glutamate excitotoxicity. Glutamate-dependent cell death results from over activation of glutamate receptors, excessive Ca2+ influx through the NMDA receptor with subsequent energy depletion and oxidative stress [15,4].

Pretreatment of embryonic neurons with clinically relevant estrogens can markedly attenuate glutamate-induced excitotoxicity and neuronal death in embryonic neurons [12,10,33,36,37]. The glutamate excitotoxicity caused by an excessive rise in [Ca2+]i via NMDA receptor activation is significantly attenuated in neurons pretreated with 17β-estradiol or conjugated equine estrogens and is coupled with a concomitant increase in survival relative to untreated neurons [12,10,33]. Nilsen and Brinton have provided evidence that maintenance of [Ca2+]i homeostasis is a pivotal component of estrogen-mediated neuroprotection which is mediated by mitochondrial sequestration of Ca2+ [31]. Recent analyses in old animals by Foster and colleagues showed that estrogen...
replacement in old rats reversed several hippocampal markers of age-related memory impairment, blocking induction of long-term depression and decreasing cytosolic calcineurin activity [17].

In women, the effects of estrogen or hormone therapy are far less consistent. Results of the Women’s Health Initiative Memory Study found that women 65 and older who received a non-cycled combination of conjugated equine estrogens and medroxyprogesterone acetate had a two-fold increase in the risk of Alzheimer’s disease relative to the placebo arm and medroxyprogesterone acetate had a two-fold increase in a non-cycled combination of conjugated equine estrogens [22,27,44]. In this study, we sought to address the issue of age and 17β-estradiol on a key component of cellular aging, regulation of Ca\(^{2+}\) homeostasis, in hippocampal neurons derived from embryonic, middle-age and old rats. Results of these analyses demonstrate striking age-related dysregulation of [Ca\(^{2+}\)]\(_i\) homeostasis that is prevented in neurons pretreated with 17β-estradiol. These data indicate that old neurons sustain the capacity to respond to 17β-estradiol and that [Ca\(^{2+}\)]\(_i\) homeostasis can be restored in both middle-age and old neurons.

2. Materials and methods

2.1. Neuron culture and additions

Our experimental model was to culture E18. middle-age (10–12 months) or old (23–24 months) male Fischer rat hippocampal neurons for 11–14 days on 12-mm glass coverslips (Assistent, Carolina Biologicals) in Neurobasal/B27 ± 5 ng/ml FGF2 (Invitrogen) [7] with a 50% medium change on day 5. Embryonic neurons were derived from mixed gender animals. The coverslips were coated with poly-d-lysine (50 µg/ml; Sigma). The incubator maintained 90% humidity, 9% oxygen and 5% carbon dioxide (Forma). During the last 2 days, vehicle or 17β-estradiol (10 ng/ml; Sigma E8875) was added from a stock solution of 1 mg/ml ethanol serially diluted into medium, with the final dilution replacing 50% of the original medium without FGF2. The investigator was not blinded to the age or estrogen treatment of the cultures.

2.2. Fura-2 intracellular calcium imaging

For calcium imaging, neurons were loaded with Fura-2-AM (5 µM) for 45 min in Krebs buffer (100 mM NaCl, 20 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mM NaH\(_2\)PO\(_4\), 4.2 mM Na\(_2\)HPO\(_4\), 10 mM glucose, 12.5 mM HEPES; pH 7.3) in the CO\(_2\) incubator, followed by de-esterification for 30 min in Krebs buffer. After mounting in a custom holder (0.8 ml) with perfusion of room temperature (22 °C) Krebs buffer at 0.6 ml/min, fluorescence was excited alternately every second for 200 ms at 340 nm and 400 ms at 380 nm (PTI Delta-Ram) through a Nikon inverted 40× quartz objective, a 450 nm dicroic and a 530 nm emission filter. Cells were imaged on a SenSys cooled CCD camera. Regions of interest of approximately 50 µm\(^2\) were selected over the soma of all neurons in the field using ImageMaster software (PTI) which also calculated the ratio of 340/380 nm images. One second pulses of glutamate (25 µM in Krebs) were delivered by solenoid activation of gravity flow (Warner Instruments, Hamden CT) at 0.2 ml/min at 2 min intervals. After 20 pulses, 7.5 µM FCCP, 3.3 µM ionomycin (Sigma) were added as a control to ensure responses to increased calcium were still measurable. Ratio values recorded with time for each neuron were exported to Quatro Pro (Corel) for analysis of peak height relative to the preceding trough and trough levels of calcium between peaks, referenced to the starting Fura-2 ratio. Calcium concentrations were estimated based on measures of \(R_{\text{max}}\) and \(R_{\text{min}}\) for our instruments [20]. Statistical evaluation by nested ANOVA was performed using Plotit (Scientific Programming, Haslet, MI) or SAS for Windows, with glutamate pulse a within group variable of age and rejection of the null hypothesis at \(p < 0.05\). Subtests were performed using Student’s \(t\)-test with Bonferroni adjustment for multiple tests.

2.3. Glutamate and β-amyloid toxicity/viability

To 25-month-old rat neurons that were cultured for 12 days was added vehicle or 10 ng/ml 17β-estradiol in a 50% medium change and incubated at 37 °C, 9% O\(_2\), 5% CO\(_2\). After 2 days, neurons were rinsed once with medium and incubated for 24 h with 0, 50 or 100 µM glutamate from a 25 mM stock solution. After rinsing twice with Hibernate A without phenol red (www.brainbitsllc.com), viable and dead cells were labeled for 5 min with 15 µg/ml fluorescein diacetate (from a 15 mg/ml stock solution) and 4.6 µg/ml propidium iodide (from a 4.6 mg/ml stock solution), followed by a rinse with Hibernate without phenol red. Twelve adjacent fields of 0.304 mm\(^2\) from each slip were counted using a Nikon 20× objective with B1A filters (excitation 470–490 nm, dichroic 510 nm, emission 520 nm) and G1B filters (excitation 541–551 nm, dichroic 580 nm, emission 590 nm). Percent survival was calculated as 100 × live cells (green)/live + dead cells (red).

For β-amyloid toxicity, 25-month-old rat neurons that were cultured for 8 days were treated for 2 days with vehicle or 10 ng/ml 17β-estradiol in a 100% medium change. The slips were rinsed once with medium and incubated for 3 days with 0, 1 or 10 µM β-amyloid (1–40) (American Peptide 62-0-78). β-Amyloid was added from a 500 µM stock that had been aged 3 days at 37 °C in PBS to promote fibrilization. Viability was determined as above.

3. Results

3.1. Culture of embryonic, middle-age and old neurons

Based on techniques developed for the isolation and regeneration of adult neurons in culture [7], we are able
to compare responses to glutamate under nearly identical culture conditions. Fig. 1 shows the healthy appearance of these cultures, with no obvious differences with age. While embryonic cultures are typically 95% neurons and 5% astrocytes, after 8–12 days in culture, adult cultures are about 80% neurons, 10% oligodendrocytes, 5% microglia and 5% astroglia [34]. A lower density of axons accompanies the lower neuron density in adult cultures. We focused our analysis on neurons within the population of cells by noting their asymmetric multi-branched processes [7]. Compared to embryonic neurons at 6.25 ± 0.33 μg protein/cm² of culture (mean ± S.E., n = 22), middle-age cultures (from 10- to 12-month-old rats) contained 7.03 ± 0.29 μg protein/cm² and old neuron cultures (from 22- to 24-month-old rats) were 6.65 ± 0.73 μg protein/cm² [34], indicating no differences with age in total protein synthesized during regeneration under common serum-free defined culture conditions. Furthermore, viability of these cultures was well maintained at 93% at 12 days, 94% at 16 days and 95% at 19 days (n = 12 fields/time). The 24-month-old Fischer rat is near its median life-span [38].

3.2. Fast intracellular calcium responses to glutamate for embryonic neurons pretreated without and with 17β-estradiol

Twenty pulses of 25 μM glutamate were applied to neurons in a flow chamber with continuous ratiometric monitoring of intracellular Fura-2 fluorescence in order to determine the magnitude of intracellular calcium responses, the ability to return to baseline (resting) calcium levels and the endurance of these responses. Each 1-s pulse was followed by
Fig. 2. Effects of 17β-estradiol on intracellular calcium levels stimulated by pulses of glutamate applied to embryonic, middle-age and old neurons. Pulses of 25 µM glutamate were applied for 1 s, followed by 2 min of washout, repeated 20 times. Neuronal soma were analyzed for Fura-2 calcium responses. (A–C) Embryonic neurons, (D–F) middle-age neurons, (G–I) old neurons. Thin black traces and symbols are untreated and wide red traces and symbols are treated with 17β-estradiol (10 ng/ml) for 2 days. (A) In one representative untreated embryonic neuron (black), glutamate elicits large rapid responses (offset by 24 s for clarity) with sharp peak heights for which the average shows little attenuation (B, open triangles) and rapid return to baseline (C, open triangles) (n = 60 neurons). In an embryonic neuron treated with 17β-estradiol (A, wide red), glutamate elicits slightly larger rapid responses for which the average sharp peak heights show even less attenuation (B, solid triangles) and almost complete ability to return to baseline (C, solid triangles) (n = 60 neurons). (D) In one representative untreated middle-age neuron (narrow black), glutamate elicits an initial large response with an average rapid attenuation of peak heights (E, open squares) and rising baseline (F, open squares) (n = 57 neurons). In a middle-age neuron treated with 17β-estradiol (D, wide red), glutamate elicits small, slower but with a consistent average peak response (E, closed triangles) with little attenuation and more reliable return to baseline (F, closed triangles) (n = 40 neurons). (G) In one representative untreated old neuron (narrow black), glutamate elicits a very large rapid response with first attenuating, then hyper-responsive peak heights at variable times, on average (H, open circles) with rapid attenuation and failure to return to baseline (I, solid squares) (n = 34 neurons). In (G), open and filled circles at zero time are mean resting levels of Fura-2 ratios for untreated and treated neurons, respectively. Statistical analyses indicate multifactor ANOVAs.
2 min of washout. Fig. 2A shows large fast calcium responses to glutamate for embryonic neurons. In response to the 1-s pulse of glutamate, intracellular calcium remains elevated for a sustained period, until buffers, intracellular stores of endoplasmic reticulum and mitochondria and plasma membrane Ca-ATPase can lower intracellular calcium. Table 1 shows a measure of peak width in seconds for the first peak, a peak width at half-height of 16 s. These rapid responses were sustained for 20 pulses with some attenuation of peak height to about half the initial ratio after 6 pulses (Fig. 2B) with a smaller increase in return to baseline before the next pulse at 120 s (Fig. 2C). Starting from resting calcium levels estimated at 144 nM, the mean first peak height is estimated at 840 nM calcium without treatment with 17β-estradiol.

When embryonic neurons were pretreated with 10 ng/ml 17β-estradiol for 2 days, peak heights were slightly higher with less attenuation (Fig. 2A and B). Neurons treated with 17β-estradiol exhibited a consistent return to initial baseline ([Ca2+]i) whereas in control neurons, a small elevation of baseline ([Ca2+]i) was apparent after the fifth glutamate pulse (Fig. 2C). Table 1 shows an even faster peak width at half-height of 14 s. Starting from resting calcium levels estimated at 140 nM, the mean first peak height is estimated at 840 nM calcium in control neurons and 1060 nM in neurons exposed to 17β-estradiol.

### Table 1

<table>
<thead>
<tr>
<th>Age of neuron donor</th>
<th>Untreated (s)</th>
<th>17β-Estradiol (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E18</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Middle-age</td>
<td>56</td>
<td>72</td>
</tr>
<tr>
<td>Old</td>
<td>104</td>
<td>46</td>
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</table>

When middle-age neurons were pretreated with 10 ng/ml 17β-estradiol, peak [Ca2+]i heights with repeated pulses of glutamate (Fig. 2D and E) were not attenuated as much and the rise in baseline (Fig. 2F) was significantly reduced, not rising two-fold above background during the 20 pulses. However, peak width (Table 1) was further increased from 56 to 72 s by pretreatment with 17β-estradiol. As a positive control and to determine the saturation properties of Fura-2 in the 20 glutamate pulse paradigm, the calcium ionophore ionomycin, together with the mitochondrial uncoupler, FCCP (to both increase influx of [Ca2+]i and to release [Ca2+]i from mitochondrial stores) were both added at the end of the 20 glutamate pulse paradigm. Exposure to these agents resulted in a large [Ca2+]i rise and concomitant increase in the Fura-2 ratiometric signal (Fig. 2D, at “pulse” position 21). The rise of [Ca2+]i to levels greater than the previous responses indicates that saturation, bleaching or leak of the Fura dye had not occurred during the 20 glutamate pulse paradigm.

### 3.3. Preservation of calcium homeostasis in middle-age neurons treated with 17β-estradiol following exposure to glutamate

Video microscopic imaging of the [Ca2+]i response shows...
Fig. 3. Fura-2 ratio images for intracellular calcium levels in old neurons in response to 25 μM glutamate: (A–C) without 17β-estradiol, (D–F) with 17β-estradiol. (A and D) Resting calcium levels. Note the lower levels for old neurons treated with 17β-estradiol in (D). (B and E) Calcium levels at the peak of response to a 1-s pulse of 25 μM glutamate. Note the increase in most cells that is much lower for neurons treated with 17β-estradiol. (C and F) Nearly 2 min after the pulse of glutamate, most neurons that were treated with 17β-estradiol have returned to baseline (F), while those treated with vehicle have a higher level of intracellular calcium reported by Fura-2. A video of these responses can be seen at www.siumed.edu/mmi/brewerestradiolcalcium.htm.
that the onset of elevated [Ca\(^{2+}\)]_i begins at the periphery of the neuronal cell body followed by a peak of [Ca\(^{2+}\)]_i at approximately 9 s following exposure to glutamate (www.siumed.edu/mmi/brewerestradiolcalcium.htm).

A comparative analysis of the images within Fig. 3A versus those in Fig. 3B demonstrates that nearly all neurons respond to the glutamate pulse with a large elevation in [Ca\(^{2+}\)]_i as indicated by an increase in the Fura-2 ratio. The rise in [Ca\(^{2+}\)]_i in the untreated control old neurons is much greater than that of old neurons pretreated with 10 ng/ml 17\(^\beta\)-estradiol (Fig. 3E). Moreover, old neurons pretreated with 10 ng/ml 17\(^\beta\)-estradiol exhibit a return to baseline [Ca\(^{2+}\)]_i comparable to that prior to exposure to glutamate (see Fig. 3F).

In contrast, untreated control old neurons exhibit an elevated [Ca\(^{2+}\)]_i following washout of glutamate indicating an inability to effectively reestablish calcium homeostasis (see Fig. 3C).

3.6. Age-related dynamics of calcium homeostasis and their regulation by 17\(^\beta\)-estradiol

Quantitative comparison of [Ca\(^{2+}\)]_i homeostasis is presented in Fig. 4. Both age and 17\(^\beta\)-estradiol exposure significantly affected resting [Ca\(^{2+}\)]_i levels. Old control neurons exhibited an average resting [Ca\(^{2+}\)]_i level of 214 ± 4 nM which was significantly higher than that of middle-age neurons at 145 ± 1 nM (p < 0.001). In contrast, old neurons exposed to 17\(^\beta\)-estradiol for 48 h exhibited a significantly lower resting [Ca\(^{2+}\)]_i of 156 ± 6 nM (p < 0.001), which was comparable to the 169 ± 1 nM [Ca\(^{2+}\)]_i level of middle-age neurons.

The reduction in resting [Ca\(^{2+}\)]_i was paralleled in the response to the first pulse of glutamate (Fig. 4B). In embryonic neurons, 25 \mu M glutamate induced a 5.8-fold rise above baseline in [Ca\(^{2+}\)]_i which was modestly potentiated in neurons pre-exposed to 17\(^\beta\)-estradiol (7.5-fold). In neurons derived from the hippocampi of middle-age rats, 25 \mu M glutamate induced a 6.4-fold rise above baseline in [Ca\(^{2+}\)]_i comparable to that induced in embryonic neurons (peak height estimated at 790 ± 120 nM [Ca\(^{2+}\)]_i (p < 0.001)). In contrast to embryonic neurons, however, middle-age neurons pre-exposed to 10 ng/ml 17\(^\beta\)-estradiol responded to glutamate with a marked attenuation in [Ca\(^{2+}\)]_i (1.5-fold above resting levels). In neurons derived from the hippocampi of old rats, 25 \mu M glutamate induced a 10-fold rise above baseline in [Ca\(^{2+}\)]_i (peak height estimated at 1050 ± 160 nM [Ca\(^{2+}\)]_i) which was profoundly attenuated in neurons pre-exposed to 17\(^\beta\)-estradiol (1.9-fold above resting levels). The attenuation of the [Ca\(^{2+}\)]_i rise in old-neurons was comparable to that of middle-age neurons (Fig. 4B). Pre-exposure to 17\(^\beta\)-estradiol, resulted in a highly significant (p < 0.001) reduction of peak heights of [Ca\(^{2+}\)]_i in both middle-age and old neurons to 95 and 186 nM [Ca\(^{2+}\)]_i above resting [Ca\(^{2+}\)]_i levels, respectively.

To determine the robustness of calcium homeostatic mechanisms in neurons derived from the hippocampus of embryonic, middle-age and old rats, we performed a series of experiments using 25 \mu M glutamate and 17\(^\beta\)-estradiol. The results are summarized in Fig. 4C. As can be seen in the figure, a significant reduction in peak heights of [Ca\(^{2+}\)]_i was observed in both middle-age and old neurons pre-exposed to 17\(^\beta\)-estradiol, with peak heights estimated at 790 ± 120 nM [Ca\(^{2+}\)]_i in middle-age neurons and 1050 ± 160 nM [Ca\(^{2+}\)]_i in old neurons (p < 0.001).

In conclusion, our data suggest that 17\(^\beta\)-estradiol can significantly modulate calcium homeostasis in neurons, particularly in the context of aging. The attenuation of calcium influx and the ability to reestablish baseline calcium levels upon glutamate exposure may contribute to the neuroprotective effects of estrogen in the aging brain.
onic, middle-age and old rat brains, we assessed the change in baseline [Ca^{2+}]_{i} following the first pulse of 25 μM glutamate. Embryonic control hippocampal neurons reestablished a basal level of [Ca^{2+}]_{i} consistent with that prior to glutamate exposure that was indistinguishable from those pre-exposed to 17β-estradiol. Baseline [Ca^{2+}]_{i} was raised by 12 ± 9 nM in embryonic neurons following exposure to 25 μM glutamate irrespective of treatment condition. Middle-age control neurons exhibited an elevated baseline [Ca^{2+}]_{i} of 74 ± 11 nM, following the first glutamate pulse indicating reduced calcium buffering capability. In contrast, middle-age neurons pretreated for 48h with 10 ng/ml 17β-estradiol exhibited a significantly lower change in baseline [Ca^{2+}]_{i} of 32 ± 7 (p < 0.0001 compared to baseline of middle-age control neurons) following exposure to 25 μM glutamate. Old control neurons exhibited the greatest change in baseline [Ca^{2+}]_{i} with an increase of 132 ± 20 nM following a single exposure to glutamate, indicating a greatly reduced calcium buffering capability. In contrast, old neurons pretreated with 17β-estradiol prior to glutamate exposure exhibited a dramatic and significantly lower baseline [Ca^{2+}]_{i} of 52 ± 9 nM (p < 0.0001 compared to baseline of old control neurons) indicating a calcium buffering capacity comparable to that of middle-age neurons (Fig. 4C).

3.7. Dose response of 17β-estradiol regulation of cellular calcium homeostasis

The above studies were conducted based on prior studies in embryonic neurons which indicated an optimal concentration of 10 ng/ml (37 nM) 17β-estradiol, an EC50 of 1–10 ng/ml [13]. We sought to determine the dose response relationship of 17β-estradiol regulation of neuronal calcium homeostasis in old neurons (Fig. 5). Results of these analyses indicated that the EC100 dose for 17β-estradiol regulation of neuronal calcium homeostasis in old neurons is three orders of magnitude more potent than that within embryonic neurons. In old neurons, 10 pg/ml (37 pM) 17β-estradiol effectively reduced the peak height and returned the baseline calcium almost to its original level (EC50 between 10 and 100 fg/ml (37–370 fM)). Significant efficacy of 17β-estradiol as low as 0.1 pg/ml (0.37 pM) was observed.

3.8. Neuroprotection of old neurons by 17β-estradiol

Fig. 6A shows that 17β-estradiol at 10 pg/ml is fully protective against continuous exposure to 50 or 100 μM glutamate, compared to a 33% loss of viability for neurons not treated with 17β-estradiol. Fig. 6B shows a 36% loss of survival caused by Aβ (1–40) which is fully prevented by a 2-day pretreatment with 17β-estradiol at 10 pg/ml.

4. Discussion

We sought to determine the integrity of Ca^{2+} homeostasis in hippocampal neurons across the age span and its regulation by 17β-estradiol. To address this issue, we exposed hippocampal neurons of three different ages, embryonic (E18), middle-age (10 months) and old (24 months), to 20 sequential pulses of 25 μM glutamate and assessed both the peak [Ca^{2+}]_{i} rise in response to each glutamate pulse and the
return to baseline [Ca\(^{2+}\)]\(i\). These analyses revealed marked age-related differences in [Ca\(^{2+}\)]\(i\) homeostasis and an advantageous prevention of dysregulation of [Ca\(^{2+}\)]\(i\) homeostasis by 17\(\beta\)-estradiol. Compared to middle-age and old neurons, embryonic neurons were best able to sustain Ca\(^{2+}\) homeostasis. In old neurons, the first peak [Ca\(^{2+}\)]\(i\) was substantially greater than that which occurred in the embryonic and middle-age neurons. The exaggerated [Ca\(^{2+}\)]\(i\) rise was followed by an immediate elevation in baseline [Ca\(^{2+}\)]\(i\) which exhibited a progressive failure to restore Ca\(^{2+}\) homeostasis. 17\(\beta\)-Estradiol pretreatment of old neurons profoundly attenuated the peak [Ca\(^{2+}\)]\(i\) rise and delayed the age-associated dysregulation of baseline [Ca\(^{2+}\)]\(i\). Considered together, these results demonstrate age-associated dysregulation of [Ca\(^{2+}\)]\(i\) homeostasis which in embryonic, middle-age and old neurons was completely prevented in the embryonic neurons and substantially attenuated in magnitude and delayed in expression in neurons pretreated with 17\(\beta\)-estradiol. The ability of 17\(\beta\)-estradiol to preserve [Ca\(^{2+}\)]\(i\) homeostasis in neurons derived from old rat hippocampi was paralleled in the survival curve of hippocampal neurons following exposure to glutamate and \(\beta\)-amyloid. Importantly, the preservation of [Ca\(^{2+}\)]\(i\) homeostasis occurred at 17\(\beta\)-estradiol concentrations well within physiological and therapeutic range. The caveat to these analyses is that many neurons, particularly from adult rat brain, do not survive the isolation and culture process and thus, the neurons upon which these analyses are based may be a select population of neurons. Mitigating this concern is the constant yield of adult neurons, irrespective of age [7]. Those middle-age and especially old neurons that have developed epigenetic compensatory changes in calcium handling may be selected for survival through the isolation process, however. Fig. 5 and our initial findings [8] indicate considerable sensitivity to glutamate and \(\beta\)-amyloid. Also, those surviving in culture may be expected to be more resistant to insults, which would imply that the observed effects of age might be more profound in vivo. Furthermore, the neurons appear equally healthy prior to exposure to a toxic insult. Our pulsed-glutamate paradigm then is not directly comparable to the case in which neurons are exposed to a persistent exposure to an increasing level of toxic insult [46].

4.1. Potential mechanisms of 17\(\beta\)-estradiol-induced preservation of [Ca\(^{2+}\)]\(i\) homeostasis

In earlier work conducted in cultured embryonic hippocampal neurons, we demonstrated a biphasic regulation of [Ca\(^{2+}\)]\(i\) by 17\(\beta\)-estradiol in which 17\(\beta\)-estradiol potentiates the [Ca\(^{2+}\)]\(i\) induced by low concentrations of glutamate and attenuates the [Ca\(^{2+}\)]\(i\) in responses to excitotoxic concentrations of glutamate [31]. A simple anti-oxidant property of estradiols seems unlikely to account for these effects [5], but involvement of a mitochondrial estrogen receptor beta is possible [43]. We investigated the mechanism by which 17\(\beta\)-estradiol induced an attenuation [Ca\(^{2+}\)]\(i\) while still promoting Ca\(^{2+}\) influx [32]. Results of our analyses indicate that both 17\(\beta\)-estradiol potentiation and attenuation of glutamate-induced [Ca\(^{2+}\)]\(i\) rise is dependent upon the Src/MAP kinase signaling cascade [33]. Further analyses demonstrated that 17\(\beta\)-estradiol-induced attenuation of glutamate-induced [Ca\(^{2+}\)]\(i\) rise is due to increased mitochondrial sequestration of Ca\(^{2+}\) in response to excitotoxic glutamate which resulted in a decrease in [Ca\(^{2+}\)]\(i\) and a concomitant rise in intra-mitochondrial Ca\(^{2+}\) content [32]. 17\(\beta\)-Estradiol-induced attenuation is temporally correlated with an increase in Bcl-2 expression, which could protect against deleterious effects of increasing mitochondrial Ca\(^{2+}\) levels [29,30]. We proposed that by increasing mitochondrial Ca\(^{2+}\) uptake capacity and the Bcl-2-induced resistance to Ca\(^{2+}\)-induced respiratory inhibition, 17\(\beta\)-estradiol limits the loss of viability initiated by neurotoxic insults. The data in the present report do not address whether 17\(\beta\)-estradiol treated neurons reduce Ca\(^{2+}\) influx or increase the sequestration of Ca\(^{2+}\) into the mitochondrial which would both be manifested by a decrease in the Fura-2 fluorescent signal. These analyses are currently underway.

While the data obtained from embryonic neurons would predict that the attenuation is due to increased mitochondrial sequestration of Ca\(^{2+}\), the findings with old neurons could be explained by a population of mitochondria less able to take up Ca\(^{2+}\). Preliminary results suggest a number of defects in mitochondrial function that persist in old neurons in culture, but equal numbers of mitochondria per neuron (Brewer, unpublished). Other factors such as a change in glutamate NMDA receptor expression [14] and or subunit composition of glutamate receptors could substantially impact the action of 17\(\beta\)-estradiol in middle-age or old neurons. Estrogen interacts with N-methyl-D-aspartate (NMDA) receptors to regulate multiple aspects of morphological and functional plasticity [10]. In the hippocampus, estrogens can increase both morphological complexity and dendritic spine density and synapse number, and NMDA antagonists block these effects. This plasticity in the hippocampus mediated by estrogen may be of particular importance in the context of aging when estrogen levels decline and cognitive function is often impaired. Analyses from the Morrison group demonstrated that NMDA receptor subunit mRNA levels were much more prominently affected by chronological age than by the reproductive status of the animals. Age-related changes were observed in NR1, NR2A, and NR2B in the ventral hippocampus and in NR1 and NR2B in the dorsal hippocampus [1,2]. An interaction between chronological age and reproductive status was found, with higher levels of NR1 mRNA seen in young animals in proestrus than in those in diestrus I (high and low estrogen levels, respectively). However, this relationship was not seen in the old rat brain. These results demonstrate that the hippocampus is subjected to age-related alterations in NMDA receptor subunit mRNA levels and that animals of different ages are influenced differently by reproductive status [3].
In addition, Adams and colleagues found that 17β-estradiol induced an increase in axospinous synapse density in young animals, but did not alter the synaptic representation of NR1, in that the amount of NR1 per synapse was equivalent across groups. However, 17β-estradiol replacement in old female rats failed to increase axospinous synapse density but did up-regulate synaptic NR1 compared with non-estrogen replaced old female animals. These data from an in vivo treatment paradigm are consistent with our in vitro mechanistic data that young and old hippocampal neurons respond differently to 17β-estradiol, with the old animals unable to mount a plasticity response to generate additional synapses, but are able to increase NMDA receptor content per synapse [2].

Based on these data, Adams and Morrison conclude that while estrogen contributes to sustaining a youthful synaptic phenotype by some measures, the old synapse differs from the young synapse in several key respects that impact plasticity in general, and endocrine influences on the synapse, in particular [1].

Results from Baudry and colleagues [6] have shown that 17β-estradiol, by activating Src and ERK/MAP kinases, enhances NMDA receptor phosphorylation and function. NR2 subunits of NMDA receptors are truncated by calpain, an effect prevented by tyrosine phosphorylation of the subunits. In old female rats, these investigators found that ovariectomy reduced ERK2 phosphorylation in brains from 3-month-old but not 24-month-old female rats. In ovariectomized rats, restoration of estrogen levels increased ERK2 and NR2 phosphorylation in young but not old animals. Calcium treatment of frozen-thawed brain sections decreased NR2 levels in both young and old female rats. This effect was absent in E2-treated young ovariectomized female rats, but was not modified in old ovariectomized female rats. These results indicate that 17β-estradiol activation of ERK2 and NR2 phosphorylation is markedly reduced in old female rats, whereas calpain-mediated truncation of NR2 subunits does not differ in young and old rats. These findings suggest that several key elements of the mechanisms involved in estrogen-mediated regulation of synaptic plasticity are altered in old animals.

Landfield’s group showed that altered [Ca2+]i is directly correlated with impaired neuronal plasticity such that elevated [Ca2+]i and frequency facilitation were negatively correlated in individual old neurons within hippocampal slices [39]. This finding led these investigators to postulate that [Ca2+]i is likely elevated in old hippocampal neurons and frequency facilitation would thus be impaired in old hippocampal neurons during the theta frequencies associated with cognitive processing. Consistent with this postulate are recent in vivo data from studies in old rats. Foy and colleagues found that 17β-estradiol suppressed the Ca2+-dependent induction of long-term depression in CA1 hippocampal neurons of old rats [42]. Later analyses by Foster et al. showed that 17β-estradiol decreased the Ca2+-activated afterhyperpolarization which is larger in old rats compared to young rat CA1 neurons [17] and is enhanced by a higher density of L-type Ca2+ channels in the old rat neuron [40]. Concomitant with the electrophysiological analyses, administration of estradiol benzoate to rats reversed age-related declines in retention of spatial discrimination and EPSP slope in hippocampal slices as well as increases in calcineurin activity [17]. Yet another mechanism of action was reported for estradiol in a rapid increase in Ca2+-ATPase activity with an EC50 of 20 pM measured in adult rat cortical synaptosomes [45].

4.2. Estrogen therapy in non-human primates and humans

How well do the data generated in rat neurons generalize to non-human primates and to humans? Recent data from the Morrison group suggest that rhesus monkeys sustain an estrogen response phenotype more consistent with young rat neurons [21]. They found that both young and old female monkeys, exhibited a comparable estrogen-inducible increase in spinophilin-immunoreactive spines relative to untreated groups. These data indicate that the 17β-estradiol-inducible increase in spine formation remains intact in the old female rhesus monkey and is lost in the old female rat. Our results in neurons derived from the old rat would predict that the loss of estrogen-inducible spines is due to a dysregulation of Ca2+ homeostasis whereas one would predict that the neurons within the old non-human primate brain are able to sustain Ca2+ homeostasis and would thus exhibit a phenotype more consistent with the embryonic rat neuron [33,39]. Importantly, our data and that from other laboratories such as the Landfield group, show that profound dysregulation of Ca2+ homeostasis can occur in old neurons that would have a direct impact on downstream consequences of Ca2+-activated signaling cascades [18,19].

The issue of application of therapeutic or preventive estrogen to elderly women faces an apparent contradiction. While several epidemiological analyses have indicated a significant reduction in the risk of developing Alzheimer’s disease in women who ever received estrogen therapy [22,27,44], results of the Women’s Health Initiative Memory Study (WHIMS) found that women 65 and older who received a non-cycled combination of conjugated equine estrogens and medroxyprogesterone acetate had a two-fold increase in the risk of Alzheimer’s disease relative to the placebo arm of the trial [55]. Three issues are immediately apparent. First, the epidemiological data apply to early prevention of AD at a mean age of menopause at 52, while the WHIMS is an extrapolation to therapy for many who may have significant existing amyloid pathology at a mean age of 74. Second, women in the epidemiological studies used a variety of hormone therapies that included cycled regimens whereas the WHIMS trial used continuous equine estrogen plus medroxyprogesterone. Our data applied estrogen for only 2 days before toxicological challenge with glutamate or Aβ. Preliminary results by one of us (RDB) indicates that hippocampal neurons exposed to E2 before Aβ exhibit significantly decreased viability as assessed by calcein viability assays in primary cultures of rat hippocampal neurons [56]. The additional results that old neurons are more sensitive to this challenge than young neurons suggests an additional level of vulnerability to Alzheimer’s disease that may be modulated by estrogen and that the estrogen response phenotype may be more consistent with human aging than has been previously considered.
greater survival than neurons treated with E2 at the time of β-amyloid insult and, most importantly, this treatment produced even less survival than those neurons exposed to Aβ alone. Together, our results suggest that estrogen therapy should be initiated before significant Aβ exposure as a preventive intervention and not therapeutically when significant pathology exists [11].

4.3. Conclusions

Data from our analyses indicate a profound loss of Ca2+ homeostasis in old neurons and are supportive of the dysregulation of Ca2+ homeostasis hypothesis of aging [41,9,24]. Middle-age and old neurons pretreated for just 2 days with physiological and therapeutically relevant concentrations of 17β-estradiol sustained Ca2+ homeostasis. In particular, 17β-estradiol delayed the age-associated dysregulation of Ca2+ homeostasis of old neurons, in part, by significantly reducing the precipitating insult which was the exaggerated [Ca2+]i rise in response to the first pulse of glutamate. These data indicate that in neurons that survive the culture process, that dysregulation of Ca2+ homeostasis which is evident in both middle-age and more dramatically in neurons from old rat brain can be prevented or significantly delayed with a relatively short course of physiologically and therapeutically relevant concentrations of 17β-estradiol therapy. These data suggest that in healthy neurons which show dysregulation of Ca2+ homeostasis, exposure to 17β-estradiol prior to excitotoxic insult can significantly attenuate the magnitude of the insult and delay adverse consequences on intracellular Ca2+ homeostasis.

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