Estradiol Reverses a Calcium-Related Biomarker of Brain Aging in Female Rats

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An increase in L-type voltage-gated calcium channel (LTCC) current is a prominent biomarker of brain aging and is believed to contribute to cognitive decline and vulnerability to neuropathologies. Studies examining age-related changes in LTCCs have focused primarily on males, although estrogen (17 β -estradiol, E2) affects calcium-dependent activities associated with cognition. Therefore, to better understand brain aging in females, the effects of chronic E2 replacement on LTCC current activity in hippocampal neurons of young and aged ovariectomized rats were determined. The zipper slice preparation was used to expose cornu ammonis 1 (CA1) pyramidal neurons for recording LTCC currents using the cell-attached patch-clamp technique. We found that an age-related increase in LTCC current in neurons from control animals was prevented by E2 treatment. In addition, *in situ* hybridization revealed that within stratum pyramidale of the CA1 area, mRNA expression of the Ca_v1.2 LTCC subunit, but not the Ca_v1.3 subunit, was decreased in aged E2-treated rats. Thus, the reported benefits of E2 on cognition and neuronal health may be attributed, at least in part, to its age-related decrease in LTCC current.

Introduction

Changes in calcium (Ca²⁺) homeostasis are prominent components of brain aging and contribute to impaired cognition and increased vulnerability to excitotoxicity and neurodegenerative diseases (Landfield, 1987; Thibault et al., 1998; Coon et al., 1999; LaFerla, 2002; Burke and Barnes, 2006; Disterhoft and Oh, 2006; Murchison and Griffith, 2007; Raza et al., 2007). Well established electrophysiological biomarkers of hippocampal aging include increases in both L-type voltage-gated calcium channel (LTCC) currents and the Ca²⁺-dependent slow afterhyperpolarization (AHP) (Thibault and Landfield, 1996; Power et al., 2002). These age-related changes in hippocampal cornu ammonis 1 (CA1) pyramidal neurons contribute to impaired synaptic plasticity and likely underlie a decrease in cognitive acuity (Deyo et al., 1989; Shankar et al., 1998; Thibault et al., 2001; Veng et al., 2003).

Most studies examining age-related changes in Ca²⁺ regulation have been conducted in male animals, although sexual dimorphism is evident in many brain regions not directly associated with reproduction, including the hippocampus (Juraska, 1991; McEwen, 1999; Cahill, 2006). The steroid hormone estrogen (17 β -estradiol, E2) plays an important role in neuroprotection and synaptic plasticity, both of which are Ca²⁺-dependent

processes (Wong and Moss, 1992; Dubal et al., 1998; Woolley, 1998; Foy et al., 1999; Hurn and Macrae, 2000; Yang et al., 2000; Bi et al., 2001; Behl, 2002; Nilsen and Brinton, 2003). In female rodents, high levels of E2 during the proestrus phase of the estrus cycle enhance hippocampal excitability and long-term potentiation (LTP) (Teyler et al., 1980; Warren et al., 1995; Good et al., 1999; Bi et al., 2001). Furthermore, this occurs simultaneously with an increase in dendritic spines and synapses (Woolley et al., 1990; Woolley and McEwen, 1992). These results suggest that the decline in E2 associated with aging and reproductive senescence may have significant effects on plasticity in the aging female brain (Yun et al., 2007; Foy et al., 2008).

Ca²⁺-related biomarkers of aging may also be targets of E2 action. For example, E2 reduces the AHP and facilitates LTCC-mediated signaling pathways involved in neuroprotection (Kelly et al., 1980; Kumar and Foster, 2002; Carrer et al., 2003; Wu et al., 2005; Kelly and Rønnekleiv, 2008). Furthermore, LTCC currents are increased in cardiac myocytes from estrogen receptor knockout (ERKO) mice (Johnson et al., 1997). Whether LTCCs are altered with aging in females in a manner similar to males and whether E2 affects LTCCs *in vivo* is unknown. Because LTCCs have a broad role in neuronal function and are altered with aging, we examined the effects of E2 on these channels.

Here, the effects of age and E2 replacement on LTCC activity and expression in hippocampal CA1 pyramidal neurons were studied in ovariectomized (OVX) rats. CA1 neurons are noted for their dynamic structure and essential role in synaptic plasticity (Spruston, 2008), both of which are affected by E2 (Gould et al., 1990; Desmond and Levy, 1997; Hao et al., 2003). To specifically investigate LTCCs in CA1 neurons, we used the hippocampal zipper slice developed by Gray and colleagues (Gray and Johnston, 1987; Gray et al., 1990) which allows unobstructed access to adult CA1 cell bodies for patch-clamp recording

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(Thibault and Landfield, 1996; Norris et al., 2008). Additionally, we used *in situ* hybridization (ISH) to study the effects of age and E2 on mRNA expression of the $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ LTCC pore-forming subunits in the CA1 region. We found that E2 treatment reversed an age-related increase in LTCC current and decreased mRNA expression of the $\text{Ca}_v1.2$ subunit in aged animals. These results may have clinical implications, given that some aspects of hippocampal brain aging in females can be reversed by *in vivo* treatment with E2.

Materials and Methods

Ovariectomy and implantation of E2 Silastic capsules

Young adult (5–6 months old) and aged (21–22 months old) female Fischer 344 (F344) rats were obtained from Harlan and housed in our animal facilities in accordance with National Institutes of Health (NIH) Guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Rats were maintained under controlled temperature and photoperiod conditions (12 h light/dark) with food and water available *ad libitum*. To reduce the contribution of dietary phytoestrogens derived from standard rodent chows (Degen et al., 2002), rats were placed on a casein-based diet (soy- and alfalfa-free diet; Purina 5K96; Purina Mills) on arrival and acclimated to this diet for 2 weeks before any manipulations. No changes in weight or food intake were observed during this period.

Rats were anesthetized using isoflurane anesthesia, and bilateral OVX was performed. OVX reduces E2 levels by ~75–85% in young adult female rats (Yang et al., 2000; Dubal and Wise, 2001). We have found that E2 levels in aged intact and aged OVX F344 rats are not significantly different and are similar to levels in young OVX rats (M. A. Curran-Rauhut and N. M. Porter, unpublished observations). All rats were immediately implanted with two subcutaneous Silastic capsules (Dow Corning; inner diameter, 1.58 mm; outer diameter, 3.18 mm; length, 20 mm for young, 30 mm for aged) containing either vehicle (sesame oil) or E2 (200 $\mu\text{g}/\text{ml}$ dissolved in sesame oil; Sigma-Aldrich). Capsules were placed dorsally on each side of the thoracic vertebrae and reliably delivered E2 for 3 weeks before being replaced under isoflurane anesthesia in animals receiving longer treatments. Animals were treated for 2–6 weeks before electrophysiological recording. Treatment durations differed because electrophysiological analyses could only be performed on one animal per day.

Electrophysiology

Zipper slice preparation. Methods for preparing partially dissociated hippocampal “zipper” slices were originally developed by Gray et al. (1990) for tissue isolated from guinea pigs and modified for use in rats, as described previously by our laboratory (Thibault and Landfield, 1996; Blalock et al., 2001; L. D. Brewer et al., 2006). The advantage of this preparation is that it provides isolated cell bodies suitable for patch clamping.

Vehicle- or E2-treated animals ($n = 4$ animals/group) were deeply anesthetized with CO_2 and then decapitated. Brains were quickly removed and briefly placed (1–2 min) into ice-cold, oxygenated (95% $\text{O}_2/5\% \text{CO}_2$) artificial CSF (ACSF; low Ca^{2+} concentration) containing the following (in mM): 1.25 KH_2PO_4 , 114 NaCl, 2.5 KCl, 2 MgCl_2 , 30 NaHCO_3 , 10 glucose, and 0.1 CaCl_2 . Hippocampi were isolated and cut into 350- μm -thick transverse slices with a McIlwain tissue chopper (Brinkmann) and placed into oxygenated, 32°C ACSF containing 2 mM CaCl_2 (Ca^{2+} -ACSF). Slices were incubated for 30 min in 3 ml of 32°C Ca^{2+} -ACSF containing 2.1 mg pronase (Calbiochem) and continuously oxygenated throughout remaining incubations. This solution was then replaced with 3 ml of 32°C Ca^{2+} -ACSF containing 1.7 mg thermolysin (protease type X; Sigma-Aldrich). After 20 min, half of the thermolysin Ca^{2+} -ACSF solution was removed and replaced with Ca^{2+} -ACSF.

After 30 min in Ca^{2+} -ACSF, slices were ready for mild dissociation. An individual slice was nicked at the edge of the CA1 layer (Fig. 1A) and placed into a 2 ml autoanalyzer cup (Fisher Scientific) containing oxygenated EGTA-ACSF (2 mM EGTA substituted for 2 mM CaCl_2). The slice was gently shaken until separation or “unzipping” of the CA1 layer was visible (Fig. 1B,C), at which time the slice was transferred to a

A. Intact slice bathed in protease solution.

B. Zipper slice: Unzipped CA1 layer with exposed neurons.

C. Zipper slice with a patch-electrode attached to a neuron.

Figure 1. Hippocampal zipper slice preparation used to expose CA1 pyramidal neurons for multichannel recording of LTCC activity with a traditional cell-attached patch pipette. **A**, Schematic of an intact hippocampal slice with the CA1, CA3, and DG regions labeled. Arrowhead indicates location where a nick is made along the CA1 region. **B**, Schematic of the partially dissociated hippocampal slice showing the unzipped CA1 region (zipper slice) with exposed pyramidal cell bodies and a patch electrode (arrow). A combination of proteases followed by mild shaking unzips the CA1 layer. **C**, Photomicrograph of a zipper slice preparation with a patch electrode (arrow) sealed to the membrane of a CA1 pyramidal neuron. The cell-attached patch electrode was used to record multichannel LTCC activity from these neurons.

perfusion-style recording chamber (Warner Instruments) containing external recording solution (see below).

Cell-attached patch-clamp recording of L-type Ca^{2+} channels. Multichannel cell-attached patch-clamp recordings of LTCCs from CA1 pyramidal neurons were performed as described previously (Thibault and Landfield, 1996; Blalock et al., 2001; L. D. Brewer et al., 2006). A “zeroing” solution was used for the external recording medium (Fox et al.,

1987) and contained (in mM) 140 K gluconate, 3 MgCl₂, 10 glucose, 10 EGTA, and 10 HEPES; pH adjusted to 7.35 with KOH and osmolarity adjusted to 300 mOsm with distilled water. The pipette solution contained (in mM) 20 BaCl₂, 90 choline Cl, 10 TEA, and 10 HEPES; pH adjusted to 7.35 with TEA–OH and osmolarity adjusted to 290 mOsm with sucrose. The pipette also contained the LTCC dihydropyridine (DHP) agonist ($\pm$) BayK8644 (0.5 μ M), which greatly increases the mean open time and open probability of the channel, resulting in patch currents dominated by this channel type (Thibault and Landfield, 1996).

Pyramidally shaped neurons in the CA1 field were identified for recording, and neurons appearing swollen or flattened were avoided. Cells were approached carefully with the recording pipette, as cell bodies were floating unsupported in the medium; a high-resistance seal was formed during contact, and recording in the cell-attached mode was achieved using standard protocols (Hamill et al., 1981). The average electrode resistance was 3.8 M Ω ($\pm$ 0.09), and there was no significant difference between groups (one-way ANOVA). The holding current and seal resistance averaged 4.8 ± 0.5 pA and 22.7 ± 1.8 G Ω , respectively, and there was no significant difference between groups (two-way ANOVA). LTCC current was evoked from a holding potential of -70 mV to 0 mV for 150 ms. Leak currents were obtained by equivalent voltage steps in the opposite direction. Fifteen consecutive sweeps from each patch were used to construct an ensemble average current from leak-subtracted currents. The total area within the ensemble average was integrated and divided by stimulus duration to obtain an average patch current. Current–voltage (I – V) relationships were determined by holding each patch at -70 mV and stepping in progressively greater $+10$ mV increments to $+40$ mV. Half-maximal activation voltages were derived by fitting data from the I – V curve to the Boltzmann equation, as described by Brewer et al. (2007) using GraphPad Prism software (GraphPad Software).

In situ hybridization

Tissue preparation. A separate group of animals of the same age and treatment groups was used for ISH studies ($n = 4$ – 5 /group). After a 6 week treatment period, animals were killed by decapitation, and trunk blood was collected for measurement of plasma E2. Brains were rapidly removed and stored at -80°C until they were sectioned for ISH. Coronal sections (12 μ m) were obtained through the hippocampal region (bregma -3.30 mm to -4.16 mm) (Paxinos and Watson, 1998). Sections were thaw-mounted onto gelatin-coated microscope slides and stored at -80°C until hybridization. Slides were prehybridized as described previously (Scott et al., 1998).

Probe preparation and hybridization. A 556 bp fragment of Ca_v1.2 cDNA (6745–7300, accession no. M59786) and a 511 bp fragment of Ca_v1.3 cDNA (6047–6557, accession no. M57682) were cloned by standard PCR methods using specific primers for each transcript (Integrated DNA Technologies). Briefly, hippocampal RNA was isolated using Trizol (Invitrogen). The RNA was then reverse transcribed according to manufacturer's instructions using random hexamers (Superscript II; Invitrogen), generating a hippocampal cDNA library. The cDNA fragment specific for Ca_v1.2 was amplified using forward (5'-CCTAATGGGTTCGT-TTCAGAAAG-3') and reverse (5'-ATCAAAACCTAGAAAACCGCAA-3') primers; the cDNA fragment specific for Ca_v1.3 was amplified using forward (5'-CTGATTGGAAGTGGAGCAGACAG-3') and reverse (5'-ATACGTCCAAGGAGCCTTTACA-3') primers. The cDNA fragments were amplified using 1 μ M of each primer, 0.2 mM dATP, dCTP, dGTP, and dTTP, 1.3 mM MgCl₂, and 1.25 U AmpliTaq DNA polymerase (Applied Biosystems). PCR products were purified using QIAquick spin columns according to the manufacturer's instructions (Qiagen). The 556 bp Ca_v1.2 fragment and the 511 bp Ca_v1.3 fragment were then ligated into pCRII (Invitrogen) and sequenced by ACGT, Inc.

The plasmids containing cloned regions of Ca_v1.2 and Ca_v1.3 cDNA were used to prepare radiolabeled cRNA probes specific for Ca_v1.2 and Ca_v1.3. The plasmids were linearized with either *Bam*HI or *Eco*RV to generate antisense and sense cRNA transcripts, respectively. Transcription reactions were performed in the presence of ³³P- α UTP (Perkin-Elmer) as described previously (Scott et al., 1998), except that the reactions included 90 pmol ³³P- α UTP in a total concentration of 12 μ M UTP. ISH using Ca_v1.2 and Ca_v1.3 probes was performed as described previ-

ously (Dowling et al., 2000), except that the hybridization buffer contained 250,000 cpm per section.

Autoradiography and signal quantitation. After ISH, all slides were arranged in x-ray cassettes and apposed to BioMax film (Eastman Kodak) for 2 weeks. ¹⁴C-labeled standards (American Radiolabeled Chemicals) were simultaneously apposed to the film to verify that the film was not overexposed. The hybridization signal was analyzed using a ScanMaker i900 (Microtek) and the public domain ImageJ program (W. Rasband, NIH). The relative abundance of Ca_v1.2 and Ca_v1.3 mRNAs was measured over the CA1 hippocampal subfields. Data were normalized by subtracting nontissue background signal from the signal measured in the CA1 area. The resulting values were averaged over four sections for each brain, with four to five animals per treatment group.

Bioassay of E2 effects

Radioimmunoassay of E2. Plasma E2 levels were measured in a subset of animals to ensure effectiveness of OVX and E2 implants. After killing, trunk blood was collected into heparinized Vacutainer tubes (BD Biosciences), immediately placed on ice, and centrifuged at 1000 g for 10 min at 4°C . Plasma was isolated, aliquoted, and stored at -20°C . E2 levels were determined by radioimmunoassay (RIA) using an Ultra-Sensitive Estradiol RIA kit (DSL-4800; Diagnostic Systems Laboratories) according to the manufacturer's instructions. Each sample was analyzed in triplicate. This assay was performed at 32.1% binding with detection limits of 5–250 pg/ml and a theoretical sensitivity of 2.2 pg/ml. The intraassay coefficient of variation (CV) was 10.9%, and the interassay CV was 16.5%. The average circulating E2 levels ranged from 5.96 to 13.57 pg/ml and were within the detection limits of the RIA kit (see Results).

Measurement of uterine weight. Uterine weight was measured in vehicle-treated and E2-treated OVX animals to determine the efficacy of the implants, as described by Adams et al. (2002). Uteri were removed by cutting at a specified distance along the uterine horns and just caudal from where the horns join. Uteri were gently blotted to remove excess fluid and weighed. Uterine weight was normalized to body weight.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Data from electrophysiological analysis, ISH, RIA, and uterine weights were analyzed using a two-way ANOVA with a Tukey *post hoc* multiple comparison. Statistical analyses were performed with SigmaStat (Systat).

Results

Systemic effects of E2

In the present study, OVX resulted in similar circulating levels of E2 (~ 6 pg/ml) in vehicle-treated young and aged control rats and were approximately twofold greater in the corresponding young and aged OVX groups treated with E2 (Fig. 2A). These replacement levels of E2 are comparable with those in previous studies and are considered to be in a low physiological range (Dubal et al., 1998). Aged female F344 rats are in an acyclic, persistent diestrus state (Estes et al., 1982; Sone et al., 2007) characterized by low E2 levels. E2 levels in 21–22-month-old F344 rats, equivalent to rats used in our study, are reported to be ~ 6 – 10 pg/ml in intact rats (Markowska and Savonenko, 2002; Bowman et al., 2006) and ~ 6 pg/ml in OVX rats (Markowska and Savonenko, 2002). By comparison, young cycling F344s have peak E2 levels of ~ 70 pg/ml during proestrus (Haim et al., 2003).

Confirming E2 action *in vivo*, uterine weight in both young and aged E2-treated animals was approximately threefold greater than that of controls (Fig. 2B). Compared with other tissues, the uterus contains a relatively high number of ERs, and thus, a bioassay of the uterotrophic effects of E2 is considered a highly sensitive test and a positive control for demonstrating E2 action *in vivo* (Adams et al., 2002; Erben et al., 2004).

Differences in animal weight were also observed in response to age and E2 treatment. As seen in the control groups, animal weight significantly increased in an age-dependent manner.

Figure 2. Bioassay of systemic effects of E2 implants. Comparison of vehicle and E2 treatment on plasma E2 levels, uterine weight, and animal weight in young and aged OVX F344 rats. **A**, E2 plasma levels were greater in E2-treated compared with vehicle-treated animals in both young and aged animals ($F_{(1,16)} = 33.6$; $p < 0.001$). **B**, Uteri were responsive to E2 implants. Uterine weight, which was normalized to body weight, increased by at least threefold in response to E2 treatment in both young and aged animals ($F_{(1,16)} = 129$; $p < 0.001$). Within the E2-treated group, uteri were larger in aged than in younger animals. **C**, Animal weight was significantly affected by age ($F_{(1,16)} = 141$; $p < 0.001$) and E2 treatment ($F_{(1,16)} = 57$; $p < 0.001$). E2 treatment resulted in a significant decrease in young and aged animals compared with their age-matched control group. Figure legend in **A** also applies to **B** and **C**. Data are mean $\pm$ SEM; post hoc analysis, ** $p \leq 0.01$.

However, E2 treatment significantly decreased the weight of both young and aged animals compared with their age-matched controls (Fig. 2C). The effect of E2 and ERs on body weight is well recognized (Wade, 1986; Heine et al., 2000) and believed to occur via multiple mechanisms, including a reduction in food intake and the expression of lipogenic genes (Cooke and Naaz, 2004; D'Eon et al., 2005). Conversely, the OVX rodent model of menopause is characterized by weight gain (Simpkins et al., 1988),

which is preventable by treatment with E2 (Meli et al., 2004). These effects on body weight in rodents are similar to those reported to occur with human menopause (Sowers et al., 2007).

Effect of E2 on LTCCs in young and aged OVX rats

The effect of chronic E2 and vehicle treatment in young and aged OVX rats on LTCC current was determined by recording LTCC activity from the cell bodies of hippocampal CA1 pyramidal neurons using the zipper slice preparation (Fig. 1C). Multichannel LTCC currents were isolated in cell-attached patch-clamp recordings by using the LTCC agonist ($\pm$)BayK8644. Representative current traces of LTCC activity from each treatment group are shown in Figure 3A. The average patch current determined from the ensembles for each treatment group is shown in Figure 3B ($n = 12$ – 15 cells/group). The average current activity of aged OVX controls increased by $\sim 70\%$ relative to the young OVX controls ($F_{(1,48)} = 4.38$, $p < 0.05$), indicating an age-related difference. However, in E2-treated aged OVX animals, LTCC currents were decreased by $\sim 45\%$ compared with the aged control group ($F_{(1,48)} = 4.55$, $p < 0.05$). Thus, LTCC currents resulting from the E2 treatment in aged animals more closely resembled currents observed in young controls. LTCC currents from E2-treated young OVX animals were reduced by $\sim 45\%$ relative to the young control group, but this reduction was not significant ($p = 0.3$).

The I – V relationship was examined for each patch to determine if age or E2 treatment altered the voltage dependence of the LTCC ($n = 10$ – 12 cells/group) (Fig. 4). The voltage at which the maximal current (0 mV) and the half-maximal activation voltages occurred were similar for all treatment groups (-18.3 to -20.0 mV). These parameters are characteristic of LTCCs and are similar to values reported previously (Thibault and Landfield, 1996; Brewer et al., 2001). Thus, E2 treatment in the aged animals reduced LTCC current activity without altering voltage dependence.

In situ hybridization

The effects of age and E2 treatment on $Ca_v1.2$ and $Ca_v1.3$ gene expression in the CA1 region of the hippocampus were determined using ISH. This approach made it possible to conduct a quantitative analysis of gene expression using film autoradiograms of the cell body layer of the CA1 region (stratum pyramidale), the same region from which patched neurons were recorded. Consistent with other studies (Herman et al., 1998; Clark et al., 2003), we found that $Ca_v1.2$ subunit expression was more robust in the dentate gyrus (DG) and CA3 regions than in the CA1, but $Ca_v1.3$ expression was more robust in the DG and more uniform in CA3 and CA1 regions (Fig. 5A).

Within the CA1 region of stratum pyramidale, E2 treatment of aged animals significantly decreased ($\sim 17\%$) $Ca_v1.2$ mRNA expression ($F_{(1,15)} = 4.3$; $p = 0.02$) (Fig. 5B). In the young animals, E2 treatment also reduced $Ca_v1.2$ expression ($\sim 9\%$), but this decrease was not significant. E2 treatment did not affect mRNA expression of the $Ca_v1.3$ subunit in either the young or aged animals compared with their age-matched controls (Fig. 5C). Additionally, there were no age-related changes in either $Ca_v1.2$ or $Ca_v1.3$ expression.

Discussion

Two potentially important findings regarding LTCC activity in female rats were identified in this study. First, similar to reports in aged male rats (Thibault and Landfield, 1996; L. D. Brewer et al., 2006), LTCC currents were increased in hippocampal CA1 neu-

rons from aged OVX rats. Aged females, therefore, appear as susceptible to changes in this prominent Ca^{2+} influx pathway. Second, LTCC currents from a similar group of aged females treated chronically with a low physiological dose of E2 were of smaller magnitude and comparable with those observed in younger female rats. These results suggest a mechanism by which LTCCs are tonically inhibited by circulating E2 in young animals. This inhibition, however, may be lost when E2 levels are very low (e.g., aging and reproductive senescence). Although intact females were not included in this study, 21–22-month-old intact and OVX F344 rats appear to have similarly low levels of E2 (Markowska and Savonenko, 2002; Bowman et al., 2006). We also found that E2 reduced LTCC activity in the young OVX females, albeit nonsignificantly. Perhaps LTCCs are affected by E2 loss, regardless of age. The impact on LTCCs, however, may be greater in aged animals because of the prolonged absence of E2 during reproductive senescence.

Given that the results of the present study show that E2 reduces LTCCs, perhaps E2 acts as an endogenous modulator of LTCCs and attenuates the impact of age-related Ca^{2+} dyshomeostasis, cognitive decline, and vulnerability to neuropathologies (Toescu et al., 2004; Disterhoft and Oh, 2006; Foster, 2007). Thus, some of the beneficial effects of E2 and hormone replacement therapy (HRT) on the brain (Wise et al., 2005; Sherwin and Henry, 2008; Simpkins and Singh, 2008; Spencer et al., 2008) may be attributed to modulation of LTCCs by E2. Our results are also consistent with studies demonstrating E2 modulation of several Ca^{2+} -dependent processes, particularly those associated with cognition (Boulware et al., 2005; Liu et al., 2008) and neuroprotection (G. J. Brewer et al., 2006; Gamerdinger et al., 2006; Zhao and Brinton, 2007). Of course, E2 exerts neuroprotective and nootropic actions by various mechanisms (Dubal and Wise, 2001; Nilsen and Brinton, 2003; Yang et al., 2004; Scharfman and Malclusky, 2005; Wu et al., 2005; Dziennis et al., 2007; Simpkins and Singh, 2008) and its effect on LTCCs represents only one component of its potentially beneficial effects (Sherwin and Henry, 2008).

LTCCs and Ca^{2+} dyshomeostasis in aging

The age-related increase in LTCC current is a biomarker of brain aging (Landfield, 1987; Foster, 2007; Thibault et al., 2007) and is associated with impaired cognitive performance in the Morris water maze (Thibault and Landfield, 1996). The increased influx of Ca^{2+} via LTCCs affects numerous Ca^{2+} -dependent processes, including the AHP, Ca^{2+} -induced Ca^{2+} release, synaptic plastic-

Figure 3. LTCC current activity from young and aged OVX rats chronically treated with either vehicle or E2. Patch currents were recorded from the cell body membranes of hippocampal pyramidal CA1 neurons using multichannel patch pipettes. **A**, Five representative current traces are shown from each treatment group. Currents were evoked by depolarizing to 0 mV from a -70 mV holding potential. Ensemble averages for each patch are also shown and were obtained from 15 depolarizations. LTCC currents from aged controls were larger than those of young controls. However, LTCC current activity was reduced in the aged animals treated with E2. **B**, Average patch currents obtained from ensemble averages for each treatment group (mean $\pm$ SEM). Aged controls had significantly larger LTCC currents than young controls. However, in the aged animals, E2 treatment significantly reduced the average ensemble currents. $*p \leq 0.05$, $n = 12$ –15 cells per group; four animals per group.

ity, and LTCC-mediated gene expression (Landfield and Pitler, 1984; Moyer et al., 1992; Deupree et al., 1993; Norris et al., 1998; Thibault et al., 2001; Deisseroth et al., 2003; Gant et al., 2006). Additionally, increased LTCCs with brain aging can lead indirectly to an increased probability of action potential failure. The increase in LTCCs enhances the Ca^{2+} -dependent AHP, reducing neuronal firing and excitability (Disterhoft and Oh, 2006; Abraham, 2008; Gant and Thibault, 2008). However, if LTCCs in aged neurons are blocked with DHPs, various forms of synaptic plasticity and cognitive performance are enhanced (Deyo et al., 1989; Norris et al., 1998; Thibault et al., 2001; Veng et al., 2003). Interestingly, a recent study of an elderly cohort with hypertension

Figure 4. *I*–*V* relationship according to age and treatment. LTCC patch currents were evoked by depolarizing in +10 mV increments from a –70 mV holding potential to +40 mV. Results are shown as average patch current (mean ± SEM). The *I*–*V* relationship was not changed by E2 treatment in either young (yg) or aged OVX animals relative to controls (cntl). The half-maximal activation voltages (approximately –18 to –20 mV) and the stimulus voltage at which maximal current amplitude occurred (0 mV) were similar for all treatment groups. However, similar to the ensemble averages (Fig. 3), currents were much greater in the aged controls than the young controls, and E2 treatment reduced currents in the aged animals. *n* = 10–12 cells per/group; four animals per group.

showed slower rates of cognitive decline among those on Ca^{2+} channel blockers versus other antihypertensive agents. Because all agents reduced blood pressure to a similar extent, cognitive preserving effects were attributed to reduced neuronal LTCC influx (Trompet et al., 2008).

In the present study, E2 reduced body weight, raising the possibility that E2 mimics the effects of caloric restriction (CR). CR reduces biomarkers of brain aging (Patel and Finch, 2002), including changes in age-related Ca^{2+} -dependent mechanisms (Hemond and Jaffe, 2005). In rodents, CR typically is initiated in young adulthood and maintained for the entire lifespan, resulting in ~40–50% body weight reduction (Turturro et al., 1999). Whether several weeks of E2 with a much smaller weight loss (8–17%) (Fig. 2C) elicit effects characteristic of lifelong CR remains to be determined. Nevertheless, CR and E2 target common nutrient-signaling pathways (D'Eon et al., 2005; Mair and Dillin, 2008), and a recent study in humans shows that even short-term CR in the elderly can improve memory (Witte et al., 2009).

Potential mechanism of E2 regulation of LTCCs

To identify a possible mechanism underlying the E2-mediated reduction in current in aged female neurons, mRNA expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ pore-forming subunits was determined. Prior studies examining either mRNA or protein expression in aged male rats have shown either no effect (Kelly et al., 2001) or an increase in one or both LTCC subunits (Herman et al., 1998; Veng and Browning, 2002; Veng et al., 2003). In the present study, ISH was used to examine mRNA expression in the stratum pyramidale of the CA1 region, the region from which we recorded LTCCs in the hippocampal zipper slices. We did not find an age-related change in mRNA expression of either $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ subunits. However, E2 treatment in aged rats selectively reduced $\text{Ca}_v1.2$ mRNA expression by ~17%, although this was not to the extent of the approximate twofold reduction in LTCC current seen with E2. It should be noted that the proportional relationship between gene expression and functional measures of LTCC activity are unknown, and E2 may affect posttranslational modifications and protein–protein interactions to modulate

Figure 5. ISH analysis of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ mRNA expression in the CA1 region of the hippocampus from control and E2-treated animals. **A**, Representative images of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ mRNA expression in the hippocampus of aged-OVX female rats treated with vehicle or E2. Intensity of $\text{Ca}_v1.2$ mRNA labeling in the CA1 region showed a small but significant reduction in response to E2 treatment compared with vehicle control. $\text{Ca}_v1.3$ mRNA was not affected by E2 treatment. Images were derived from film autoradiograms after ISH in which cRNA probes were applied to coronal sections. Sense controls for cDNA probes were applied to adjacent sections and produced a negligible hybridization signal (data not shown). **B**, E2 treatment significantly decreased $\text{Ca}_v1.2$ expression levels in aged animals (**p* = 0.02) but not young animals. mRNA levels in the CA1 region were measured using optical density of film autoradiograms. **C**, E2 treatment did not significantly affect $\text{Ca}_v1.3$ expression levels in either young or aged animals. Data are mean ± SEM; *n* = 4–5 animals/group.

channel function (Davare and Hell, 2003; Kobayashi et al., 2007; Norris et al., 2008). Furthermore, the fact that E2, but not aging, affected mRNA expression suggests that aging and E2 modulate this channel via different mechanisms. Nonetheless, our results suggest that $\text{Ca}_v1.2$ is a likely target of E2 action in the brain and that E2's mechanism of action may involve, at least in part, a suppression of LTCC mRNA expression.

$\text{Ca}_v1.2$: a target of E2

It is interesting that E2 reduced expression of $\text{Ca}_v1.2$ in aged females, because this LTCC subtype has distinct properties from

$\text{Ca}_v1.3$ (Catterall et al., 2005). For example, $\text{Ca}_v1.2$ is selectively blocked by lower concentrations of DHPs (Catterall et al., 2005) and can be internalized during excessive activation (Green et al., 2007). Additionally, a phosphorylation-dependent increase specifically in the activity of $\text{Ca}_v1.2$ (Davare and Hell, 2003) contributes to age-related increases in LTCCs and Ca^{2+} influx in male rats (Thibault and Landfield, 1996; Thibault et al., 2001). A subpopulation of $\text{Ca}_v1.2$ s also form signaling complexes in neuronal membrane caveolae (Balijepalli et al., 2006), sites of membrane-bound ERs (Luoma et al., 2008). Together, these results suggest that a high degree of regulation has evolved to control LTCC activity and maintain optimal intracellular concentrations of Ca^{2+} .

Further support for a distinct role of $\text{Ca}_v1.2$ comes from studies of LTP. LTP is dependent on Ca^{2+} influx via NMDARs and LTCCs (Grover and Teyler, 1990) and consists of early and late LTP phases. Recently, Huang and Kandel (2006) described a novel form of late-LTP that steadily increases with age, is elicited by low-frequency stimulation, and is blocked by DHPs. Furthermore, this form of late-LTP may be an important underlying factor contributing to age-related memory impairment. Interestingly, $\text{Ca}_v1.2$ may participate in late-LTP and spatial learning (Moosmang et al., 2005). Given that E2 can reduce LTCCs and target $\text{Ca}_v1.2$ s in aged female brain, E2 may prevent an age-related increase in a form of synaptic plasticity associated with memory impairment. Indeed, we have observed that E2 treatment decreases LTCC-dependent LTP and improves cognitive performance in mid-aged female rats (J. T. Rogers and N. M. Porter, unpublished observations). Furthermore, the E2-related reduction in LTCCs may shift the balance between NMDAR and LTCC forms of LTP to favor the NMDAR-dependent form. Our results, together with others, suggest this may lead to enhanced LTP in the aging female brain exposed to E2 (Foy et al., 2008).

Because *in vivo* treatment with E2 was chronic, genomic mechanisms likely contributed to the E2 effects. Further support comes from ERKO mice which have larger LTCC currents in isolated cardiac myocytes (Johnson et al., 1997). Although we cannot rule out a rapid effect of E2 (Kelly and Rønnekleiv, 2008), LTCC recordings were obtained from hippocampal zipper slices bathed in media lacking E2. Rapid modulation of LTCCs has been reported *in vitro*, and depending on conditions, E2 can either stimulate (Wu et al., 2005; Sarkar et al., 2008) or inhibit Ca^{2+} influx (Mermelstein et al., 1996; Kurata et al., 2001; Boulware et al., 2005; Ullrich et al., 2007). These results suggest complex E2 regulation of LTCCs that may be concentration-, cell type-, age-, and even gender-dependent. Nonetheless, our study addresses a key issue regarding E2 action in the aged brain, whether E2 retains the ability to modulate some targets after a prolonged absence (Morrison et al., 2006). The present results suggest that E2 may prevent or reduce age-related Ca^{2+} dyshomeostasis by regulating LTCCs and offer some degree of neuroprotection even at advanced age. Although this late administration may not represent optimal timing for treatment initiation (Suzuki et al., 2007; Brinton, 2008; Sherwin and Henry, 2008), our studies also demonstrated benefit derived from a low physiological dose of E2. With continuing refinement of dosage in humans, HRT may safely attenuate CNS complications of menopause and pose minimal risk to an appropriately identified female population. Whether E2 has a unique gender-specific role in modulating LTCCs *in vivo* is unknown, although the results of Johnson et al. (1997) suggest otherwise. The development of nonfeminizing E2 analogs (Simpkins et al., 2005) or brain selective E2-like compounds (Zhao et al., 2007) may provide an opportunity to use

these agents broadly (Toung et al., 1998), regardless of gender, to take advantage of potential age-reversing E2 effects.

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