Hypersensitivity of the hippocampal CA3 region to stress-induced neurodegeneration and amyloidogenesis in a rat model of surgical menopause

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Females who enter menopause prematurely via bilateral ovariectomy (surgical menopause) have a significantly increased risk for cognitive decline and dementia. To help elucidate the mechanisms underlying this phenomenon, we used an animal model of surgical menopause, long-term (10-week) bilateral ovariectomy in female rats. Herein, we demonstrate that long-term oestrogen deprivation dramatically increases sensitivity of the normally resistant hippocampal CA3 region to ischaemic stress, an effect that was gender-specific, as it was not observed in long-term orchietomized males. Furthermore, the enhanced damage to the CA3 region correlated with a worse cognitive outcome after ischaemic stress. Long-term ovariectomized rats also displayed a robust hyperinduction of Alzheimer’s disease-related proteins in the CA3 region and a switch in amyloid precursor protein processing from non-amyloidogenic to amyloidogenic following ischaemic stress. CA3 hypersensitivity also extended to an Alzheimer’s disease-relevant insult, as the CA3 region of long-term ovariectomized rats was profoundly hypersensitive to the neurotoxic effects of amyloid-β1–42, the most amyloidogenic form of the amyloid-β peptide. Additional studies revealed that CA3 region hypersensitivity, Alzheimer’s disease-related protein induction, and amyloidogenesis are mediated by a NADPH oxidase/superoxide/c-Jun N-terminal kinase/c-Jun signalling pathway, involving both transcriptional and post-translational mechanisms. In addition, while 17β-oestradiol replacement at the end of the long-term oestrogen deprivation period could not prevent CA3 hypersensitivity and amyloidogenesis, if 17β-oestradiol was initiated at the time of ovariectomy and maintained throughout the 10-week oestrogen deprivation period, it completely prevented these events, providing support for the ‘critical window’ hypothesis for oestrogen replacement therapy benefit. Collectively, these findings may help explain the increased risk of cognitive decline and dementia observed in women following surgical menopause, and they provide increased support that early 17β-oestradiol replacement is critical in preventing the negative neural effects associated with bilateral ovariectomy.
**Introduction**

Menopause is a natural part of reproductive senescence in females and has a median onset of 51 years of age (Kato et al., 1998; Rocca et al., 2011). The menopausal transition is characterized by gradual ovarian follicle depletion, cessation of menstruation, and a dramatic decrease in serum levels of the ovarian-derived steroid hormone, 17β-oestradiol (Kato et al., 1998). Intriguingly, recent evidence suggests that premature or early menopause is associated with several negative neurological outcomes. Along these lines, recent studies from the Mayo Clinic Cohort Study of Oophorectomy and Ageing revealed that females who enter menopause prematurely by bilateral oophorectomy (surgical menopause) have a significantly increased risk of cognitive decline and dementia (Rocca et al., 2007, 2010; Rivera et al., 2009; Shufelt et al., 2011). The mechanisms underlying these increased risks are currently poorly understood. It has been proposed that these negative outcomes could be due to premature and prolonged 17β-oestradiol deficiency. In potential support of this possibility, females who underwent bilateral oophorectomy before the age of 49 but received oestrogen therapy through to the age of 50 years or older did not experience an increased risk of cognitive impairment or dementia (Rocca et al., 2010, 2011). Nevertheless, it remains unclear how 17β-oestradiol deficiency actually contributes to these detrimental neurological outcomes. To gain insight on this critical issue, our laboratory and others have been studying the effects of long-term oestrogen deprivation (LTED) upon the brain in animal models of surgical menopause. We recently found that sensitivity of the hippocampal CA1 region to 17β-oestradiol neuroprotective effects is lost after LTED (Zhang et al., 2011). Furthermore, while the focus of those studies was on the CA1 region, we noted that the normally resistant hippocampal CA3 region appeared to become more damaged from ischaemic stress after LTED (Zhang et al., 2009). We hypothesized that enhanced hippocampal CA3 hypersensitivity to stress may explain the increased risk of cognitive decline and dementia following surgical menopause, especially if it could be shown to be gender-dependent and could be extended to Alzheimer’s disease-relevant stressors and hyperinduction of Alzheimer’s disease-related proteins. Therefore, the purpose of the current study was to (i) establish whether CA3 hypersensitivity to ischaemic stress is gender-specific; (ii) determine the mechanisms underlying the CA3 hypersensitivity and whether it involves hyperinduction of Alzheimer’s disease-related proteins; (iii) establish whether the CA3 hypersensitivity extends to Alzheimer’s disease-relevant insults; (iv) determine whether CA3 hypersensitivity correlates with a decrease in cognitive function; and (v) establish whether 17β-oestradiol treatment can prevent the CA3 hypersensitivity, and whether a critical window exists for its effect.

**Materials and methods**

**Animals and global cerebral ischaemia**

Adult (3-month-old) Sprague Dawley female rats (Harlan) were bilaterally ovariectomized. Placebo or 17β-oestradiol Alzet mini-pumps (0.025 mg; 21 day release) were implanted subcutaneously in the upper mid-back region at the time of ovariectomy, and global cerebral ischaemia (GCI) was performed 1 week later (short term). The dose of 17β-oestradiol used produces serum 17β-oestradiol levels of ~10–15 pg/ml, which represents physiological low dioestrus I levels of 17β-oestradiol (Zhang et al., 2008). In some experiments, LTED was performed, in which animals were ovariectomized 10 weeks before placebo or 17β-oestradiol mini-pumps were implanted, and GCI was performed 1 week later. Finally, some animals with LTED had 17β-oestradiol replacement initiated immediately and maintained throughout the 10 weeks of ovariectomy. In some studies, 3-month-old adult Sprague Dawley male rats were bilaterally gonadectomized and GCI performed 1 week or 11 weeks later. For GCI, all animals (except sham control) underwent 4-vessel occlusion GCI performed as described previously (Zhang et al., 2008). Briefly, 24 h after electrocauterization of the vertebral arteries, the common carotid arteries were occluded with aneryxym clips to induce 10 min of forebrain ischaemia. Animals that lost their righting reflex within 30 s and whose pupils were dilated and unresponsive to light during GCI were selected for the experiments. After 10 min the clips were removed, and blood flow through the arteries was confirmed before the wound was sutured. Rectal temperature was maintained at 36.5 to 37.5 °C throughout the experiment with a thermal blanket. Sham animals underwent identical procedures except that the common carotid arteries were simply exposed but not occluded. The detailed experimental design for the studies is presented in Supplementary Fig. 1.

**Drug administration**

NADPH oxidase competitive antagonist, gp91ds-tat, or its scrambled peptide (AnaSpec Inc) were dissolved in saline at a concentration of 20 ng/μl. Five microlitres of the solutions were bilaterally infused into the lateral ventricles (anterior/posterior 0.8 mm; medial/lateral 1.5 mm; dorsal/ventral –3.5 mm) of the LTED 20 min before induction of GCI. For intracerebroventricular injections, anaesthetized rats were placed on ear bars of a stereotaxic instrument, and the skull was exposed through an incision on the midline of the scalp. The drug was infused at a flow rate of 1 μl/min with a Hamilton microsyringe. Following injection, the needle was left in situ for 5 min before the complete 2 min retraction, after which the incision was closed using wound clips. The JNK inhibitor SP600125 was dissolved in PPCES vehicle (30% PEG-400/20% polypropylene glycol/15% cremophor EL/5% ethanol/30% saline) as described previously (Zhang et al., 2008), and was treated by tail vein injection at a dose of 10 mg/kg 20 min before induction of GCI. For the amyloid-β1–42-induced neurotoxicity study, full-length amyloid-β1–42 or scrambled amyloid-β1–42 (AnaSpec Inc) was dissolved in 35% acetonitrile solution, diluted with 0.1 M PBS to a concentration of 500 μM, and incubated for 18 h at 37°C ( Ryu et al., 2004). Amyloid-β1–42...
(1 nmol in 2 μl) was injected bilaterally to the coordinates targeting midway between the CA3 and CA1 regions (anterior/posterior −3.3 mm, medial/lateral ± 3.3 mm, dorsal/ventral −3.6 mm).

**Histological analysis**

Histological examination of the ischaemic brain was performed by NeuN and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining as described previously (Zhang et al., 2009). Briefly, 7 days after ischaemic reperfusion, the animals were deeply anaesthetized with chloral hydrate and underwent transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, post-fixed overnight with paraformaldehyde, cryoprotected with 30% sucrose until they sank, and frozen sections (20 μm each) were made in series in the coronal plane of the dorsal hippocampus (~2.5–4.5 mm posterior from Bregma, ~100 sections per brain). To prevent double counting of cells, every fifth section was collected and used for staining. Staining for NeuN was performed using a mouse anti-NeuN monoclonal antibody (1:500, Millipore Bioscience) as described previously (Zhang et al., 2008). Images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss). NeuN-positive pyramidal cells with intact and round nuclei were counted as surviving cells. TUNEL staining was performed on free-floating coronal sections using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions. Slides for negative control were incubated with the label solution without terminal transferase for TUNEL. For quantitative analyses, the number of surviving neurons, and TUNEL-positive cells per 250 μm length of medial CA3 pyramidal cell layer was counted bilaterally in five representative sections per animal. Cell counts from the right and left hippocampus were averaged to provide a single value for each section, and the counts of five sections were averaged to provide a single value for each animal. A mean ± standard error (SE) was calculated from the data in each group (n = 5–8). Statistical analysis was performed as described below.

**Immunofluorescence staining**

Coronal brain floating sections from rats at 3 h or 7 days reperfusion were prepared as described above. Briefly, sections were incubated with 10% donkey serum for 1 h at room temperature in PBS containing 0.1% Triton X-100, followed by incubation with appropriate primary antibodies overnight. The following primary antibodies were used in different combinations: anti-NeuN (1:500, MA8377, Millipore); anti-amyloid-β(1-42) (1:500, #700254) and anti-BACE1 (1:200, AH80241) from Invitrogen Corporation; anti-phospho-JNK (1:50, sc-12882) and anti-phospho-clun (1:50, sc-7981) from Santa Cruz Biotechnology, anti-phospho-amyloid precursor protein (1:400, #3823, Cell Signaling Technology), anti-PHF1 (1:200; gift from Dr. Peter Davies). Sections were then washed four times at room temperature, followed by incubation with Alexa Fluor® 594/647/488 donkey anti-mouse/rabbit/goat secondary antibody (1:500; Invitrogen) for 1 h at room temperature. After washes, sections were mounted with water-based mounting medium containing anti-fading agents. Confocal images were captured on an LSM510 Meta confocal laser microscope (Carl Zeiss) using either a ×5 or ×40 oil immersion Neofluor objective (NA, 1.3) with the image size set at 1024 × 1024 pixels. The captured images were viewed and analysed using LSM510 Meta imaging software. At least five representative sections per animal were utilized for immunostaining. Each treatment group consisted of four to seven animals and the typical staining was selected for presentation.

**Brain homogenization and western blotting**

For brain tissue preparation, rats were decapitated under deep anaesthesia at 3 h after reperfusion. Whole brains were removed, and the hippocampal CA3 regions were micro-dissected and immediately frozen in dry ice. Tissue homogenization was performed as previously described (Zhang et al., 2008). Western blotting was performed as previously described (Zhang et al., 2008) using the following antibodies: NOX2 (1:1000, Abcam); phospho-tau (1:5000; PHF1; gift from Dr. Peter Davies); phospho-amyloid precursor protein (1:400, #3823, Cell Signalling Technology); amyloid precursor protein C-Terminal (recognizes C-83/C-99 fragments, 1:4000, A8717, Sigma Aldrich); amyloid precursor protein (recognizes full-length protein at 100 kD, 1:1000, #36-6900, Invitrogen); p-JNK (sc-12882), JNK (sc-572), p-clun (sc-7981), clun (sc-45), BACE1 (sc-10748) and β-actin (sc-81178) were all from Santa Cruz Biotechnology and used at a 1:200 dilution. Following western blotting, bound proteins were visualized using the Odyssey Imaging System (LI-COR Bioscience), and semi-quantitative analysis of the bands was performed with the ImageJ analysis software (Version 1.30v; NIH). Band densities for the indicated phospho-proteins were normalized to the corresponding band densities for total proteins, and the indicated total proteins were normalized relative to β-actin. A mean ± SE was calculated from the data for graphical presentation and statistical comparison.

**In situ hybridization**

Locked nucleic acid (LNA)-modified oligonucleotide probes were digoxigenin (DIG)-labelled by miRCURY LNA™ technology, Exiqon. The sequences used in study were /5 ’DigN/TGCAACAGCCAGTAAGTAGA/3’Dig_N/ for NOX2 probe, /5 ’DigN/CCACCTGTGCTAGTGATGT/3’Dig_N/ for BACE1 probe. Scrambled oligonucleotides were used as negative controls. In situ hybridization was performed according to a protocol described previously (Obernosterer et al., 2007). Briefly, the fixed coronal brain sections from rats at 3 h reperfusion were treated for 5 min with 5 μg/ml proteinase K in 0.1 M PBS. The sections were placed in PBS and incubated in prehybridization solution for 6 h at room temperature. The locked nucleic acid DIG-labelled probes (1 pM) were denatured by heating to 80 °C for 5 min and quickly placed on ice. Afterward, the sections were incubated with probe mix overnight at 60 °C. After hybridization, the slides were soaked in pre-warmed 5 saline-sodium citrate and incubated in ×0.2 saline-sodium citrate at 60 °C for 1 h. Finally, slides were incubated in B1 solution (0.1 M Tris pH 7.5, 0.15 M NaCl) at room temperature for 10 min. Then, sections were blocked with 5% bovine serum albumin for 1 h at room temperature and incubated with anti-DIG-alkaline phosphatase antibody (1:2000) in blocking solution at 4 °C overnight. The sections were washed three times with B1 solution and equilibrated for 10 min in B3 solution (0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl2) at room temperature. Finally, the colouring reaction was developed using BCIP/NBT substrate solution (Invitrogen) in the dark, and staining was visualized using standard light microscopy. For quantitative analysis of Nox2 messenger RNA expression, optical density of in situ staining was analysed using Image J analysis software (Version 1.30v; NIH), and a mean ± SE was calculated for each treatment group, which consisted of four to five animals each and five sections per animal. Statistical analysis was performed as described below, and results were expressed as percentage change compared with the short-term sham value.
Morris water maze

At Days 7, 8 and 9 after reperfusion, the Morris water maze task was performed to test spatial learning and memory as described previously (Raz et al., 2010; Yang et al., 2010). The maze consisted of a black, circular pool with a black, circular platform (9 cm diameter) placed 2 cm below the water line in the centre of one quadrant. The swimming path of each rat was monitored by an overhead video camera connected to a personal computer and analysed by an automated tracking system. Briefly, at 7 days post-ischaemia, rats were placed in the water facing the wall at one random start location out of four (north, south, east and west), located equidistant from one another around the rim of the pool. Rats were then allowed to find the submerged platform within 90 s, and remain on the submerged platform for 20 s. If the rat failed to find the hidden platform within the allotted time, it was placed on it for 20 s. The procedure was repeated from each of the four start locations. The escape latency, time to reach the hidden platform, and swimming speed were recorded. Two sessions of the four trials were conducted on the first testing day, within a 4 h interval, and the first session was considered as a training procedure. Formal testing was conducted daily on the next two testing days. Four hours after the last hidden platform testing, a 90 s memory probe trial was performed by removing the platform from the tank. The rat was placed in the water at the same random start location, and time spent in the quadrant of the pool, which previously contained the platform, was used to assess the level of learning and memory on the given task.

NADPH oxidase activity and superoxide production assay

NADPH oxidase activity and superoxide production were determined as described previously (Zhang et al., 2009). NADPH oxidase activity was calculated as relative light unit changes per microgram of protein. Superoxide production was measured using a LumiMax Superoxide Anion Detection kit (Stratagene). A mean ± SE was calculated from the data collected in each group for graphical depiction and was expressed as fold change versus sham control group (Zhang et al., 2009; Raz et al., 2010).

BACE1 and cJun/AP-1 activity assays

To measure BACE1 activity, 20 μg each of CA3 total protein samples were incubated with 50 μM of a fluorescence conjugated peptide substrate [Mca-EVKDAEF-K(Dnp), #60268, AnaSpec Inc.] in 0.15 M of acetate buffer (pH 5) at 37°C for 2 h. The substrate cleavage was measured using a fluorescence luminometer (LS-55, Perkin–Elmer) at excitation of 325 nm and emission of 393 nm. A mean ± SE was calculated in each group and expressed as fold change versus sham control in short term group. The cJun/AP-1 activity was performed using an ELISA TransAM® kit (Active Motif, #46096) according to manufacturer’s instructions.

Analysis of endogenous amyloid-β levels

Levels of amyloid-β in the hippocampal CA3 were measured using amyloid-β1–40 ELISA Kit (KMB3481) and amyloid-β1–42 ELISA Kit (KMB3441) (Invitrogen) according to the protocol provided by the manufacturer. The plates were read on a spectrophotometer at 450 nm, and the data based on the standard curve were expressed as fold changes as sham control in short term group.

Statistical analysis

Statistical analysis was performed using either one-way or two-way analysis of variance (ANOVA), followed by Student-Newman-Keuls post hoc tests to determine group differences. When groups were compared with a control group (e.g. sham), Dunnett’s test was adopted for post hoc analyses after ANOVA. When only two groups were compared, a Student’s t-test was used. Statistical significance was accepted at the 95% confidence level (P < 0.05). Data were expressed as mean ± SE.

Results

Long-term oestrogen deprivation leads to enhanced hippocampal CA3 apoptosis-like neuronal cell death and cognitive impairment after ischaemic stress

In a previous study, we reported that the normally resistant hippocampal CA3 region appeared to become more damaged in animals with LTED (10-week oestrogen deprivation) following GCI (Zhang et al., 2009), although this effect was not quantified. In the current study, we sought to confirm and quantify this effect in the female hippocampus, and establish whether the effect was gender-specific. We also sought to determine whether the increased hippocampal CA3 damage could be due to enhanced apoptotic-like delayed neuronal death. As shown in Fig. 1A–D, immunohistochemistry for the neuronal marker, NeuN, revealed that whereas 10-min GCI did not significantly damage the CA3 region at 7 day reperfusion in female rats with short-term oestrogen deprivation (STED; short-term 17β-oestradiol deprived, 1-week oestradiogen deprivation), it caused extensive damage to the CA3 region of rats with LTED. In addition, staining for TUNEL, a marker of apoptosis, revealed that there is significantly enhanced apoptosis in the CA3 region of rats with LTED compared with rats with STED after GCI (Fig. 1C and D) and that the enhanced neuronal cell death in the CA3 region could not be prevented by 17β-oestradiol treatment initiated at the end of the LTED period (% surviving neurons, 15.38 ± 6.87 in 17β-oestradiol versus 20.50 ± 8.51 in placebo, n = 8). We next determined whether the hypersensitivity of the CA3 region to ischaemic stress following long-term gonadectomy was gender-specific. As shown in Fig. 1, staining for NeuN and TUNEL (Fig. 1B and D) and quantification (Fig. 1A and C) revealed that surviving neurons and apoptotic-like cells in the CA3 were not significantly different between short-term and long-term gonadectomy male rats following GCI. This finding demonstrates that, in contrast with female animals, long-term gonadectomy in male animals does not induce CA3 hypersensitivity to ischaemic stress. The results thus indicate that CA3 hypersensitivity following long-term gonadectomy is a gender-specific phenomenon that only occurs in females. We next examined the effect of LTED upon functional outcome following GCI by examining spatial learning and memory in female rats at Days 7, 8 and 9 after...
reperfusion using the Morris water maze (Fig. 2A–D). Figure 2A illustrates the paradigm used for treatment, ischaemia, and behavioural testing. Figure 2B shows representative traces from maze latency trials and probe trials on Day 9, while Fig. 2C and D shows results for escape latency (Fig. 2C) and quadrant occupancy (Fig. 2D). These results show that following GCI, rats with LTED had worse cognitive function as compared to rats with STED, with rats with LTED demonstrating significantly longer escape latencies (Fig. 2C) and spending significantly less time in the quadrant where the platform was located (Fig. 2D). Intriguingly, while 17β-oestradiol was capable of improving/preserving cognitive function in rats with STED after GCI, this beneficial effect was lost in rats with LTED (Fig. 2C and D).

**Activation of a NADPH oxidase-superoxide-JNK/c-Jun signalling pathway mediates hippocampal CA3 region hypersensitivity to ischaemic stress following long-term oestrogen deprivation**

We next sought to elucidate the mechanisms underlying the CA3 hypersensitivity to ischaemic stress in rats with LTED. Previous work has demonstrated that activation of NOX2 NADPH oxidase plays a critical role in ischaemic damage to the vulnerable hippocampal CA1 region (Zhang et al., 2009). Because the CA3 region is resistant to ischaemic stress in rats with STED, we hypothesized that NOX2 expression and activation in the CA3 may be low and not induced by GCI, thus explaining its relative resistance to ischaemic stress. We further hypothesized that LTED may alter the CA3 region such that ischaemic stress leads to induction of NOX2, thereby contributing to enhanced oxidative stress damage and neuronal cell death. Figure 3A reveals that, indeed, NOX2 protein levels are very low in the CA3 region of sham rats with STED and not induced by GCI or regulated by 17β-oestradiol. Sham rats with LTED also show low Nox2 messenger RNA and protein levels (Supplementary Fig. 2A–C). However, in contrast to rats with STED, rats with LTED displayed enhanced neuronal expression of NOX2 protein levels in the CA3 region following 3h reperfusion (Fig. 3A and B). This effect appears to be exerted at the messenger RNA level, as further work using in situ hybridization showed that Nox2 messenger RNA was robustly increased in the CA3 region of placebo and 17β-oestradiol-treated rats with LTED at 3h reperfusion as compared with rats with STED (Supplementary Fig. 2A and B). We next examined activation of NOX2 NADPH oxidase, since it is known that NOX2 activation leads to generation of the reactive oxygen species superoxide, which can be converted to highly damaging reactive oxygen species such as hydroxyl radical and peroxynitrite (Kawano et al., 2007; Kunz et al., 2007). Previous work showed that NADPH oxidase activation and superoxide levels in the CA3 of rats with STED are very low and not significantly affected by either GCI or 17β-oestradiol treatment (Zhang et al., 2009). In contrast, in the
current study, rats with LTED exhibited a robust, 4 to 5-fold increase in both NADPH oxidase activity and superoxide levels in the CA3 region following 3 h reperfusion, which was not prevented by delayed 17β-oestradiol treatment (Fig. 3C).

C-Jun N-terminal kinase (JNK) is a stress-activated kinase that has been implicated to play an important role in neuronal apoptosis and amyloidogenic processing (Ozawa et al., 1999; Guan et al., 2005; Colombo et al., 2009; Quiroz-Baez et al., 2009), and it can be induced by NADPH oxidase-derived superoxide (Blatt et al., 2008; Soberanes et al., 2009). JNK is activated by dual phosphorylation at Thr183 and Tyr185 residues (Thevenin et al., 2011), and JNK activation subsequently leads to phosphorylation (and activation) of its downstream transcription factor target, cJun, at residues Ser63 and Ser73 (Weston and Davis, 2007). Using phospho-specific antibodies, we thus examined activation of JNK/cJun in the CA3 region of female rats with STED and LTED. As shown in Fig. 3D and E, both p-JNK and p-cJun levels in the CA3 region were low in rats with STED and unchanged by 17β-oestradiol treatment or ischaemia. In contrast, rats with LTED showed a marked induction of p-JNK and p-cJun levels in the CA3 region after 3 h reperfusion, and delayed 17β-oestradiol treatment was incapable of preventing this event.

To determine whether activation of JNK and cJun was due to NADPH oxidase activation, we administered a competitive NOX2 NADPH oxidase inhibitor, gp91ds-tat (91ds-tat), or a scrambled control peptide (Scr). As shown in Fig. 3E, while the scrambled control peptide had no effect upon JNK and cJun activation, the NOX2 inhibitor gp91ds-tat strongly attenuated both JNK and cJun activation in the CA3 region of rats with LTED. In addition, LTED female rats also displayed robust cJun/AP-1 activity at 3 h reperfusion in the CA3 region after ischaemic stress, an effect that was attenuated by administration of the JNK inhibitor SP600125 or by the NOX2 NADPH oxidase inhibitor gp91ds-tat, indicating that activation of the JNK/cJun signalling pathway lies downstream of NOX2 (Fig. 3F). Finally, administration of either gp91ds-tat or SP600125 markedly reversed the enhanced neuronal cell death seen in the CA3 region of female rats with LTED at 7 day reperfusion following GCI (Fig. 3G), demonstrating the importance of...
NADPH oxidase and JNK signalling in the enhanced susceptibility of CA3 hippocampal neurons to cell death. Further studies also revealed that JNK inhibitor administration significantly reversed the cognitive decline seen in rats with LTED after GCI (Supplementary Fig. 2D and E).

Robust induction of BACE1 in hippocampal CA3 neurons of rats with long-term oestrogen deprivation following ischaemic stress

Since cJun is a well-known transcription factor for the β-secretase, BACE1, which cleaves amyloid precursor protein at the N-terminal region of the amyloid-β domain (Tamagno et al., 2005; Guglielmotto et al., 2009), we hypothesized that LTED could lead to dysregulation of Alzheimer’s disease proteins in the CA3 region following ischaemic stress. Using western blot analysis, it was revealed that BACE1 protein levels are consistently low in the CA3 region of rats with STED and not significantly induced by ischaemic stress (Fig. 4A). In contrast, ischaemic stress resulted in robust induction of BACE1 in rats with LTED at 3 h reperfusion, an effect that was dependent upon JNK activation, as treatment with a JNK inhibitor markedly attenuated the observed BACE1 elevation. Further work revealed that p-JNK and p-cJun are highly induced and co-localized with BACE1 in CA3 neurons of LTED, but not STED, rats following 3 h ischaemic stress, further suggesting a causal relationship between JNK/cJun activation and BACE1 induction (Fig. 4B). In further support of a causal role of JNK/cJun signalling in BACE1 induction, measurement of BACE1 activity at 3 h reperfusion using a cleavable fluorescent substrate assay confirmed robust induction of BACE1 activity in the CA3 region of rats with LTED, but not STED, after ischaemic stress, an effect that was significantly attenuated by administration of the JNK inhibitor SP600125 (Fig. 4C). Finally, in situ hybridization demonstrated that only rats with LTED had marked induction of BACE1 messenger RNA in the CA3 region after 3 h ischaemic
stress, which was significantly attenuated by administration of SP600125 (Fig. 4D and E).

Amyloid precursor protein phosphorylation is markedly enhanced in the CA3 region of rats with long-term oestrogen deprivation after ischaemic stress

Recent evidence suggests that phosphorylation of amyloid precursor protein at Thr668 by JNK enhances amyloid-β generation by facilitating BACE1 cleavage of amyloid precursor protein (Colombo et al., 2009). We therefore examined whether LTED affects amyloid precursor protein phosphorylation. As shown in Fig. 5A, dual immunohistochemistry revealed that p-JNK and amyloid precursor protein Thr668 are significantly co-localized in CA3 neurons of LTED, but not rats with STED after ischaemic stress. Western blot analysis confirmed that amyloid precursor protein Thr668 phosphorylation was low in CA3 of rats with STED and unchanged by GCI (Fig. 5B). Importantly, amyloid precursor protein phosphorylation was also low in sham rats with LTED, which were not subjected to ischaemia. However, in animals with LTED subjected to ischaemic stress (placebo, vehicle), amyloid precursor protein phosphorylation was robustly elevated at 3 h reperfusion. Treatment with the JNK inhibitor SP600125 (SP) markedly attenuated the elevation of amyloid precursor protein phosphorylation in animals with LTED after GCI (Fig. 5B), implicating a role for JNK in amyloid precursor protein phosphorylation. Additional studies revealed that there is also a robust hyperphosphorylation of the microtubule-associated protein, tau in the CA3 neurons, of rats with LTED, but not STED at 3 h reperfusion, an effect that was...
found to be dependent upon JNK activation (Supplementary Fig. 3A and B). Furthermore, phospho-tau was shown to be highly co-localized in p-JNK-positive neurons in the CA3 of rats with LTED, but not STED at 3 h reperfusion (Supplementary Fig. 3C).

Enhanced amyloidogenic processing in the CA3 region of rats with long-term oestrogen deprivation after ischaemic stress

Proteolytic processing of amyloid precursor protein is divided into amyloidogenic and non-amyloidogenic pathways (Lichtenthaler and Haass, 2004). The non-amyloidogenic pathway involves amyloid precursor protein cleavage by $\alpha$-secretase, which cleaves within the amyloid-\(\beta\) domain and thus precludes amyloid-\(\beta\) peptide generation. Amyloid precursor protein cleavage by $\alpha$-secretase yields a C-terminal fragment (C-83) and a soluble amyloid precursor protein fragment (sAPP$\alpha$). In the amyloidogenic pathway, however, the $\beta$-secretase BACE1 cleaves amyloid precursor protein to generate an N-terminal soluble domain and a membrane-tethered C-terminal domain (C-99). Subsequently, insoluble amyloid-\(\beta\) is released by $\gamma$-secretase cleavage within the transmembrane domain of amyloid precursor protein. To determine the effect of LTED on amyloid precursor protein processing, we thus examined the C-99/C-83 ratio in the hippocampal CA3 region in sham animals and in animals that experience 3 h ischaemic reperfusion. As shown in Fig. 5C, sham and placebo (placebo, ischaemic) rats with STED and sham rats with LTED display a low C-99/C-83 amyloid precursor protein ratio in the CA3 region, which is indicative of active non-amyloidogenic amyloid precursor protein processing. In contrast, placebo and vehicle-treated
ischaemic rats with LTED exhibit a robust increase in the C-99/C-83 ratio, which suggests enhanced amyloidogenic amyloid precursor protein processing. Intriguingly, the increase in amyloidogenic amyloid precursor protein processing in rats with LTED after GCI appears to involve mediation by JNK, as treatment with the JNK inhibitor SP600125 (SP) prevented the C-99/C-83 ratio increase. Increased amyloidogenic processing of amyloid precursor protein would be expected to lead to enhanced amyloid-β formation. Examination of the most common isoforms found in Alzheimer’s disease, amyloid-β1–40 and amyloid-β1–42, indeed revealed a robust elevation of both isoforms in the CA3 of LTED, but not STED, rats at 3 h reperfusion (Fig. 5D). The induction of amyloid-β1–42 at 7 h reperfusion in the CA3 of female rats with LTED was shown to co-localize with TUNEL, a marker of apoptosis (Fig. 5E), suggesting that amyloid-β may play a role in the enhanced CA3 neuronal cell death in rats with LTED after GCI. Furthermore, administration of either a NOX2 (91ds-tat) or a JNK inhibitor (SP600125) markedly attenuated the amyloid-β1–42 induction and the number of TUNEL-positive cells observed in the CA3 region following 7 d ischaemic reperfusion (Supplementary Fig. 4A and B), which further suggests a role for NOX-JNK signalling. We next examined whether CA3 hypersensitivity in rats with LTED extends to a non-ischaemic, Alzheimer’s disease-relevant insult by administering amyloid-β1–42 oligomers directly into the CA3 region of female rats with STED and LTED. The results showed that infusion of amyloid-β1–42 (1 nmol) induced ~30% loss of CA3 neurons in rats with STED at ischaemic Day 7, while rats with LTED, in contrast, showed a much greater sensitivity, with ~80% loss of CA3 neurons (Fig. 5F). Increased CA3 sensitivity to amyloid-β neurotoxicity after LTED could help explain the increased risk of cognitive decline and dementia in females following surgical menopause.

Continuous 17β-oestradiol replacement initiated at the onset of long-term oestrogen deprivation prevents BACE1 induction, amyloid-β induction and CA3 hypersensitivity to ischaemic stress

As delayed 17β-oestradiol replacement initiated at the end of LTED was incapable of preventing the hypersensitivity of the CA3 region to ischaemic stress and Alzheimer’s disease protein induction, we sought to determine whether early low-dose 17β-oestradiol replacement initiated at the beginning and maintained throughout the LTED period would prevent the CA3 hypersensitivity. As shown in Fig. 6A and B, early 17β-oestradiol replacement initiated at the time of ovariectomy and maintained throughout the 10-week oestrogen deprivation period prevented the CA3 hypersensitivity to ischaemic stress, as evidenced by an increase in the number of NeuN-positive cells in the CA3 region and decrease in the number of TUNEL-positive cells in the CA3 region at 7 days after GCI. Note also that early 17β-oestradiol treatment abolished the robust induction of amyloid-β (1–42), NOX2, p-JNK and BACE1 in the CA3 region that was typically observed following GCI in rats with LTED (Fig. 6A–D), which likely explains its ability to abolish the CA3 hypersensitivity and amyloid-β induction after GCI in rats with LTED.

Discussion

Using an animal model of surgical menopause (LTED), the current study demonstrates that the normally resistant hippocampal CA3 region becomes hypersensitive to ischaemic stress in a gender-specific manner following long-term gonadectomy, leading to robust Alzheimer’s disease protein induction and amyloidogenic processing, increased neurodegeneration, and poorer cognitive outcome after GCI. The finding of a poorer cognitive outcome in rats with LTED after ischaemic stress is consistent with the purported critical role of the hippocampal CA3 region in cognitive function. In fact, previous work has shown that the hippocampal CA3 region contains extensive recurrent collaterals and is considered an auto-association network that is critical for storage and retrieval of information, particularly with regard to working memory (Florian and Roulet, 2004; Kesner, 2007). An additional important observation of our study was that the CA3 hypersensitivity in animals with LTED also extended to a non-ischaemic Alzheimer’s disease-relevant stressor, as LTED animals displayed a profound hypersensitivity to the neurotoxic effects of amyloid-β1–42, the most amyloidogenic form of the amyloid-β peptide. It is proposed that LTED-induced hypersensitivity to both amyloid-β1–42 and ischaemic stress, along with a profound post-ischaemic hyperinduction of Alzheimer’s disease proteins and amyloidogenic processing in the CA3, may explain, in part, the purported 2-fold increased risk of cognitive decline and dementia observed in women following surgical menopause (Rocca et al., 2007; Rivera et al., 2009; Phung et al., 2010).

Based on the results of our studies, the following mechanistic schema is proposed to explain the increased CA3 hypersensitivity to ischaemic stress in rats with LTED (Fig. 7). As shown in Fig. 7, hypoestrogenicity and ischaemic stress lead to a robust induction of NADPH oxidase and superoxide in the CA3 region, with a corresponding profound induction and activation of the stress activated protein kinase, JNK. Once activated, JNK subsequently phosphorylates tau and clun, a transcription factor that induces expression of BACE1 (Tamagno et al., 2005), which cleaves amyloid precursor protein to yield amyloid-β. Intriguingly, JNK also enhances phosphorylation of amyloid precursor protein at residue Thr668 (Colombo et al., 2009; Quiroz-Baez et al., 2009), which further enhances BACE1-induced cleavage of amyloid precursor protein to amyloid-β. A positive feedback loop is also proposed, in which clun induces transcription of NOX2, thus contributing to its over-expression and perpetuating CA3 region hypersensitivity. In support of JNK/clun signalling exerting positive feedback regulation on NOX2, administration of a JNK inhibitor was shown to block the elevation of NOX2 in the CA3 of rats with LTED following ischaemic stress (Supplementary Fig. 2C). Finally, CA3 neuronal cell death and cognitive impairment are proposed to be mediated, at least in part, by overproduction of amyloid-β (Fig. 7), which is well known to be neurotoxic (Lambert et al., 1998; Shankar et al., 2008; Ono et al., 2009; Brouillette et al., 2012).
It is important to note that sham rats with LTED did not display any increased neuronal cell death in the CA3, nor did they exhibit any enhancement of NOX2, superoxide, JNK/Jun activation or Alzheimer’s disease-related protein induction. This is consistent with sham rats with LTED not demonstrating any significant decline in cognitive function. Along these lines, CA3 alterations in the NOX2-JNK signalling pathway, as well as amyloidogenesis, neuronal cell death, and cognitive decline were only observed after ischaemic stress. This suggests that ovarian 17β-oestradiol is an important regulator of hippocampal CA3 neuron sensitivity to stressors. The mechanisms of how 17β-oestradiol regulates cell sensitivity to stress is unclear, but we recently reported that 17β-oestradiol can prevent acetylation of the stress sensor p53 in the hippocampus (Raz et al., 2011). The importance of this effect is evidenced by previous findings showing that acetylation of p53 leads to its stabilization and enhances cells’ sensitivity to subsequent stressors (Ito et al., 2002).

The findings of our study also provide support for the ‘critical window’ hypothesis of oestrogen replacement beneficial effect (also known as the ‘critical period’ hypothesis), which suggests that 17β-oestradiol must be given at peri-menopause (e.g. close to the time of 17β-oestradiol deprivation) in order to yield maximum neurological benefit (Sherwin, 2003, 2007, 2009; Maki, 2006; Scott et al., 2012). Along these lines, our study found that low-dose 17β-oestradiol administered at the end of the LTED period was incapable of reversing the hyperinduction of NOX2-JNK-Jun signalling, amyloidogenesis and CA3 neuronal damage after GCI. However, 17β-oestradiol initiated early at the time of LTED initiation and maintained throughout the LTED period completely prevented the NOX/JNK signalling pathway/Alzheimer’s disease protein hyperinduction and CA3 hypersensitivity to ischaemic stress. Our finding that 17β-oestradiol replacement initiated early after 17β-oestradiol deprivation was neuroprotective is consistent with previous reports in humans that 17β-oestradiol replacement prior to the age of 50 in surgical menopause patients may alleviate the increased risk for cognitive decline and dementia in women who enter menopause prematurely due to bilateral oophorectomy (Rocca et al., 2010, 2011). In addition, the recent 10-year follow-up of the Women’s Health Initiative (WHI) also provided support for the critical window hypothesis since researchers found oestrogen (conjugated equine oestrogens) alone to be beneficial for cardiovascular disease and overall mortality if treatment was initiated before 60 years of age (LaCroix et al., 2011). In the same study, oestrogen’s benefit

Figure 6 Neuroprotection in hippocampal CA3 neurons, and the prevention of p-JNK, BACE1 and NOX2 induction by early continuous 17β-oestradiol (E2) replacement for 10 weeks in rats with LTED. (A) Representative staining of NeuN, TUNEL and amyloid-β1–42 of CA3 region from sham (Sh), and animals treated with 17β-oestradiol or placebo (Pla) immediately after ovariectomy for the entire 10-week period. The rats were subjected to sham or 10 min ischaemia followed by 7 days of reperfusion. (B) Cell-counting study shows the number of surviving neurons (% changes of sham) and TUNEL-positive cells per 250 μm length of medial CA3 region. Magnification: × 40. Scale bar = 50 μm. n = 6–7 in each group. #P < 0.05 versus placebo group. (C and D) Western blot analyses of the indicated CA3 proteins at 3 h ischaemic reperfusion from sham rats with LTED, and LTED rats treated with 17β-oestradiol/placebo immediately at the time of ovariectomy for the full 10 weeks. Data are means ± SE from four rats in each group. *P < 0.05 versus placebo-treated group.

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Figure 7 Summary diagram of the proposed mechanisms underlying CA3 hypersensitivity, Alzheimer’s disease protein induction, neuronal death and behavioural deficits following cerebral ischaemia in rats with LTED. LTED plus stress (cerebral ischaemia) leads to hyperinduction of NOX2 NADPH oxidase activity and superoxide elevation, resulting in the activation of stress-responsive JNK/c-Jun signalling pathway in hippocampal CA3 neurons. JNK pathway activation induces the gene expression of BACE1 as well as amyloid precursor protein (Thr668) phosphorylation, which together promotes amyloid-β expression following ischaemic stress. JNK/c-Jun activation also initiates a positive-feedback regulation of NADPH oxidase and superoxide production by inducing NOX2 expression. Finally, Tau hyperphosphorylation resulting from JNK activation, together with amyloid-β generation and other possible factors induced by JNK/c-Jun signalling, leads to enhanced CA3 neuronal cell death and memory deterioration following cerebral ischaemia in rats with LTED. APP = amyloid precursor protein.

appeared to wane with increasing age and time after onset of menopause, until conjugated equine oestrogens trended towards detrimental (LaCroix et al., 2011). The mechanisms underlying the critical window are not fully understood, but recent work by our group (Zhang et al., 2011) found that hippocampal Erα levels decrease significantly after LTED due to a CHIP-mediated proteosomal degradation, which may help explain the decreased sensitivity of the hippocampus to 17β-oestradiol after LTED. A similar decrease of hippocampal Erα has also been reported following natural ageing (Adams et al., 2002; Mehra et al., 2005; Zhang et al., 2011).

While the current study yields potential insight into the increased risk of cognitive decline and dementia in surgical menopause patients and provides support for the critical window hypothesis of hormone replacement therapy benefit, there are several caveats to consider. For instance, the mechanisms proposed to mediate CA3 hypersensitivity to stress were observed in a rodent model of surgical menopause. It is currently unclear whether the same cascade of events will ensue in females who enter menopause prematurely due to an organic cause, such as premature ovarian failure. It should also be mentioned that low-dose 17β-oestradiol replacement was used in our study, with the 17β-oestradiol being delivered both continuously and subcutaneously. While higher 17β-oestradiol doses were not examined in the current study, in a previous study we found that continuous high dose 17β-oestradiol treatment also lost its ability to exert neuroprotection in the hippocampal CA1 region after LTED (Zhang et al., 2011). From a clinical perspective, low dose 17β-oestradiol replacement is generally considered more optimal, as it is less likely to exert negative side effects. Indeed, low dose, transdermal 17β-oestradiol replacement therapy, which is most similar to our replacement paradigm, has begun to gain favour in the clinical setting since it bypasses liver metabolism and is reportedly associated with less adverse side effects (Renoux et al., 2010; Scott et al., 2012). However, it should be noted that not all studies show a loss of 17β-oestradiol brain sensitivity after LTED and that several factors may influence the critical window for 17β-oestradiol benefit. For instance, 17β-oestradiol has been shown to still be able to enhance spatial memory in aged rats with LTED if administered weekly beginning at 3 months, but not 12 months after ovariectomy (Gibbs, 2000). Similarly, and unlike continuous low-dose 17β-oestradiol treatment, acute high dose 17β-oestradiol administration has been shown to still be able to enhance synaptic transmission and exert neuroprotection in aged rats with LTED (Inagaki et al., 2012). Furthermore, 17β-oestradiol was reported to still be capable of regulating hippocampal CA1 long-term potentiation at 15 months after ovariectomy, but the effect was lost at 19 months post-ovariectomy (Smith et al., 2010). As a whole, these findings suggest that context, dose and LTED duration may be key variables that lead to differing reports on tissue sensitivity to 17β-oestradiol following LTED, and that these variables are important points to consider when discussing a critical window for 17β-oestradiol benefit.

In conclusion, the findings of the current study demonstrate that the normally resistant female hippocampal CA3 region becomes hypersensitive to ischaemic stress damage after LTED through a mechanism that involves hyperinduction of a NOX2-superoxide-JNK-Jun signalling pathway. The CA3 hypersensitivity is associated with a profound induction of Alzheimer’s disease-related proteins, robust amyloidogenesis, and a poorer cognitive outcome following GCI. Furthermore, CA3 hypersensitivity after LTED also extended to the Alzheimer’s disease-relevant insult, amyloid-β1–42. Collectively, these findings may help explain the increased risk of cognitive decline and dementia observed in women following surgical menopause, and they provide increased support that early 17β-oestradiol replacement is critical to preventing the negative neural effects associated with LTED.

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Supplementary material
Supplementary material is available at Brain online.
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