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NEUROPROTECTION AGAINST EXCITOTOXIC BRAIN INJURY IN MICE AFTER OVARIAN STEROID DEPLETION

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Abstract

Ovarian steroid hormones influence not only seizure phenomena, but also the neuronal cell death that follows. In the present study, we applied two models of ovarian steroid loss, ovariectomy and chemically-induced ovarian failure, to evaluate kainate-induced seizure activity and the susceptibility of hippocampal neurons to seizure-induced neurodegeneration. Young adult female FVB/NJ mice were ovariectomized with (OVX+E, n=6) or without (OVX, n=8) estrogen replacement. A separate group of females received the ovotoxin, 4-vinylcyclohexene diepoxide (VCD, n=8) to deplete ovarian follicles. Mice underwent kainate-induced status epilepticus and were evaluated for seizure activity (3 hours) and delayed hippocampal neuronal injury (7 days). While there were no differences in latency or duration of severe seizures among control, OVX and VCD-treated mice, OVX+E mice exhibited seizures of a significantly longer duration. However, both VCD-induced ovarian failure and OVX led to a dramatic reduction in the extent of excitotoxic cell death, with slightly greater effects observed in VCD-treated mice. Estradiol administration to OVX mice also exerted a significant neuroprotective effect against kainate-induced cell death. These results support and extend earlier findings suggesting that the hormonal milieu may have differential effects on seizure susceptibility that are separate and distinct from those influencing hippocampal neuronal vulnerability. Collectively, these findings highlight the complex interactions among the loss of ovarian steroid hormones, estrogen replacement, seizures, and seizure-induced cell death.

Keywords

Estrogen; Progesterone; 4-Vinylcyclohexene diepoxide; Ovariectomy; Neuroprotection

1. Introduction

Epilepsy is a neurodegenerative disorder that afflicts more than 50 million people (Browne and Holmes, 2001). While the prevalence of epilepsy is similar for men and women, it is estimated that nearly 1,000,000 women of childbearing age in the United States currently suffer from epilepsy (Jacobs et al., 2001). Epilepsy in women presents special problems resulting from the complex and significant interplay of seizure disorders with reproductive function (Zahn et al., 1998). In particular, the ovarian cycle influences seizures in many women with epilepsy. Seizure frequency can vary across the menstrual cycle (catamenial epilepsy), during

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puberty when ovarian cyclicity is first established, or at menopause when cyclicity ceases (Bauer, 2001; Morrell, 2002; Herzog et al., 2004; Maguire et al., 2005).

Similarly, studies in young adult animals have demonstrated that gonadal hormones can influence both seizure initiation and propagation. The effects of gonadal steroids on seizure susceptibility have been shown in adult ovariectomized female rats in several studies (Gazzaley et al., 1996; McEwen, 1996; MacLusky et al., 1997; Edwards et al., 1999; Hoffman et al., 2003). In particular, progesterone exerts anticonvulsive effects in both acute and chronic epilepsy models (Spiegel and Wycis, 1945; Landgren et al., 1978; Nicoletti et al., 1985; Bäckström and Rosciszewska, 1997; Herzog, 1999; Hoffman et al., 2003), while estrogen, by contrast, lowers the seizure threshold and facilitates seizures (Buterbaugh, 1989; Frye, 1995; Murphy et al., 1998; Wooley and Schwartzkroin, 1998; Frye and Bayon, 1999; Wooley, 1999; Hoffman et al., 2003). Estrogen has been shown to potentiate the behavioral effects of kainic acid (Azcoitia et al., 1999a; Hoffman et al., 2003) and facilitates kindling from the anterior neocortex (Buterbaugh, 1989).

In contrast to estrogen's pro-convulsant effects, substantial evidence from *in vivo* animal studies has indicated that estradiol can act as a neuroprotectant against hippocampal neuronal loss induced by excitotoxins (Azcoitia et al., 1999a; 2001; Reibel et al., 2000). Pretreatment with estradiol attenuates hippocampal damage in the hilus of the dentate gyrus and in the CA1 and CA3 hippocampal regions following severe long-lasting seizures (Reibel et al., 2000; Velišková et al., 2000; Galanoupoulou et al., 2003; Hoffman et al., 2003; Kalkbrenner and Standley, 2003).

Ovariectomy (OVX) is the most common animal model for studying the effects of ovarian steroids on brain function. However, OVX causes a precipitous decline in estrogen and progesterone, eliminates all ovarian secretions, and severs important nerve connections (Aguado and Ojeda, 1984). Recently, a chemically-induced model of ovarian steroid loss has been developed. In 2002, Mayer et al. reported that the industrial chemical 4-vinylcyclohexene diepoxide (VCD), selectively kills the small primordial and primary ovarian follicles in rats and mice, leading to a gradual decline in reproductive activity. Importantly, evidence for VCD's lack of toxicity to other tissues or organ systems was published in early work by the National Toxicology Program (1986, 1989). Thus, VCD treatment provides an important opportunity to study how a gradual decline in ovarian hormones modifies normal and pathologic processes in the brain. However, the effects of the VCD-induced model of ovarian failure on seizure susceptibility and seizure-induced cell loss have yet to be evaluated. In the present study, we compared the effects of OVX and VCD on seizure susceptibility and seizure-induced cell death in female mice induced by the kainate model of temporal lobe epilepsy. Our data suggest that the loss of ovarian steroid hormones plays an important functional role in neuronal viability following excitotoxic injury.

2. Results

Effect of VCD on ovarian cycles

VCD has previously been shown to disrupt estrous cyclicity by acting as an ovarian toxin, resulting in the loss of primary and primordial follicles (Springer et al., 1996a, b; Borman et al., 1999; Kao et al., 1999). As shown in Figure 1A, all vehicle-treated females maintained regular cycles throughout the study, averaging 4.9 ± 0.2 days. VCD treatment caused a gradual disruption of estrous cyclicity with VCD-treated females averaging cycle lengths of 8.5 ± 0.9 days (Fig. 1A). Ovarian failure, as assessed by a persistent state of diestrus, was observed 26.2 ± 3.2 days after the onset of dosing. Likewise, in post-mortem histologic sections of the ovary, VCD treatment significantly depleted the number of oocytes (Fig. 1B). Ovaries from vehicle-treated female controls averaged 2.2 ± 0.7 oocytes/mm². By contrast, VCD-treated females had

only 0.1 ± 0.1 oocytes/mm² ($p < 0.05$). However, the area of the ovarian sections in VCD females (0.6 ± 0.1 mm²) was not significantly different from that of controls (0.9 ± 0.1 mm², $p > 0.05$). Thus, in agreement with previous studies in other strains of mice, (Mayer et al., 2002; Thompson et al., 2002; Devine et al., 2004; Hu et al., 2006), VCD-induced follicular depletion caused ovarian failure in FVB/NJ female mice.

Effect of ovarian steroid loss on seizure parameters

Administration of KA induced seizures in VCD- and vehicle-treated female mice, with a behavioral progression as previously reported (Schauwecker and Steward, 1997; Schauwecker, 2000, 2002a, b). Between 35 and 45 minutes after injection, all mice were rearing and exhibited continuous tonic-clonic seizures that lasted, on average 77 ± 4.9 minutes. Furthermore, VCD-treated mice did not differ significantly from vehicle controls with regard to seizure latency (Fig. 2A) or seizure duration (Fig. 2B).

As with VCD and vehicle females, OVX and SHAM females displayed generalized continuous tonic-clonic convulsions. Likewise, OVX and SHAM mice did not differ significantly with respect to seizure latency (Fig. 3A) or seizure duration (Fig. 3B).

Effect of estrogen replacement on seizure parameters

Following KA injection, the latency to first seizure was not affected by estrogen replacement in OVX mice (Fig. 3A). As with OVX or VCD-induced ovarian depletion, seizure latency in OVX+E females was comparable to that in SHAM or vehicle-treated females. However, estrogen replacement in OVX mice significantly increased seizure duration, relative to that in ovary-intact females or OVX females without hormone replacement (Fig. 3B).

Effect of VCD and OVX on seizure-induced cell death

Consistent with previous studies in mice (Schauwecker and Steward, 1997; Schauwecker, 2000; Schauwecker et al., 2000; Schauwecker, 2002a,b), administration of KA to ovary-intact SHAM or vehicle-treated mice led to the degeneration and loss of CA3 pyramidal neurons and hilar neurons, and sporadic loss of CA1 pyramidal neurons. In accordance with previous studies (Nadler and Cuthbertson, 1980; Nadler et al., 1980; Sperk et al., 1983; Ben-Ari et al., 1984), cells within the dentate granule cell layer and area CA2 of Ammon's horn were spared. In contrast, both VCD-treated and OVX females demonstrated significantly less cell death, compared with their respective controls. Cell damage scores for VCD-treated females averaged 1.57 ± 0.4 versus 3.0 ± 0.1 in vehicle controls (Fig. 4A). Similarly, ovariectomy reduced cell damage scores (2.28 ± 0.6) compared with SHAM females (3.0 ± 0.1 , $p < 0.05$). Quantitative analysis of subfield group means revealed that OVX and VCD mice lost on average twenty-five fold fewer CA3 pyramidal neurons and twenty-four or twenty-fold fewer hilar neurons, respectively, as compared to either SHAM or vehicle-treated mice (Fig. 4B). Importantly, the amount of protection conferred by OVX or VCD treatment did not differ in all three subfields. Furthermore, within the dentate hilus and area CA1, the extent of protection against KA-induced cell death was no different between OVX and VCD treatment versus naïve (non-KA treated) mice (Fig. 4B). While all 3 subfields in VCD and OVX mice displayed a dramatic decrease in the extent of cell death, the CA3 region appeared the most susceptible to cell death (~23% cell loss as compared to ~5% in area CA1 and ~10% in the dentate hilus).

Sections from each of four groups of females (vehicle, SHAM, OVX, and VCD), sacrificed 7 days following kainate injection and processed for cresyl violet are shown in Figure 5. As demonstrated in Fig. 5, following kainate administration to SHAM mice, a dramatic reduction of pyramidal neurons in area CA3 and area CA1 was observed. Similarly, a significant reduction (absence of stain) was also noted within the dentate hilus. In contrast, representative

sections from both OVX and VCD groups showed a significant attenuation in the extent of cell loss throughout all hippocampal subfields.

Estrogen replacement in OVX females exerted significant protective effects against kainate-induced cell death in all 3 hippocampal subfields, as compared to Sham-OVX mice. Nonetheless, cell loss in OVX+E females was more extensive compared with that in OVX females (Fig. 4B). In particular, OVX+E mice consistently displayed a 30–45% cell loss among hippocampal subfields, which was significantly greater than the cell loss observed in VCD or OVX groups in area CA3, dentate hilus, or area CA1, respectively (Fig. 4B).

3. Discussion

The studies described examine the interplay of epileptogenesis and loss of ovarian steroid hormones. We compared chemically-induced ovarian steroid loss and ovariectomy to begin to relate changes in seizure patterns with ovarian function. The present data reveal a novel interaction between ovarian steroid hormones and seizure-induced excitotoxic cell death in a mouse model of temporal lobe epilepsy. In female mice, loss of ovarian steroid hormones, through ovariectomy or administration of the ovariotoxic, VCD, substantially reduced seizure-induced excitotoxic cell death, as compared with ovary-intact female mice. Moreover, estrogen replacement in OVX mice also protected the hippocampus against excitotoxic cell death, albeit to a lesser extent. The results of these studies suggest that the combined loss of progesterone and estrogen is capable of protecting the hippocampus from seizure-induced brain damage compared with ovary-intact females.

Furthermore, the present study demonstrates that the effects of OVX and VCD are equivalent in terms of hippocampal cell death. Relative to Sham-OVX or vehicle-treated mice, KA-induced cell death was profoundly reduced with the combined loss of progesterone and estrogen. These results are indeed surprising as recent studies have suggested that estrogen is neuroprotective in both young and aging animals. Thus, we would have predicted that the loss of estrogen should exacerbate kainate-induced cell damage. In fact, we saw the opposite effect: loss of ovarian steroid hormones reduced hippocampal damage from kainate-induced seizures. Substantial evidence from *in vivo* animal studies has indicated that estradiol can act as a neuroprotectant against hippocampal neuronal loss induced by excitotoxins (Azcoitia et al., 1999b; 2001; Reibel et al., 2000). Animal studies suggest that the beneficial effect of 17 β -estradiol may also be extended to status epilepticus-induced hippocampal damage. In particular, pretreatment with estradiol attenuates hippocampal damage in the hilus of the dentate gyrus and in the CA1 and CA3 hippocampal regions following severe long-lasting seizures (Reibel et al., 2000; Velišková et al., 2000; Galanoupoulou et al., 2003; Hoffman et al., 2003; Kalkbrenner and Standley, 2003). Results from our studies demonstrated that OVX +E females had significant neuroprotection, as well, against kainate-induced cell death, at least compared with ovary-intact females. On the other hand, estrogen replacement in OVX females actually enhanced neuronal cell loss relative to OVX or VCD-treated females.

Together, these observations highlight the diversity of hormonal stimuli produced in the ovary. They further suggest that the neuroprotective effects of estradiol may depend on the concentration of estradiol and/or the intensity of the neuronal insult. While a variety of evidence demonstrates that estradiol can enhance neuronal survival, the minimal cell loss in OVX or VCD females implies that other ovarian secretory products (protein hormones, androgens, progestagens, and other estrogens) may counteract the neuroprotective effects of estradiol. Likewise, our observation of increased hippocampal cell loss in OVX+E females relative to OVX females suggests that estrogen neuroprotection may depend on the dose and duration of steroid deprivation and steroid exposure, as well as the type of neuronal insult. In this regard,

acute estradiol exposure may have a more pronounced neuroprotective effect, compared with the chronic physiologic estrogen replacement of the present study.

Our results also suggest that while the combined loss of progesterone and estrogen has little effect on seizure behavior, estrogen replacement in OVX females prolongs the duration of KA-induced seizures. The majority of animal studies have demonstrated that sex steroid hormones influence both seizure initiation and propagation (Gazzaley et al., 1996; McEwen, 1996; MacLusky et al., 1997; Edwards et al., 1999; Hoffman et al., 2003). Our results demonstrating no significant difference in seizure susceptibility (determined by measures of latency to first seizure and duration of severe seizures) in the absence of ovarian steroids are in agreement with previous studies. In particular, a recent study by Scharfman and MacLusky (2006) determined that the incidence of status epilepticus, latency to first seizure, and number of severe seizures did not differ significantly between OVX and Sham-OVX rats administered pilocarpine. In the present study, we compared two models of reproductive dysfunction: chemically-induced ovarian depletion through repeated administration of VCD versus surgically-induced acyclicity via ovariectomy. Regardless of the mode of induction, a reduction in ovarian steroid levels was without effect on the onset, duration or severity of seizures.

Previous studies in ovariectomized adult female rats have demonstrated that sex steroid hormones influence both seizure initiation and propagation (Gazzaley et al., 1996; McEwen, 1996; MacLusky et al., 1997; Edwards et al., 1999; Hoffman et al., 2003). In particular, estrogen has been touted as a proconvulsant neurosteroid in several animal models of epilepsy, including amygdala kindling and pentylenetetrazol administration in ovariectomized rats (Hom and Buterbaugh, 1985). Our studies in OVX + E mice support this finding in that we observed a significantly longer duration of seizures in OVX + E mice as compared to Sham-OVX, OVX, VCD- or vehicle-treated mice.

In summary, the present study fits into the current reexamination of how the ovarian hormonal milieu may modify neural function in response to different insults. Our data indicate that, compared with ovary-intact females, a reduction in ovarian steroid levels can reduce damage to the hippocampus without reducing seizure activity. Clearly, the manner in which ovarian steroids produce alterations in ictal activity and act as effective neuroprotectants is complex. Cumulative evidence from both basic science and clinical research suggests that estrogens can ameliorate symptoms and exert significant neuroprotective effects against a variety of neurodegenerative pathologies (Bedard et al., 1977; Paganini-Hill, 1995; Henderson, 1997; Cyr et al., 2002; Brinton, 2004; Currie et al., 2004). However, additional studies are needed to better characterize the modes of action of ovarian steroids in the brain. In particular, it will be important to determine the mechanism whereby each of the steroids influences seizure activity and the resultant neuronal injury.

4. Experimental Procedure

4.1 Animals

Forty-eight female FVB/NJ mice, eight to ten weeks of age, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed separately. We used the FVB/NJ strain due to our previous observations that C57BL/6J mice are nearly resistant to seizure-induced cell death following kainate administration (Schauwecker and Steward, 1997). Mice were maintained on a 12L:12D cycle with food and water available *ad libitum*.

4.2 VCD treatment

Previous studies have determined that repeated treatment of rats and mice with the industrial chemical 4-vinylcyclohexene diepoxide (VCD) selectively destroys primordial and primary

ovarian follicles (Bormon et al., 1999; Kao et al., 1999; Springer et al., 1996a,b). Two groups of females (n=8 each) were weighed and received injections of VCD (320 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) or cottonseed oil vehicle daily for 15 days. This dose of VCD induces persistent diestrus within 4 weeks (data not shown). Mice were monitored using daily vaginal cytology to determine ovarian failure.

4.3 Ovariectomy and estrogen replacement

Additional females underwent OVX (n=8) or sham OVX (SHAM, n=12) via dorsal incision under tribromoethanol anesthesia (240 mg/kg, i.p.). The sham operation consisted of the same incision with palpation of the ovaries. To examine the effects of estrogen on susceptibility to kainate-induced seizures and seizure-induced cell death, two groups of OVX females (n=6/group) received a 5 mm Silastic implant (o.d. 2.125 mm, i.d. 1 mm, Dow Corning, Midland, MI) sc immediately after ovariectomy. OVX+E females received an implant with 1:1 17 β -estradiol (E2) and cholesterol; OVX females received a blank implant. This E2 implant is designed to maintain plasma E2 at physiological levels for female mice and produces physiologic responses in terms of neuroendocrine function and progesterone receptors in the brain (Jacob et al., 2001).

4.4 Kainate model of temporal lobe epilepsy

Females for each of the five groups listed above were treated with KA (20 mg/kg in saline sc; Diagnostic Chemical, Ltd., Charlottetown, PEI) to produce sustained seizures (status epilepticus; SE) characteristic of temporal lobe epilepsy. At 20 mg/kg, KA produces consistent seizures in FVB/NJ mice with a mortality rate of <25% (Schauwecker and Steward, 1997); most deaths occur within 48 hours. For VCD and vehicle-treated females, SE was induced 4 weeks after the end of daily injections when all VCD-treated females were acyclic (in a persistent state of diestrus). OVX and SHAM females received KA one week after surgery. One week after seizure induction, mice were sacrificed for histologic evaluation of the brain and ovaries. One group of females (n=6) were naïve to kainate and were injected with an equivalent amount of saline.

4.5 Vaginal cytology

Estrous cyclicity was determined by monitoring of vaginal cytology after dosing with VCD or vehicle. Daily vaginal smears were collected each morning by vaginal lavage and stained with cresyl violet. Estrous cycle stage was determined by two independent observers according to the methods of Nelson et al. (1992) using the following criteria: 1) proestrus – predominantly round, nucleated epithelial cells, few cornified epithelial cells or leukocytes; 2) estrus – predominantly cornified epithelial cells with no prominent nucleus; 3) metestrus – predominantly leukocytes and cornified epithelial cells; and 4) diestrus – predominantly leukocytes, some nucleated epithelial cells, occasional parabasal cells, and mucus. Ovarian failure in VCD-treated mice was assigned when vaginal cytology indicated >15 days of persistent diestrus.

4.6 Assessment of KA-induced seizure activity

Following KA injection, mice were monitored for 3–4 hours to assess the severity and length of behavioral seizures. The behavioral progression of KA-induced seizures was rated and recorded using Racine's classification (1972) as follows: Stage 1: immobility; Stage 2, forelimb and/or tail extension, rigid posture; Stage 3, repetitive movements, head bobbing; Stage 4, rearing and falling; Stage 5, continuous rearing and falling; Stage 6, severe tonic-clonic seizures. Only those mice exhibiting at least 45 minutes of continuous stage 4 seizures were included in this study, as previous studies have suggested that there is a direct relationship

between the generation of epileptiform activity and the extent of damage in hippocampal subfields (Sperk et al., 1983; Ben-Ari, 1985; Sperk, 1994).

4.7 Tissue preparation

Mice were deeply anesthetized with Avertin and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) seven days following KA or saline administration. To evaluate KA-induced neural damage in VCD, vehicle, OVX, OVX+E, SHAM, and KA-naïve females, brains were removed and post-fixed overnight, followed by cryoprotection in 30% sucrose for at least 12–18 hours. Horizontal (40 μ m) frozen sections were cut on a sliding microtome and collected as free-floating sections in 0.1M phosphate buffer (pH 7.4) until processed for light-microscopic histology. To quantify ovarian follicular depletion, ovaries from VCD- and vehicle-treated females were trimmed of fat, post-fixed in Bouin's fixative, embedded in paraffin, serially sectioned at 5 μ m, and stained with Masson's trichrome stain.

4.8 Ovarian histology

Quantification of ovarian follicles was by unbiased stereologic methods modified from those of Pedersen and Peters (1968) and Smith et al. (1990). Briefly, 2 pairs of adjacent ovarian sections separated by at least 80 μ m were examined under a 40x objective, and traced using a drawing tube by an observer blinded to treatment group. Oocytes sectioned through the cell nucleus were identified in the reference section, and in the adjacent lookup section. Only those oocytes in the reference section that did not also appear in the lookup section were counted. This was repeated using the look-up section as the reference section, doubling the number of observations. The number of oocytes per mm², and the means ($\pm$ SEM) in 5 VCD and 6 vehicle-treated females were analyzed for significant differences by Student's t-test using Statview software (SAS Institute, Cary, NC). Significance was set at $p < 0.05$.

4.9 Neural histology

Neural degeneration was visualized using a modified Fink-Heimer stain and by staining with Fluoro-Jade C, according to our previously published methods (Schauwecker and Steward, 1997; Schauwecker, 2000; Schauwecker et al., 2000; Schauwecker, 2002a, b; Santos and Schauwecker, 2003), seven days following kainate administration. In addition, every sixth section was processed for cresyl violet staining to assess cell loss and morphology.

One series of sections was stained with a modification of the selective Fink-Heimer silver stain, which stains degenerating fibers, synaptic terminals and cell bodies (Nadler and Evenson, 1983). Briefly, sections were washed in distilled water, pretreated in a solution containing 4.5% sodium hydroxide and 0.06% ammonium nitrate for 5 min, and then incubated for 10 min in an impregnating solution containing 5.4% sodium hydroxide, 6.4% ammonium nitrate, and 0.3% silver nitrate. Sections were then washed three times for a total of 5 min in a solution containing 28.5% ethanol, 1.2% ammonium nitrate, and 0.5% sodium bicarbonate, and then transferred to a developing solution containing 0.5% citric acid, 5% formalin, 9.5% ethanol, and 1.2% ammonium nitrate. Sections were rinsed briefly, mounted onto gelatinized slides, dehydrated through graded alcohols, cleared in xylene and coverslipped.

An additional series of sections was stained with Fluoro-Jade C, a fluorescent marker for dying neurons, according to a method outlined previously (Schmued et al., 2005). Briefly, horizontal sections were mounted onto gelatin-coated slides and allowed to air-dry. Slides were then immersed in absolute alcohol for 3 min, followed by 70% ethanol for 2 min and distilled water for 2 min. The slides were then transferred to 0.06% potassium permanganate for 15 min. After rinsing with distilled water for 2 min, the slides were then incubated for 30 min in 0.001% Fluoro-Jade C solution (Chemicon, Temecula, CA) made in 0.1% acetic acid. Slides were

rinsed briefly in water, allowed to air-dry for several minutes, cleared in xylene, and coverslipped with nonfluorescent mounting media (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Tissue sections were examined with an epifluorescent microscope equipped for visualizing fluorescein isothiocyanate.

4.10 Morphological assessment of neuronal damage

To provide a global picture of treatment effects on cell loss throughout the hippocampus, neuronal degeneration was evaluated in sections stained with cresyl violet. The number of degenerating neurons in both the right and left hippocampus from every sixth section (240 μ m separation distance) in CA3, CA1, dentate hilus and dentate gyrus, was estimated and a histological damage score was assigned on a 0–3 grading scale according to the following criteria: grade 0, absence of pyknotic cells; grade 1.0, mild (<25% of cells pyknotic); grade 2.0, moderate (<50% of hippocampal neurons pyknotic); and grade 3.0, extensive (>50% of cells pyknotic) according to a previously defined scale (Fujikawa et al., 1994; Fujikawa, 1995; 1996; Morrison et al., 1996; Schauwecker et al., 2004). All grading was performed by an observer blinded to the treatment groups.

Values for right and left hemispheres were averaged for each mouse. For the hippocampus, scores from sections were averaged and used for calculating group values. Means ($\pm$ SEM) in VCD, vehicle, OVX and SHAM mice were analyzed for significant differences by one-way ANOVA with post-hoc comparison by Student Newman-Keuls test. Data from OVX and OVX +E females were compared by Student's T-test. For all statistical comparisons, $p < 0.05$ was considered significant.

4.11 Quantitative analysis of hippocampal cell loss

Subsequently, to determine the susceptibility of individual hippocampal subfields to neurotoxic insult, we counted neurons in Nissl-stained sections. Quantitative analysis of hippocampal cell loss was performed by an observer blinded to the treatment groups using unbiased stereological methods on cresyl violet-stained sections according to previously published protocols (Schauwecker and Steward, 1997; Schauwecker et al., 2000; Santos and Schauwecker, 2003). The number of Nissl-stained neurons was counted in both the right and left hippocampus in horizontal sections and averaged into single values for each animal. Neurons counts were made in areas CA3, CA1, the dentate hilus, and the dentate granule cell layer. Counting was initiated within the ventral hippocampus at the first point where hippocampal subfields could be easily identified. This level corresponded to horizontal section 54, based on the atlas of Sidman et al. (1971). Hippocampal subfields were based on the Franklin and Paxinos (1997) classification, and only cells with an evident nucleus and nucleolus were included in the counts. Counts within each of the hippocampal subfields were obtained by examining horizontal sections at 400X magnification using grid morphometric techniques. Briefly, an ocular grid (214 $\times$ 163 μ m) was placed over the hippocampal region of interest and all cells with visible nuclei containing a nucleolus within the grid were counted. Neuronal numbers within the grid were evaluated from six sections (240 μ m separation distance) at three different focal planes, and stereologic analysis was performed with the aid of ImagePro Plus 4.5 software (Media Cybernetics, Silver Spring, MD), and a motorized Z-stage (Optiscan; Prior Scientific, Fairfax, VA). Final cell counts were expressed as the percentage of cells as compared to intact mice. Results were assessed statistically by one-way ANOVA using the computer program, SigmaStat (Jandel Scientific, San Rafael, CA), and intergroup differences were analyzed by Newman-Keuls post hoc test.

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Abbreviations

ANOVA	analysis of variance
E	estrogen
E2	17- β estradiol
KA	kainic acid
OVX	ovariectomy
S.E.M	standard error of the mean
SHAM	sham ovariectomy
VCD	4-vinylcyclohexene diepoxide

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Fig. 1. VCD causes a dose-related inhibition of estrous cyclicity and induces follicular depletion. A. Daily administration of 320 mg/kg VCD for 15 days eliminated estrous cyclicity by day 11. Note the significant increase in average days per cycle in VCD-treated mice, as compared to normally cycling ovary-intact mice. ($F=29.513$; $P=0.002$). B. VCD also significantly depleted oocytes in ovarian sections, compared with vehicle-treated control females. Bars represent mean $\pm$ S.E.M. * $P<0.05$.

Fig. 2. Histograms of seizure parameters in vehicle-treated versus VCD-treated mice following kainic acid-induced status epilepticus. A. Data represent latency to severe stage 4 seizures in minutes (mean $\pm$ S.E.M.). No significant differences between treatment groups were observed. B. Duration of severe stage 4 seizures in minutes was not significantly different among VCD- versus vehicle-treated mice. C. Seizure severity did not differ among VCD- versus vehicle-treated mice. Data represent the seizure parameters for at least 8 mice for each condition.

Fig. 3. Histograms of seizure parameters in Sham-OVX, OVX, and OVX + E mice following kainate-induced status epilepticus. A. Latency to severe stage 4 seizures in minutes (mean $\pm$ S.E.M.) was not significantly different between treatment groups. B. Data represent seizure duration scores in minutes (mean $\pm$ S.E.M.). Note that a significant difference in seizure duration was observed in OVX + E mice following kainate treatment. C. Seizure severity was not significantly different between treatment groups. * $P < 0.05$ as compared to Sham OVX + KA and OVX + KA.

Fig. 4.

Kainate-induced cell death is substantially reduced in two models of reproductive dysfunction. A. Data represent neuronal damage scores (in arbitrary units, mean $\pm$ S.E.M.) for Sham-OVX, OVX, VCD-treated, vehicle-treated, and OVX + E female mice ($F=16.657$; $P<0.001$). The extent of neuronal damage was indexed using a histological damage score. B. Differences in cell loss in all 3 hippocampal subfields were observed between the two ovary-intact groups (Sham-OVX and vehicle-treated) and two models of ovarian output loss (OVX, VCD) or OVX + E mice. Differences in the extent of cell loss were most obvious within area CA3 ($F=47.246$; $P<0.001$), but significant differences were also observed within area CA1 ($F=35.738$; $P<0.001$) and the dentate hilus ($F=20.515$; $P<0.001$). Data represent the mean $\pm$ S.E.M. of 8 mice/

treatment. Bars with the same letter subscripts are not significantly different. CA1 and CA3, hippocampal subfields; Hilus, dentate hilus. * $P < 0.05$ for

Fig. 5. Kainate-induced cell death is reduced in OVX and VCD-treated mice as compared to Sham-OVX and vehicle-treated mice. Low magnification cresyl violet staining of horizontal sections through the hippocampus and high-magnification cresyl violet staining of area CA3, dentate hilus, and area CA1. Note the significant amount of cell damage in representative sections from KA-treated mice and dramatic reduction in the extent of cell loss in OVX or VCD-treated mice. CA1 and CA3, hippocampal subfields; H, dentate hilus. Scale bars: left panels, 750 μ m; right panels, 100 μ m.