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## Ovarian steroid modulation of seizure severity and hippocampal cell death after kainic acid treatment

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### Abstract

To determine whether maintained estrogen or progesterone levels affect kainic acid (KA) seizure patterns or the susceptibility of hippocampal neurons to death from seizures, ovariectomized Sprague–Dawley rats were implanted with estrogen pellets, 0.1 or 0.5 mg, that generated serum levels of  $42.4 \pm 6.6$  (mean  $\pm$  SEM) and  $242.4 \pm 32.6$  pg/ml or one to six capsules of progesterone that generated serum levels of  $11.00 \pm .72$  to  $48.62 \pm 9.4$  ng/ml. Seven days later, the rats were administered KA (8.5mg/kg, ip) and scored for seizure activity; 96 h later, the rats were killed and their brains processed for localization of neuron nuclear antigen (NeuN), a general neuronal marker. The hippocampus was scored for spread (the number of separate regions showing cell loss), and the area within the CA fields occupied by NeuN immunoreactivity was measured (indicating surviving neurons). Administration of estrogen or progesterone (independent of dose) significantly reduced mortality from KA seizures. Progesterone reduced seizure severity in animals that received one to four implants; compared with controls, no difference in seizure severity was noted for animals with six progesterone implants. The reduced seizures in progesterone-treated animals were accompanied by a reduction in the spread of hippocampal damage ( $r^2 = 0.87$ ;  $P < 0.05$ ). Likewise, in progesterone-treated rats, neuron survival and reduction in seizure scores were correlated ( $r^2 = 0.76$ ;  $P < 0.0001$ ). Estrogen had no effect on seizure severity ( $P > 0.05$ ), but reduced both the spread ( $P < 0.05$ ) and degree of neuronal loss ( $P < 0.05$ ). Indeed, in the estrogen-treated rats, neuronal death was significantly lower than that observed in progesterone-treated animals with equally severe seizures ( $P < 0.05$ ). These data are consistent with the hypothesis that progesterone produces its effects by reducing seizures, whereas estrogen has little beneficial effect on seizure behavior but protects the hippocampus from the damage seizures produce.

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**Keywords:** Estrogen; Progesterone; Neuroprotection; Excitotoxicity; Limbic seizures; Epilepsy; Neuronal nuclear antigen

### Introduction

Complex partial seizures involve the limbic system and comprise the most common form of epilepsy. In women, the pattern of complex partial seizures is influenced by the hormonal changes that occur across the menstrual cycle (Herkes et al., 1993; Herzog et al., 1997; Morrell, 1992, 1999; Murri and Galli, 1997; Schachter, 1988). Increased seizure incidence is observed in the menstrual phase, when both estrogen and progesterone levels are low, as well as in the follicular phase, when estrogen levels are on the rise. By

contrast, decreased seizure incidence is noted during the luteal phase when progesterone levels are high relative to estrogen. In animals, estrogen administration decreases while progesterone increases seizure thresholds (Beyenburg et al., 2001; Buterbaugh, 1987, 1989; Buterbaugh and Hudson, 1991; Edwards et al., 1999; Hom and Buterbaugh, 1986); these differential steroid effects are used to explain the cycle-dependent changes in seizure patterns in women. Indeed, the effects observed with progesterone form the basis for progesterone treatment of women with catamenial epilepsy (Bauer, 2001; Bonuccelli et al., 1989; Herzog, 1995; Herzog et al., 1997; Holmes et al., 2001; Morrell, 1992, 1999; Murri and Galli, 1997).

Limbic system seizures, when persistent, increase the risk of permanent damage to the hippocampal formation

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(Kalviainen et al., 1998; Mathern et al., 1998; Moshe, 1998; Salmenpera et al., 2001; Tasch et al., 1999; Theodore et al., 1999). Thus, an understanding of the hormonal effects on limbic seizures and the damage they produce is critical to the design of rational treatments. In animals, the use of the toxin kainic acid, an excitatory amino acid analog, produces limbic seizures that damage neurons in the hippocampal formation and surrounding structures, particularly CA1, CA3, hilus, and entorhinal cortex, while sparing CA2 and the dentate gyrus (Ben-Ari et al., 1980; Gayoso et al., 1994; Jarrard, 1983; Lothman and Collins, 1981; Olney et al., 1979, 1986; Sperk, et al., 1983). Progesterone treatment reduces limbic seizures in a variety of experimental models (Edwards et al., 1999; Frye and Bayon, 1998; Frye and Scalise, 2000; Tauboll and Lindstrom, 1993) but it is unclear if the steroid has any neuroprotective effects of its own apart from its effects on seizure activity per se.

For estrogen, a few studies suggest that despite the potential for increased seizures, estrogen may reduce neuronal death from seizures (Azcoitia et al., 1999; Veliskova et al., 2000). However, those studies only used injected steroid (which produces variable hormone levels) and doses that often exceeded the physiological range. Thus, it is unclear if the effects of estrogen are dose-dependent. To fill those gaps, the studies presented in this manuscript sought to determine if maintained physiological levels of either estrogen or progesterone affected kainic acid (KA) seizure patterns and if these hormones could alter the relationship between seizure severity and brain injury.

## Methods

### *Animal treatment*

Adult female Sprague–Dawley rats (200–225 g) were maintained on 12:12 light:dark cycle (lights on at 3:30 EST). After a 1-week acclimation, rats were anesthetized with Metofane and ovariectomized under sterile conditions; 7 days later they were implanted with blank silastic capsules ( $n = 18$ ; capsule length = 40 mm; OD = 0.125mm; ID = 0.078mm); estrogen pellets (estradiol-17 $\beta$ , 0.5mg/21 day,  $n = 6$ ; or 0.1 mg/21 day,  $n = 8$ ; Innovative Research of America, Sarasota, FL); or 1 ( $n = 3$ ), 2 ( $n = 9$ ), 4 ( $n = 6$ ), or 6 ( $n = 8$ ) silastic capsules containing crystalline progesterone (capsule length = 40 mm; OD = 0.125mm; ID = 0.078 mm; Sigma, St. Louis MO) designed to achieve progesterone plasma levels of 10–60 ng/ml.

### *Behavioral testing*

Seven days after implants or pellets were inserted, half the control animals and the steroid-replaced animals were administered KA at a dose of 8.5 mg/kg, ip. The remaining control rats received an equal volume of saline vehicle. The behavior of the animals was monitored by an individual

blind to the animal treatment for 6 h following saline or KA injection and assigned a score for seizure behaviors using the following scale modified from Lothman (Lothman and Collins, 1981):

1 = minor behaviors such as catatonia, wet dog shakes (WDS), scratching, sniffing, and head bobbing;

2 = minor behaviors + chewing and salivation, rearing without loss of balance;

3 = minor behaviors + chewing and salivation, rearing with ataxia;

4 = biclonus seizure activity; and

5 = death. Following the observation period the animals were returned to the vivarium.

### *Tissue preparation and neuroanatomical analysis*

Ninety-six hours after injection of KA or vehicle, each animal was anesthetized with an overdose of pentobarbital (100 mg/kg, ip), a blood sample was removed directly from the heart, and the animals were perfused transcardially with saline containing 2% sodium nitrite, followed by fixation with 2.5% acrolein in 4% paraformaldehyde in 0.05 M phosphate buffer, pH 6.8 (Hoffman et al., 2001). The brains were removed, sunk in 30% sucrose, and sectioned at 25  $\mu$ m on a Leica freezing microtome (Bannockburn, IL). The sections were placed into antifreeze cryoprotectant solution (Watson et al., 1986) and stored at  $-20^{\circ}\text{C}$  until immunocytochemical localization of NeuN (a neuron-specific marker that stains nuclei and frequently dendrites and soma (Mullen et al., 1992; Wolf et al., 1996)) was initiated.

Brain sections from each animal (1:6 series) that contained the entorhinal cortex and hippocampal formation were processed for NeuN immunoreactivity using standard immunocytochemical techniques (Hoffman et al., 2001). Following immunocytochemical staining, the slides were coded and examined for the number of separate regions that showed neuron loss (“spread of damage”). This parameter was chosen due to the variability in sites of hippocampal damage in less severely affected rats with respect to which particular CA subfield showed damage. Second, neuronal survival was assessed by measuring the area occupied by NeuN immunoreactivity within the CA subfields at the coronal level where the CA regions showed maximal length. To accomplish this, an image of the section was captured with a 4X objective on a Nikon Eclipse 800 microscope using a Sensys digital camera (Biovision Technologies, Exton, PA). With IP Spectrum software (Scanalytics, Fairfax, VA) operating on a Power Computing Macintosh computer, the CA area (in square micrometers) occupied by NeuN immunoreactive structures was determined. Reductions in NeuN area reflected the degree of neuron loss. To control for slight variation in hippocampal orientation, the total CA length was determined for each section and the NeuN area measurements were normalized for CA length (NeuN area/ $\mu\text{m}$  length).

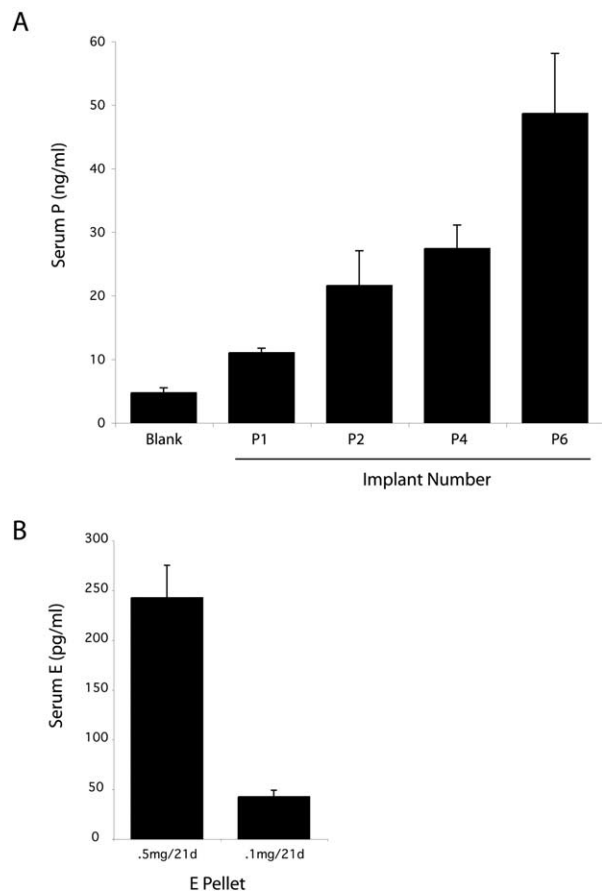


Fig. 1. (A) Serum levels of progesterone (P). Each bar represents the mean  $\pm$  SEM for serum levels of P in nanograms per milliliter for animals that were ovariectomized and received blank capsules (pooling animals that later were administered saline with animals that received KA) or one, two, four, or six capsules containing P. (B) Plasma  $\beta$ -estradiol (E) levels (mean  $\pm$  SEM) in picograms per milliliter for ovariectomized animals implanted with estradiol pellets containing either 0.5 mg/21 day release or 0.1 mg/21 day release. Ovariectomized animals had E values below detection in the assay.

#### Radioimmunoassay for estrogen and progesterone

Atrial blood was collected at the time of perfusion to determine serum progesterone and estrogen concentrations for the various treatment paradigms. After 2 h at room temperature to allow for clot formation, serum was separated by centrifugation. Samples were stored at  $-20^{\circ}\text{C}$  until radioimmunoassays were initiated. For estradiol, samples were first extracted with diethyl ether. Serum progesterone and estrogen concentrations were determined using the Diagnostics Products Corporation Coat-A-Count kit (Estradiol, TKE25; Progesterone, TKPG2; Los Angeles, CA).

#### Statistical analysis

Significant differences among treatment groups were analyzed using the nonparametric Kruskal–Wallis test fol-

lowed by post hoc Mann–Whitney  $U$  tests;  $P < 0.05$  was considered significant. Correlations between the various measures were assessed using a simple regression analysis and GB-Stat software;  $P < 0.05$  was considered significant.

## Results

### Hormone levels

The placement of one to six progesterone implants resulted in serum progesterone levels averaging from 11 to 48 ng/ml (Fig. 1A); these values are all within physiological ranges. Since the placement of two or four implants produced essentially the same progesterone levels, the data from those two groups were pooled in subsequent analyses. The 0.5 mg/21 day and 0.1 mg/21 day estrogen pellets produced hormone levels of  $242.4 \pm 32.6$  pg/ml and  $42.4 \pm 6.6$  pg/ml, respectively (Fig. 1B). Only the lower dose was within the physiological range.

### Mortality

One striking feature of animals replaced with estrogens or progesterone was a reduction in the mortality rate. Seven of the 24 animals (29.2%) that received blank capsules and KA died, whereas for E, only 4 of 48 animals (8.3%) died and for P, 3 of 34 animals (8.8%) died. There was no obvious relationship of mortality to dose of replaced hormone (Table 1).

### Seizure severity

Seizure severity was significantly reduced in progesterone-treated, but not estrogen-treated, rats (Fig. 2A). This reduction in seizure severity by progesterone was dose-dependent. In animals with one or two to four progesterone implants, seizure scores were significantly reduced compared with ovariectomized animals treated with KA that received blank capsules (for P1,  $P = 0.05$ ; for P2–4,  $P < 0.0005$ ). No significant difference in seizure severity was noted in animals that received six implants compared to blanks ( $P > 0.05$ ). As a result, animals that had only mild

Table 1  
Mortality after kainic acid seizures

Treatment group	No. died	Total	%
Blank + KA	7	24	29.2
E-0.1 mg/21 d + KA	2	25	8.0
E-0.5 mg/21 d + KA	2	23	8.7
E Combined	4	48	8.3
P1 + KA	0	6	0.0
P2–4 + KA	2	18	11.1
P6 + KA	1	10	10.0
P Combined	3	34	8.8

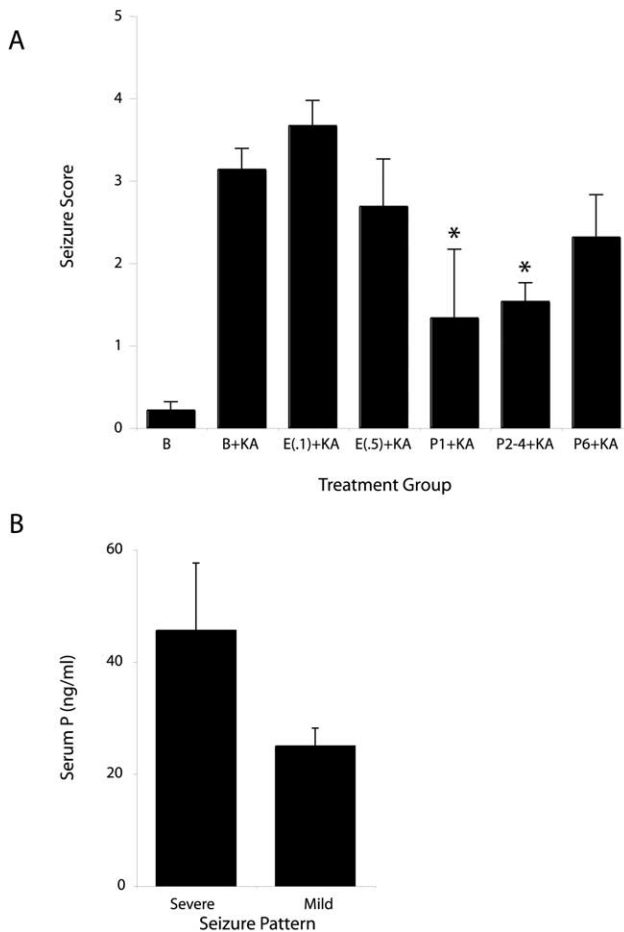


Fig. 2. (A) Effects of hormone treatment on KA-induced seizures. Animals fitted with one or two to four P implants showed significantly lower seizure scores ( $*P \leq 0.05$ ) than animals treated with KA that received no hormone treatment (B+KA). In contrast, animals that received six P pellets showed no suppression of KA seizures as did animals fitted with E pellets. Values represent the mean  $\pm$  SEM. (B) Comparison of plasma P levels in animals which displayed either severe (score  $> 2.0$ ) or mild (score  $\leq 2.0$ ) seizures. P-implanted animals with severe seizures had significantly higher P levels than P-treated animals that had mild seizures ( $P < 0.02$ ).

seizures in the progesterone-treated group (i.e., seizure scores  $\leq 2.0$ ) had significantly lower plasma P values (mean plasma P =  $25.0 \pm 3.2$ ) than animals treated with P that had severe seizures with scores  $> 2.0$  (mean plasma P =  $45.6 \pm 12.0$ ;  $P < 0.05$ ; Fig. 2B).

#### Number of areas showing neuronal loss: spread of damage

On average, approximately three hippocampal areas showed neuronal loss in control animals after KA treatment (Fig. 3). This typically included the entorhinal cortex (not shown), CA1, CA3, or the hilus (Fig. 4B compared with controls, Fig. 4A). CA2 and the dentate gyrus were generally spared. The spread of damage after KA administration varied significantly with the level of seizure activity (Fig. 5A;  $r^2 = 0.45$ ;  $P < 0.02$ ).

In P-treated animals, the spread of damage induced by KA administration was independent of plasma P level (Fig. 3). Rather, in P-treated animals, spread of damage was directly influenced by seizure severity. Animals displaying a high level of seizure activity (score  $> 2$ ) after KA had a greater number of areas showing neuronal loss than animals with lower seizure activity (score  $\leq 2$ ) (Fig. 4C–E and Fig 5B,  $r^2 = .665$ ;  $P < .0001$ ; Fig. 6).

In estrogen-treated animals, fewer hippocampal regions showed signs of neuron loss after KA treatment than controls (Fig. 3,  $P < 0.05$ ; Fig. 4F and G) despite the persistence of seizures (Fig. 2A). Therefore, unlike progesterone, the effect of estrogen on the spread of damage was not dose-dependent (Fig 3;  $P > 0.05$ ) nor was it correlated with seizure severity (Fig. 5C;  $r^2 = 0.049$ ,  $P > 0.05$ ). Thus, even when animals displayed severe seizures following KA, estrogen administration (at either high or low doses) limited the number of hippocampal regions that showed any neuronal damage (Fig. 6).

#### Loss of neurons in CA subfields: area occupied by NeuN immunoreactivity

Compared with saline-treated control animals (blank capsules), KA-treated control rats with high seizure activity had significant losses of NeuN in the CA subfields ( $p < 0.005$ ; Fig. 7). Overall, the losses in NeuN immunoreactivity in the Blank + KA group were highly correlated with seizure score (Fig. 8A;  $r^2 = 0.47$ ,  $P < 0.02$ ).

In animals treated with P, the animals with high seizure scores also had severe reductions in NeuN compared with controls ( $*P < 0.005$ ; Fig. 7). The NeuN losses in P-treated

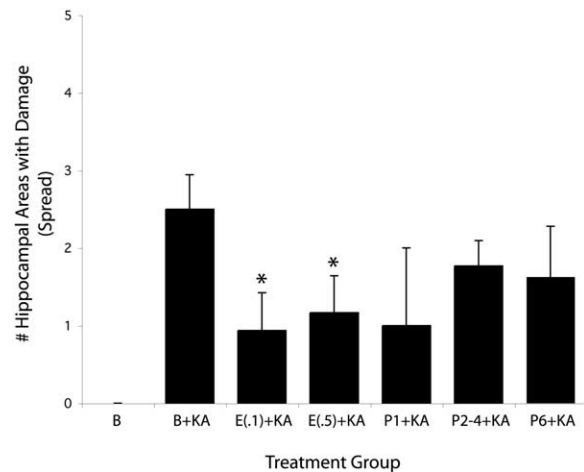
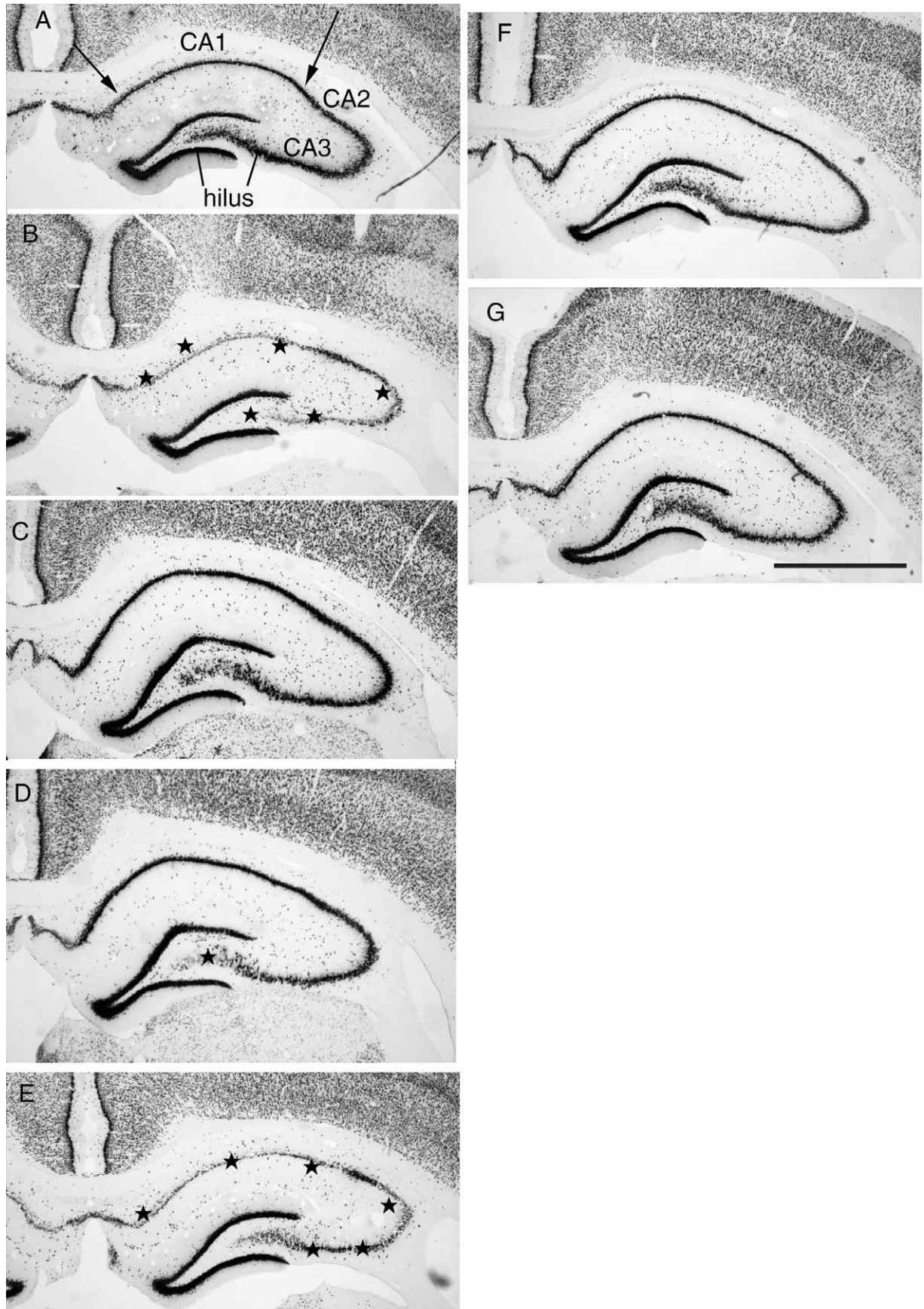


Fig. 3. Effects of hormonal treatment on the spread of damage to the hippocampal formation after KA treatment. Values represent the number of hippocampal formation regions (mean  $\pm$  SEM) showing neuronal injury after KA treatment. P did not produce significant dose-dependent effects on spread of damage. Both low and high E treatment produced a significant reduction ( $*P \leq 0.05$ ) in the number of regions showing neuronal damage after KA treatment despite the failure of the steroid to reduce seizure severity.



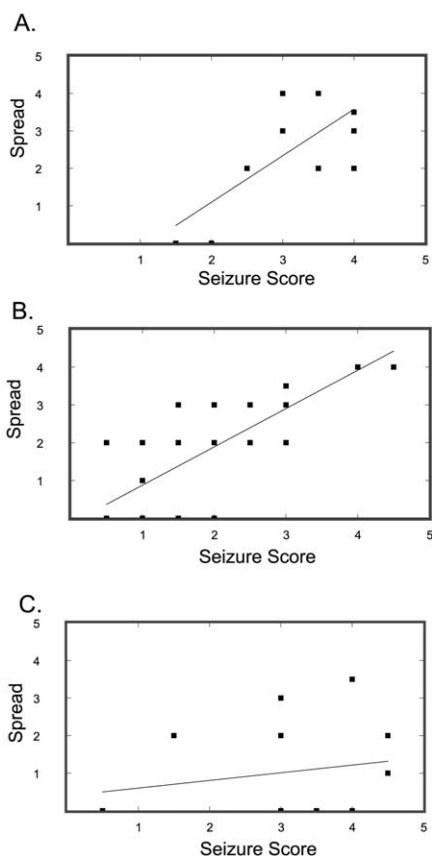


Fig. 5. Correlation of seizure score and spread of damage in KA-treated ovariectomized rats with (A) blank implants, (B) progesterone, or (C) estrogen replacement. The spread of damage was significantly correlated with seizure scores in animals with blank ( $P < 0.02$ ) and P ( $P < 0.001$ ) implants but in E-treated rats this relationship was lost ( $P > 0.05$ ). Thus, despite increased seizures, damage to the hippocampus did not spread to many areas after E replacement.

animals with mild seizures after KA were significantly less than those seen in the severely affected P-treated rats ( $P < 0.001$ ). Similar to what was noted in control animals, the losses of NeuN immunoreactivity within the CA subfields after KA treatment in P treated rats were significantly correlated with the seizure scores ( $r^2 = 0.343$ ,  $P < 0.005$ ; Fig. 8B). Thus, the higher the seizure score, the greater the degree of neuronal loss.

For estrogen-treated animals, no significant losses of NeuN immunoreactivity after KA treatment were noted. This was true even in animals displaying high seizure scores. As a result, estrogen-treated animals displaying severe seizures had significantly more NeuN-immunoreactive

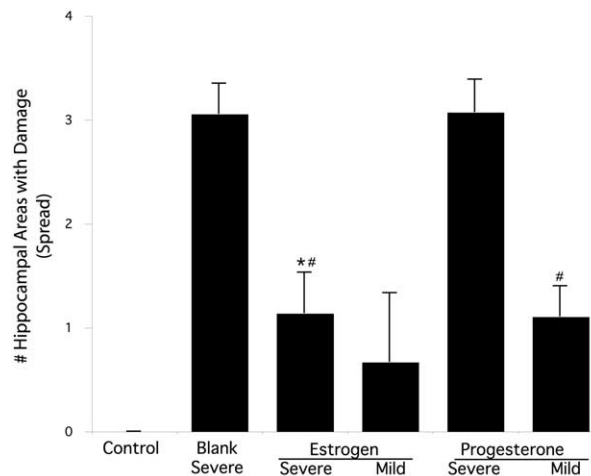


Fig. 6. Effects of estrogen or progesterone on spread of damage from KA in animals exhibiting mild (behavioral score  $\leq 2$ ) or severe (behavioral score  $> 2$ ) seizures. Animals treated with E that displayed severe seizures showed significantly fewer regions of neuronal damage after KA than severely affected animals that either did not receive hormone replacement ( $*P < 0.005$ ) or received P treatment ( $\#P < 0.01$ ). The spread of damage in these E-treated rats was no different from that seen in animals that had only mild seizures. The spread of damage in severely affected rats that were treated with P was no different from that seen in severely affected KA-treated control animals (Blank, Severe).

neurons than was seen in the animals treated with progesterone that had equally high seizure scores (Fig. 7;  $P < 0.05$ ). No correlation was observed between seizure scores and NeuN immunoreactivity in the estrogen-treated rats (Fig. 8C;  $r^2 = 0.018$ ;  $P > 0.05$ ).

## Discussion

The results of these studies indicate that progesterone produces its effect principally by reducing seizure behavior; by contrast, estrogen has little beneficial effect on seizure behavior but is capable of protecting the hippocampus from seizure damage. The majority of animal studies examining the effects of gonadal steroids on seizures reported that seizure susceptibility/activity was reduced by progesterone (Backstrom et al., 1985; Beyenburg et al., 2001; Edwards et al., 1999; Nicoletti et al., 1985). In addition, in women with catamenial epilepsy, the luteal phase, when progesterone levels are high relative to estrogen, is associated with a lower seizure incidence than the menstrual and follicular phases where estrogen and progesterone are both low or

Fig. 4. Hippocampal neuron patterns. Micrographs of the hippocampus from (A) control saline-treated ovariectomized rat with blank capsules shows the CA1, CA2, CA3, hilus, and dentate gyrus. (B) Blank + KA rat displaying a seizure score of 3.5 has marked neuronal losses in CA1, CA3, and the hilus (\*). This same animal also possessed damage to the entorhinal cortex (not shown). (C) P + KA-treated rat with a seizure score of 1.5 shows only slight neuron loss in the hilus; all other regions are normal. (D) P + KA-treated rat with a seizure score of 2.5 shows clear evidence of hilar neuronal loss (\*); (E) P + KA-treated rat with a seizure score of 3.5 had damage to the hippocampal CA fields (\*) that are quite similar to those in the blank + KA rats. (F) E-treated rat with a seizure score of 1.5 shows little or no hippocampal damage; (G) E treated rat with a seizure score of 3.5 also shows little evidence of neuronal loss.

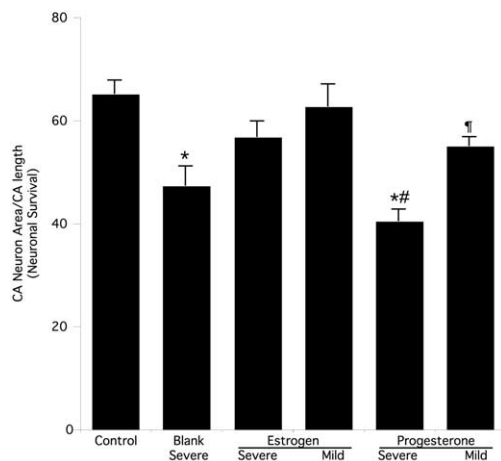


Fig. 7. Effects of estrogen or progesterone on neuron loss in KA-treated animals exhibiting mild (behavioral scores  $\leq 2$ ) or severe (behavioral scores  $> 2$ ) seizures. Ovariectomized animals that received blank capsules and showed severe seizures after KA had significant loss of NeuN immunoreactivity compared with control rats ( $*P < 0.005$ ). Losses of NeuN immunoreactivity in P-treated rats were significantly different from control rats only when seizures were severe ( $*P < 0.005$ ). Thus P-treated animals with high seizures had significantly more neuron losses than P-treated rats with mild seizures ( $\#P < 0.001$ ). In contrast, E-treated rats with either severe or mild seizures showed no significant differences in NeuN immunoreactivity (NeuN area/CA length) compared with controls.

estrogen is elevated relative to progesterone (Bauer, 2001; Bonuccelli et al., 1989; Herkes et al., 1993; Herzog et al., 1997; Lundberg, 1997; Morrell, 1992, 1999; Murri and Galli, 1997; Schachter, 1988; Zimmerman, 1986). Thus, it would logically follow that progesterone should reduce brain damage from seizures simply because there are fewer seizures. Indeed, in P-treated animals that exhibited reduced seizure severity, brain damage was reduced. What was surprising is that progesterone was only effective in reducing seizures at low physiological ranges. High doses of progesterone failed to reduce seizures or prevent brain damage.

Progesterone is metabolized to 3- $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one (allopregnanolone), a potent allosteric modulator of the GABA<sub>A</sub> receptor (Baulieu et al., 1996). Several studies have suggested allopregnanolone acting at the GABA<sub>A</sub> receptor is the mechanism whereby progesterone attenuates seizure activity (Beyenburg et al., 2001; Frye and Scalise, 2000; Frye et al., 1998; Galli et al., 2001; Morrell, 1992; Murri and Galli, 1997). Frye (1995), reported that subcutaneous administration of allopregnanolone 3 h prior to perforant path stimulation significantly reduced both seizure severity and the resulting hippocampal neuronal loss. What is difficult to explain is why higher plasma levels of progesterone failed to alter seizures or the brain damage from them. Levels of allopregnanolone and GABA receptor activity in vitro and in vivo are positively correlated with progesterone levels (Barbaccia et al., 1996; Corpechot et al., 1993), raising the expectation that increases in plasma progesterone should result in increased seizure suppression. In women suppression of brain excitability by progesterone is

not universally effective. George and co-workers measured CSF levels of progesterone and its metabolites in women and their relationship to affective disorders (George et al., 1994). In that study, increased levels of allopregnanolone did not always accompany the increased progesterone levels seen during the luteal phase of the cycle, suggesting that biological variation in metabolism of progesterone could explain the variable effects of the steroid on seizures. Herzog (1995) too noted that of his female patients treated with progesterone, 28% did not respond to progesterone treatment. While that study did not speculate on the lack of effect in those women, altered conversion to allopregnanolone could be responsible. In our rats whether prolonged exposure to high, but not low, progesterone levels is capable of interfering with the synthesis of allopregnanolone remains to be determined.

It is also possible that after long-term exposure to allopregnanolone, the GABA receptor fails to respond or is downregulated. Studies examining GABA receptor function after withdrawal from chronic allopregnanolone or progesterone in pseudopregnant rats show that the receptor is desensitized and that expression of the alpha 4 subunit of

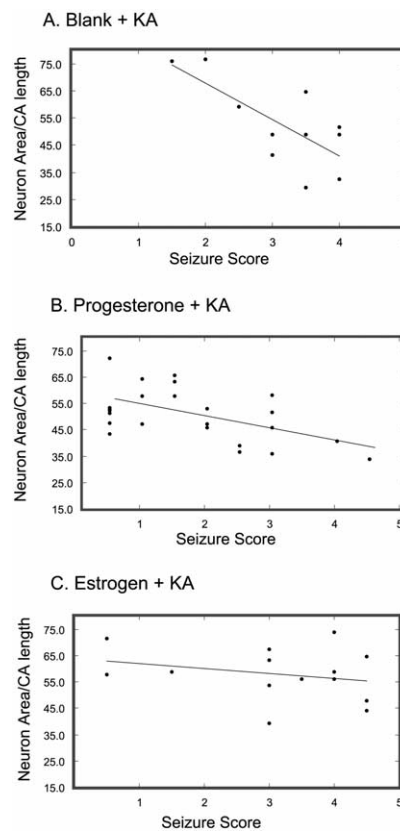


Fig. 8. Correlation of seizure severity and NeuN immunoreactivity in KA-treated ovariectomized rats with (A) blank implants, (B) progesterone, or (C) estrogen replacement. The NeuN immunoreactivity was negatively correlated with seizure scores in KA-treated animals with blank ( $P < 0.02$ ) and P ( $P < .005$ ) implants. In E-treated rats this relationship was lost ( $P > 0.05$ ) owing to the fact that even when seizures were severe, neuron losses were reduced.

the GABA<sub>A</sub> receptor is reduced (Smith, 2002; Smith et al., 1998). The levels of the steroids were both relatively high prior to withdrawal. Since the studies did not examine GABA receptor function during pseudopregnancy when the hormone levels were still elevated, it is possible that the desensitization actually occurred prior to withdrawal. Indeed, *in vitro*, prolonged exposure of cortical neurons to allopregnanolone abolishes the potentiation of GABA<sub>A</sub> receptors by altering the allosteric interactions of allopregnanolone with the benzodiazepine binding sites (Friedman et al., 1993). Subsequent studies of cortical neurons *in vitro* determined that attenuation of GABA<sub>A</sub> receptor function was due to reduction in GABA receptor binding due to alterations in both beta and alpha receptor subunit expression (Yu et al., 1996). *In vivo* prolonged treatment with either progesterone or allopregnanolone produces similar desensitization of the GABA receptor and it was proposed that similar alterations in GABA<sub>A</sub> receptor subunit expression were responsible (Gulinello et al., 2001; Wohlfarth, et al., 2002). While none of the studies varied the dose of progesterone or allopregnanolone, if the desensitization of the GABA receptor only occurs in conditions of high steroid levels, such changes in the GABA<sub>A</sub> receptor composition/function could explain why low but not high doses of progesterone reduced seizures after KA.

In the present study, administration of estrogen had no effect on KA seizure severity. A large number of studies *in vivo* and *in vitro* demonstrate that the steroid increases after discharge patterns and seizure thresholds (Backstrom et al., 1985; Buterbaugh, 1987, 1989; Buterbaugh and Hudson, 1991; Edwards et al., 1999; Gu and Moss, 1996; Hom and Buterbaugh, 1986; Hom et al., 1993; Kubo et al., 1975; Maggi and Perez, 1985; Nicoletti et al., 1985; Smith et al., 2002; Stitt and Kinnard, 1968; Teyler et al., 1980; Wong and Moss, 1991; Woolley, 2000). Thus, one would predict that under conditions of high estrogen concentrations KA would yield the strongest stimulatory effects thereby increasing the potential for excitotoxic brain damage. Yet, despite the persistence of seizures, estrogen replacement reduced mortality from KA seizures and significantly limited the overall spread of damage and amount of neuronal loss. Earlier studies examining hormonal effects on KA-induced brain damage are mixed. In intact females, injection of KA on proestrus (when estrogen levels are maximal) still produced substantial brain damage (Azcoitia et al., 1999). However, in that same study, animals that were ovariectomized and replaced with estrogen but not progesterone showed protection from damage, provided the steroid was administered 2 days prior to onset of seizures. Subcutaneous injections of 0.2  $\mu$ g estrogen administered 48 and 24 h prior to KA reduced hippocampal brain damage (Veliskova et al., 2000), as did supraphysiological doses of estrogen (150  $\mu$ g/rat) administered before or along with KA (Azcoitia et al., 1998, 1999). It would appear from these studies that reduction in damage from KA seizures by estrogen administration may not be strictly dose-dependent, but fluctuating

hormone levels after steroid injection make that conclusion tenuous. Our data demonstrate more clearly that maintained doses of E in either the physiological range or supraphysiological range protect neurons from damage.

How estrogen protected the hippocampus from damage is not immediately clear. Initially it was thought that KA cell death was exclusively necrotic and there would be little basis for estrogen interfering in that process. More recent studies demonstrate that delayed cell death accompanied by DNA laddering (normally associated with apoptosis) after KA induced seizures (Fujikawa et al., 2000; Kondo et al., 1997; Kondratyev and Gale, 2001; Liu et al., 2001; Pollard et al., 1994a, 1994b; Venero et al., 1999). The proapoptotic molecule Bax is upregulated following KA seizures and concomitantly the prosurvival molecule Bcl-2 is downregulated (Gillardon et al., 1995). In a variety of models of neuronal injury estrogen upregulates expression of Bcl-2 (Choi et al., 2001; Dubal et al., 1999; Harms et al., 2001; Pike, 1999; Sawada et al., 2000; Singer et al., 1998) and, if acting similarly in our studies, this mechanism could explain estrogen's protective effects. There are also studies suggesting that antioxidant effects seen with high doses of estrogen protect neurons from cell death (Bae et al., 2000; Behl et al., 1995, 1997, 2000; Culmsee et al., 1999; Emilien et al., 2000; Goodman et al., 1996; Green et al., 1998; Green and Simpkins, 2000; Gridley et al., 1998; Inestrosa et al., 1998; Keller et al., 1997). The pellets we used generated plasma levels of estrogens high enough to produce antioxidant effects. The high progesterone levels in our animals should also have exerted antioxidant effects (Goodman et al., 1996); however, high levels of progesterone did nothing to protect the animals from seizures or neuronal cell death.

In summary, our data indicate that low but not high doses of progesterone reduce seizures and, in so doing, reduce damage to the hippocampus. Estrogen, on the other hand, at either physiological or supraphysiological levels, reduces neuronal cell death from seizures, but has little effect on seizure severity. It will be important to determine the mechanism whereby each of the steroids influences seizure activity and the ensuing brain injury. These results also raise the question of what effect combinations of both steroids might have. Possibly, when E + P are administered together, P would suppress seizure activity, but should some seizures still persist, E would then reduce the potential for brain damage. Studies examining this issue are currently underway.

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