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Seizure susceptibility in intact and ovariectomized female rats treated with the convulsant pilocarpine

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Abstract

Despite numerous neuroendocrinological studies of seizures, the influence of estrogen and progesterone on seizures and epilepsy remains unclear. This may be due to the fact that previous studies have not systematically compared distinct endocrine conditions and included all relevant controls. The goal of the present study was to conduct such a study using pilocarpine as chemoconvulsant. Thus, age and weight-matched, intact or ovariectomized rats were tested to determine incidence of status epilepticus and to study events leading to status. Intact female rats were sampled at each cycle stage (proestrus, estrus, metestrus, or diestrus 2). Convulsant was administered at the same time of day, 10:00–10:30 a.m. Statistical analysis showed that there was a significantly lower incidence of status on the morning of estrus, but differences were attenuated in older animals. Ovariectomized rats were distinct in their rapid progression to status. These results show that the incidence of status in female rats following pilocarpine injection, and the progression to pilocarpine-induced status, are influenced by reproductive state as well as age. The hormonal milieu present specifically on the morning of estrus appears to decrease susceptibility to pilocarpine-induced status, particularly at young ages. In contrast, the chronic absence of reproductive steroids that characterizes the ovariectomized rat leads to a more rapid progression to status. This dissociation between incidence vs. progression provides new insight into the influence of estrogen and progesterone on seizures.

Keywords

Estrogen; Progesterone; Allopregnanolone; Status epilepticus; Estrus; Estrous cycle; Aging

Introduction

In many women with epilepsy, seizures do not occur randomly but cluster in association with specific stages of the ovarian cycle. This condition, termed ‘‘catamenial’’ epilepsy, is believed to result from the actions of circulating ovarian steroid hormones on the brain. The mechanisms responsible for hormonal modulation of seizure susceptibility, however, remain poorly understood. One way to investigate this problem is to use laboratory animal models, in which interactions between seizures and the ovarian hormones can be studied under more controlled

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conditions than is possible in human populations. Numerous studies over the last few decades have identified sex and cycle-dependent differences in seizure susceptibility in rodents. These studies have used various methods to induce seizures, including kindling, electroshock, and chemoconvulsant administration (Bujas et al., 1997; Buterbaugh, 1989; Edwards et al., 1999; Finn and Gee, 1994; Frye and Bayon, 1998; Frye et al., 1998; Hoffman et al., 2003; Hom and Buterbaugh, 1986; Hudson and Buterbaugh, 1991; Kalkbrenner and Standley, 2003; Matejovska et al., 1998; Nicoletti et al., 1985; Pericic and Bujas, 1997; Persinger et al., 1988; Pesce et al., 2000; Schwartz-Giblin et al., 1989; Slamberova and Vathy, 2000; Tan and Tan, 2001; Tominaga et al., 2001; Valente et al., 2002; Velisek et al., 1999; Wahnschaffe and Loscher, 1992; Woolley, 2000). However, these studies have failed to provide a clear picture of the relationship between ovarian hormone levels and seizure susceptibility. Hence, significant questions remain concerning the roles of these hormones in regulating seizure induction, propagation, and subsequent seizure-associated sequelae, such as neuronal damage.

Of the principal ovarian steroids, estrogen has been suggested to be primarily proconvulsant, whereas it is commonly assumed that progesterone is anticonvulsant. This perspective is based on studies beginning in the 1950s, in both laboratory animals and women with epilepsy, showing that estrogens can exacerbate seizures while progestins decrease seizure frequency (Backstrom, 1976; Logothetis and Harner, 1960; Logothetis et al., 1959; Terasawa and Timiras, 1968). Subsequent work, however, has shown that proconvulsant effects of estrogen, as well as anticonvulsant effects of progesterone, may depend to a large extent on the specific experimental conditions used. For example, although there have been repeated demonstrations that estrogen is proconvulsant (Buterbaugh, 1989; Matejovska et al., 1998; Nicoletti et al., 1985), there is also evidence that this is not the case (Hoffman et al., 2003; Hom and Buterbaugh, 1986; Hudson and Buterbaugh, 1991; Slamberova and Vathy, 2000; Velisek et al., 1999; Woolley, 2000). Furthermore, while estradiol may protect neurons from seizure-induced neuronal damage (Azcoitia et al., 1998; Veliskova et al., 2000), this protective effect may not be mediated by a decrease in seizure severity (Hoffman et al., 2003; Reibel et al., 2000).

One of the major difficulties in evaluating the role of the ovarian steroids is that results from different laboratories have not been consistent. A possible reason for this is that most studies have used ovariectomized rats, treated with a variety of different hormone replacement regimens. These regimens cannot easily be compared because both estrogen and progesterone may have variable effects depending on the dose and duration of treatment (Edwards et al., 1999; Kalkbrenner and Standley, 2003; Schwartz-Giblin et al., 1989; Tominaga et al., 2001).

Intact cycling female rats have also been used to examine the influence of the ovarian steroids on seizures, but this has not necessarily clarified the ways ovarian steroids influence seizures. Again, this may be due at least in part to inconsistencies in the experiments (time of day for the studies, age and body weight of the animals). Most studies have also focused on a few specific endpoints, without consideration of other potential factors that may contribute to seizure susceptibility. For example, in one recent study, susceptibility to pilocarpine-induced seizures was compared in ovariectomized and intact rats, but the intact rats only included animals at the estrous stage of the female reproductive cycle (the ‘‘estrous cycle’’; Valente et al., 2002). While ovariectomized animals appeared to have a shorter latency to seizures than intact rats, general conclusions regarding the role of the ovarian hormones could not be drawn, since other stages of the reproductive cycle were not included. Other stages could potentially be similar to ovariectomized rats, for example. Tan and Tan (2001) reported increased seizure susceptibility at proestrus compared to estrus. Studies of ovariectomized rats treated with proestrous levels of estradiol also support the hypothesis that seizure susceptibility may be increased at proestrus (Edwards et al., 1999). However, proestrous rats may not *always* be more susceptible to seizures relative to rats examined at other times of the estrous cycle (Finn and

Gee, 1994; Pesce et al., 2000; Wahnschaffe and Loscher, 1992), for reasons that presently remain unclear.

The results of the study by Valente et al. (2002) were also surprising because the incidence of status epilepticus was reported not to differ significantly between estrus and ovariectomized rats (Valente et al., 2002). This finding contrasts with data from other studies that have indicated a decreased seizure duration at estrus to seizures induced by the chemoconvulsant kainic acid (Frye and Bayon, 1998; Frye et al., 1998), although seizure threshold in response to other convulsants besides pilocarpine and kainic acid was least at estrus relative to the following day, metestrus (Finn and Gee, 1994).

The present study was undertaken in an attempt to resolve some of this uncertainty, via a systematic comparison of the chemoconvulsant sensitivity of ovariectomized rats and intact rats at different stages of the estrous cycle. Rather than utilize rats treated with different hormone regimens, we focused on the comparison of ovariectomized to intact cycling animals, because cycling rats would provide the best insight into the physiological hormonal parameters that may be most important in regulating seizure susceptibility, free of the possible problems of interpretation associated with hormone replacement. All animals were administered convulsant at the same time of day, mid-morning, to avoid the potential confound of variability due to the circadian rhythm. Furthermore, mid-morning comparisons of cycling rats allowed optimal comparisons of physiological changes of reproductive steroids (e.g., estradiol is elevated during the morning on proestrus but progesterone is not, allowing specific insight into the influence of physiological levels of estradiol). Since age and body weight may also influence the response to convulsants (Bujas et al., 1997; Darbin et al., 2004; Hunter et al., 1989; Pericic and Bujas, 1997; Persinger et al., 1988; Turski et al., 1989), we included animals from a range of ages and body weights in each reproductive condition, to determine whether age and/or body weight might affect the outcome.

Pilocarpine was chosen as the test chemoconvulsant, for several reasons. First, pilocarpine has the ability not only to elicit single seizures but also a state of continuous, severe seizures (status epilepticus). This is potentially important because single seizures and status epilepticus could be regulated differentially by circulating hormones. Second, status epilepticus is associated with some degree of mortality, allowing the potential to compare effects of hormones on mortality after status epilepticus. Third, pilocarpine-induced status epilepticus has an onset that is distinct, and therefore measurable with confidence. Other chemoconvulsants, such as kainic acid, can be used to induce status epilepticus; but the onset of status is less easily discriminated, and therefore the measurement of latency to status epilepticus is more prone to error. Finally, choosing pilocarpine allowed us to directly address previous studies that have concluded that ovarian hormones reduce the sensitivity to this chemoconvulsant drug (Valente et al., 2002).

Materials and methods

Animals and animal care

Guidelines for animal care and use met the standards set by the New York State Department of Health and the National Institutes of Health. All efforts were made to minimize the number of animals that were necessary for statistically valid results and to minimize animal discomfort.

Adult female Sprague–Dawley rats (Taconic) were housed 2 per cage to avoid stress related to isolation. Cycling females were housed with another cycling female; ovariectomized females were housed with another ovariectomized female. They were provided food (Purina 5001) and distilled water ad libitum in a temperature (68–74°F) and humidity (40–70%) controlled environment with a 12 h light cycle (lights on, 07:00 h). At least 2 weeks prior to convulsant administration, rats were transferred to a room where cages of female rats were placed between

cages of male rats. This arrangement was used because it was empirically determined that rats had more consistent 4-day cycles when they were housed between cages of males. Opaque-sided cages (Fisher Scientific) with corn-cob bedding (W.F. Fisher) were used for housing.

Terminology

Designation of cycle stage is based on the terminology of Freeman (1975) and a 4-day estrous cycle for a sexually-mature Sprague–Dawley rat. The first day, the day of proestrus, begins with a rapid rise in serum estradiol, followed in the afternoon and evening by a surge in progesterone. Progesterone peaks in the late evening, at the time of ovulation, then declines rapidly (Freeman, 1975). The next day, the day of estrus (referred to as ‘‘estrus’’ in the Results) is not to be confused with behavioral estrus, a time of sexual receptivity, which is often used in the literature to refer to the latter part of the day of proestrus, when estrogen is still elevated and progesterone is rising.

On the morning of the second day of the estrous cycle, the day of estrus, serum progesterone and estradiol are both low (Freeman, 1975). The next day of the cycle, the third day, begins a 2-day period called diestrus. The first day of diestrus (termed ‘‘metestrus’’ in the Results) is accompanied by a modest and transient elevation in progesterone during the middle of the day (Freeman, 1975). During the second day of diestrus (‘‘diestrus 2’’ in the Results), serum values of estradiol and progesterone are low early in the day, but subsequently, estradiol begins a slow rise in preparation for the next ovulation.

Vaginal cytology and confirmation of hormone levels by radioimmunoassay (RIA)

Cycle stage was determined by daily vaginal examination with a light microscope at 10× magnification. Evaluation of vaginal epithelial cells was conducted as previously described (Edwards et al., 1999). Only animals showing at least three consecutive 4-day cycles were used for experiments on intact rats. For determination of hormone levels in cycling female rats, a group of animals in the same age and weight range as those used for pilocarpine treatment were used. They were housed under identical conditions as described above, and were anesthetized with CO_2 at approximately 10:00–11:30 a.m. After loss of consciousness, trunk blood was collected after decapitation; serum was collected and frozen at −20°C for subsequent radioimmunoassay, described elsewhere (Scharfman et al., 2003).

Serum hormone levels were measured as previously described (Scharfman et al., 2003). Briefly, serum testosterone (T) and progesterone (P) were measured by RIA using commercial kits (Coat-a-count kits Cat #TKTT1 for T, TKPG1 for P, purchased from Diagnostic Products Corporation, Los Angeles, CA) according to the kit instructions. Serum sample volumes were 50 μl. To correct for any potential blank effect due to components of rat serum, the standards for the assay also contained 50 μl of a serum pool obtained from ovariectomized rats. The minimum detectable concentrations of hormone, defined as being greater than the 95% upper confidence limit for the zero standard, were 0.1 ng/ml for P and 10 pg/ml for T.

Serum estradiol (E) levels were also measured using commercial RIA kits (coat-a-count RIA kit KE2D1; Diagnostic Products Corp, Los Angeles, CA), with one important modification to the assay procedure. Direct use of this kit, which is designed for use with human samples, results in overestimation of estradiol levels as a result of interference from components of rat serum (MacLusky, unpublished observations). Correction for this interference cannot be reproducibly accomplished by adding an equivalent volume of serum from ovariectomized rats to the assay standards. However, the interference can be completely eliminated by solvent extraction of the estradiol prior to assay. The assay standards and rat serum samples (100 μl/sample) were therefore extracted prior to assay with 2 ml anhydrous ethyl ether. The ether extracts were dried under a stream of air. The dry extracts were then reconstituted into 100 μl

of the human serum zero calibration standard from the RIA kit and assayed according to kit instructions. The minimum detectable concentration of estradiol was 2 pg/ml.

Hormone assays were performed in one group of animals to interpret the findings in the pilocarpine-treated cohort of animals. These assays allowed us to confirm that serum hormone levels in our colony of rats were consistent with previous reports for the Sprague–Dawley strain (Freeman, 1975). Both the group used for hormone determination and the pilocarpine-treated cohort were treated identically in terms of housing, food, lighting, methods for vaginal cytologic evaluation, and involved the same investigators. Hormone assays on the animals receiving pilocarpine were not conducted because of the likelihood that the stress of taking substantial blood samples, and effects of blood loss, would influence the responses to pilocarpine administration.

Ovariectomy and sham-ovariectomy

Only animals with at least two normal 4-day estrous cycles were used for ovariectomy and sham-ovariectomy, to ensure that reproductive function was normal in female rats that were ovariectomized. Ovariectomy and sham-ovariectomy procedures were identical except that, in the latter case, ovaries were not ligated and removed. Animals were initially injected i.p. with ketamine (70 mg/kg) and xylazine (7 mg/kg) until loss of consciousness and loss of any response to a pinch of the tail. The lower back was shaved at the midline with an animal hair shaver and a single rostral–caudal incision (2 in. long) was made with a sterile scalpel. Fascia was separated from the skin to expose the lateral peritoneum above the ovary on one side. A 1/2-in. incision of the peritoneum was made and forceps were used to remove the ovary from the abdominal cavity. Sterile sutures were used to tie the ovary at its junction with the uterus. The ovary was then cut away from the uterus, and the uterus was allowed to settle back into the abdominal cavity. The peritoneal incision was sutured, and the entire procedure was repeated on the other side to remove the second ovary. Subsequently, the midline skin incision on the back was closed with wound clips or skin adhesive (TissueMend) and swabbed in Betadine solution. Animals were placed in their home cage under a heat lamp until recovery a few hours later. They were then housed in pairs until experimentation.

Pilocarpine administration

On the day of pilocarpine administration, females were weighed at approximately 08:30 h and cycle stage was confirmed by vaginal cytological examination. Animals were then placed in individual clear cages with fresh bedding and food and water ad libitum. Animals were positioned on a lab bench beside the shelves where they were normally housed. No more than 7 animals were tested at one time. Different endocrine states were included in each test group, so that animals in different endocrine states could be simultaneously compared. At approximately 09:30 h, an experimenter blinded to endocrine state (i.e., cycle stage, ovariectomy, or sham surgery) injected each rat with 1 mg/kg atropine methylbromide i.p. Thirty minutes later, animals were injected by the same experimenter with 350 mg/kg pilocarpine hydrochloride i.p., and subsequent measurements and observations were made by the same experimenter.

Latencies were measured from the time of pilocarpine injection or the first abnormal behavior following pilocarpine administration. The ‘‘first abnormal behavior’’ described in the Results refers to lowering of the entire body to the bottom of the cage, including chest and head, while maintaining open eyes, without accompanying movement. This behavior has only been observed after pilocarpine administration or administration of another chemoconvulsant, such as kainic acid. In some cases, facial automatisms and hypersalivation were observed as the first abnormal behavior. These behaviors always preceded more severe seizures, rather than following seizures of stages 1–5, which were defined by the Racine scale (Racine, 1972).

However, it should be noted that electrographic events associated with such behaviors have not been studied, and therefore the phrase ''first abnormal behavior'' is used rather than ''first seizure.'' The number of stage 5 seizures prior to status refers to the number of individual stage 5 seizures that had a clear termination, and occurred prior to status. Status epilepticus was defined as the time when a stage 5 seizure did not cease, i.e., stage 5 seizures became continuous. One hour after the onset of status epilepticus, animals were injected with 5 mg/kg diazepam i.p. Approximately 6 h later, they were injected subcutaneously with 2.5 ml dextrose-lactate Ringer's solution. Mortality was defined as death within 24 h after the onset of status epilepticus. This included either abrupt respiratory arrest during status, or cessation of respiration in the subsequent 24 h.

Statistical analysis

Data were analyzed using Statview (SAS Institute, Cary N.C.). Comparisons of the frequency of seizures and mortality in different reproductive conditions were performed using χ^2 analysis and Fisher's Exact Test. Seizure latency data were first tested for homogeneity of variance using Bartlett's test: where significant inhomogeneity of variance was present, this was eliminated by log transformation of the results. Inter-group comparisons were performed by one-way ANOVA followed by the Bonferroni–Dunn post-hoc test procedure. Comparisons between all treatment groups and a single, specific cycle stage were performed using Dunnett's test. Correlations between seizure latencies and age or body weight were analyzed by bivariate least squares regression. The contributions of age, body weight, and reproductive state to the incidence of status epilepticus were analyzed by stepwise multiple regression analysis, coding reproductive state as a numeric variable (1–6, for each of the 6 reproductive states examined). For the multiple regression analysis, a variable was considered to be significantly correlated with the endpoint of interest if the corresponding F value for removal of the variable from the regression model had a P value < 0.05. Conversely, variables were not considered correlated if the corresponding F statistic to enter the variable into the model was not statistically significant, by the same criterion. A criterion for statistical significance of $P < 0.05$ was adopted for all analyses.

Results

Subjects

Of the 98 animals treated with pilocarpine, 66 were intact cycling female rats, 19 were ovariectomized, and 13 were sham-ovariectomized. RIA values for intact cycling female rats were based on 77 additional animals that were housed and treated identically to those that were administered pilocarpine. RIA measurements confirmed the estimation of cycle stage predicted on the basis of vaginal cytology (Fig. 1), providing mean estimates of serum hormone levels that are consistent with previous published data for cycling female rats (Freeman, 1975).

Status epilepticus

Incidence of status—The incidence of pilocarpine-induced status in the 6 experimental groups (proestrus, estrus, metestrus, diestrus 2, ovariectomized, sham-ovariectomized) is shown in Fig. 2. Animals that were administered pilocarpine on the morning of estrus had a low incidence of status epilepticus relative to the proestrous and ovariectomized groups (Fig. 2, Fisher's Exact Test, $P < 0.05$). However, there were no significant differences between any other stage of the cycle and the ovariectomized animals in this analysis, which included all animals tested, regardless of age or body weight.

The ages and body weights of each experimental group are shown in Fig. 3. Analysis of variance revealed no significant differences between the groups in mean age at the time of pilocarpine administration ($F = 1.234$; df 5,92; $P = 0.299$). Significant differences were observed in mean

body weight (ANOVA; $F = 11.18$; df 5,92; $P < 0.0001$) reflecting the markedly higher body weight of the ovariectomized animals, expected because of the loss of the anorectic effect of estrogen (Nance and Gorski, 1978). Visual inspection of the data suggested that the incidence of status might be higher in the older animals (Fig. 3, cf. filled vs. open symbols, respectively data for animals that entered status vs. those that did not). To test this hypothesis, stepwise multiple regression analysis was performed with incidence of status as the dependent variable and age, body weight, and reproductive state as potentially correlated variables. The incidence of status was found to be significantly dependent on both age (F to remove 9.30; df 2,95; $P < 0.001$) and reproductive state (F to remove 6.23; df 2,95; $P = 0.003$), but not on body weight (F to enter 0.255; $P > 0.5$). Analysis of covariance with incidence of status as the dependent variable also revealed a significant dependence of status on reproductive state ($F = 3.772$; df 5,74; $P = 0.004$) with a significant interaction between reproductive state and age ($F = 4.468$; df 5, 74; $P = 0.001$). Post-hoc analysis confirmed that the incidence of status was significantly lower at estrus than in any other group (Dunnett's test, $P < 0.05$) with no significant differences between the other reproductive states.

In summary, pilocarpine-induced status on the morning of estrus was relatively infrequent, demonstrating an influence of reproductive state on the susceptibility to status. There was also a marked effect of age (the incidence of status being higher in the older animals) and a strong interaction between age and reproductive state, reflecting the fact that in older animals the apparent protective effect of estrus was diminished. Thus, when all data were included, regardless of age or body weight, there was a significantly lower incidence of status at estrus, but only relative to proestrous or ovariectomized rats. However, estrus was significantly distinct from all groups when age was taken into account.

Mortality after status—As shown in Fig. 2, there was no significant difference in mortality among animals that had status epilepticus (χ^2 test, $P > 0.1$), possibly because of the small numbers of animals that died in each group (for example, only 2 of 17 animals in the estrus group had status). In animals that were treated with pilocarpine, but did not progress to status, there was no mortality in any of the groups.

Ovariectomy vs. sham-ovariectomy—To compare ovariectomized with sham-ovariectomized rats, sham-operated animals were allowed to recover for 1 week and were then examined daily by vaginal cytology to ensure that they resumed regular 4-day estrous cycles. This was critical to ensure that sham surgery did not influence ovarian function. Initially, we tested the hypothesis that sham surgery might have affected pilocarpine sensitivity using sham-operated animals selected on the morning of metestrus ($n = 9$). Metestrus was chosen because any relative increase or decrease in seizure susceptibility would be most easily detected; at estrus, by contrast, any decrease in susceptibility might not be apparent. Only animals tested at least 15 days after surgery were included. No significant differences in the incidence of status were demonstrated between ovariectomized rats, sham-ovariectomized rats at metestrus, and intact cycling rats tested at metestrus (Fisher's Exact Test, $P > 0.5$ for all comparisons), suggesting that surgery does not influence susceptibility to status if it occurred >15 days before convulsant administration.

A few additional sham-ovariectomized rats were tested at other cycle stages (proestrus, $n = 3$; estrus, $n = 1$), with similar results. When the data from these animals were pooled with the sham-operated rats at metestrus, the combined data were not significantly different from results in either ovariectomized animals or intact animals at metestrus (Fig. 2, Fisher's Exact Test, $P > 0.5$). Therefore, for all other analyses, results for all sham-ovariectomized animals were pooled.

Time between ovariectomy and pilocarpine administration did not significantly influence seizure susceptibility. The recovery periods between ovariectomy and pilocarpine administration ranged from 15–100 days (mean ± SEM., 38.31 ± 6.37 days after ovariectomy, $n = 19$). For this range of recovery periods, the incidence of status was not significantly affected by the delay between ovariectomy and pilocarpine treatment ($F = 3.73$; df 1,19; $P = 0.07$).

Progression to status epilepticus

To determine whether the events leading up to status epilepticus may also be influenced by reproductive state, we measured the latency to status, the latency to the first abnormal behavior that occurred after pilocarpine administration, the latency to the first stage 5 seizure, and the number of stage 5 seizures prior to the onset of status epilepticus.

Latency to status epilepticus and the first stage 5 seizure—The total latency to status, i.e., the time between pilocarpine injection and the onset of status) was not significantly different across groups (Fig. 4C1 $F = 1.57$; df 5,34; $P = 0.195$). However, examination of the latency from the time of pilocarpine administration to the first abnormal behavior suggested variability in the initial period after pilocarpine administration (Fig. 4A). Therefore, we tested the hypothesis that age and/or body weight underlie some of the variability. Stepwise linear regression analysis revealed a highly significant dependence of the latency to the first abnormal behavior on body weight (F to remove 13.62; df 1,94; $P < 0.001$; Fig. 4B1), but no significant dependence on either age (F to enter 0.001; $P > 0.9$; Fig. 4B2) or reproductive state (F to enter 0.204; $P > 0.5$).

These observations suggested that body weight might have significantly affected latency to the first abnormal behavior, possibly due to increased body fat near the site of pilocarpine injection. This could have distorted the measurement of latency to status, particularly for the ovariectomized animals, since these were significantly heavier than the other groups. Therefore, we examined the latency to status using time from the first abnormal behavior as the starting point, making the assumption that the first sign of abnormal behavior probably reflects the approximate time when the drug has entered the brain and attained a sufficient concentration in the brain to influence CNS function. Using this approach, latency was significantly dependent on reproductive state ($F = 2.73$; df 5,34; $P = 0.036$), largely because of a significantly shorter latency to the onset of status in ovariectomized animals (Fig. 4C2).

Similarly, evaluation of the latency from pilocarpine injection to the first stage 5 seizure did not reveal significant differences across groups (Fig. 5A, black bars; $F = 1.76$; df 5,33; $P = 0.148$), but when the data were reanalyzed using the first abnormal behavior as the starting point, ovariectomized rats had a significantly shorter latency (Figs. 5B–C; $F = 4.08$; df 5,33; $P = 0.005$).

In summary, the first abnormal behavior appeared to be delayed in ovariectomized animals, an effect that correlated with their higher body weight. Thus, when latency was measured from the first abnormal behavior to either the first stage 5 seizure or to the onset of status, differences could be detected among experimental groups. Latencies were considerably faster in ovariectomized rats vs. intact animals. Although diestrus 2 animals also appeared to be slightly delayed to their first abnormal behavior (Fig. 4A), this was not statistically significant (Bonferroni–Dunn test; $P > 0.05$).

Other measures that define the progression to status—Progression to status is not a single, homogeneous event. It is likely that there are many steps and control points that result in the first seizures and ultimately seizures that do not terminate (status). To probe the later stages in this progression, we examined the interval between the first stage 5 seizure and the onset of status. No significant differences were observed across experimental groups for

interval between the first stage 5 seizure and status (Fig. 5C; $F = 0.7$; df 5,34; $P = 0.627$). This suggests that the more rapid development from first abnormal behavior to status in ovariectomized rats (as described in the preceding section) resulted from effects that occurred early in the progression to status, rather than in later stages of this process.

Another index of progression to status is whether the first stage 5 seizure is followed by many other individual seizures before status eventually develops, or whether the first stage 5 seizure does not terminate (i.e., it represents the initiation of status itself). The latter occurred most often in ovariectomized rats (30%, 3/10; Fig. 5D). Interestingly, this has never been observed in our laboratory in male rats (unpublished data). However, the small numbers of female rats in which this occurred, in all groups, preclude meaningful statistical analysis.

For those animals that had status, there were no significant differences in the percent of animals within each group that had at least 1 stage 5 seizure (Fig. 6A; χ^2 test, $P = 0.527$) or the number of stage 5 seizures that occurred before status (Fig. 6B; $F = 0.293$; df 5,33; $P = 0.913$).

Pilocarpine-treated rats that did not have status epilepticus—Several of the experimental animals treated with pilocarpine had some seizures that could be classified as stages 1–4, or even had stage 5 seizures, but did not reach status epilepticus. For animals that had status, and those that did not, Fig. 6A compares the percentage of animals within each group that had at least 1 stage 5 seizure. This was not distinct within the animals that had status, as stated in the previous paragraph. For animals that did not have status, ovariectomized animals and sham-ovariectomized animals appeared to exhibit stage 5 seizures less frequently than other groups (Fig. 6A). However, the mean number of stage 5 seizures did not vary significantly (Fig. 6B; ANOVA; $F = 0.985$; df 5,49; $P = 0.437$).

Table 1 shows other data for the animals that did not have status. There were no significant differences in the latency to the first abnormal behavior, the latency to the first stage 5 seizure, or the interval between the latency to the first abnormal behavior and the first stage 5 seizure. It should be noted, however, that very large differences in these parameters would be required for statistical significance, because there were very few stage 5 seizures (usually 0–2) in animals that did not have status.

Discussion

Susceptibility to status

There was a low incidence of pilocarpine-induced status on the morning of estrus, demonstrating an influence of reproductive state on the susceptibility to status. This effect was pronounced in the younger animals and appeared to be attenuated with age.

Why would there be a low incidence of status on the morning of estrus? At this stage of the cycle, both estrogen and progesterone are low in the serum, as is the case in diestrus 2 and ovariectomized rats; but incidence of status clearly was lower at estrus only. These results suggest that serum estrogen and progesterone levels *at the time of pilocarpine injection* are not predictive of the susceptibility to status. A key element may be the recent history of hormonal changes during the 8–10 h prior to pilocarpine administration. Indeed during the day before estrus, the animal has experienced a surge of gonadotropin releasing hormone that could profoundly influence the brain. This may lead to changes in the metabolism of pilocarpine, uptake of pilocarpine, and other potentially relevant pharmacological effects that would influence the response of the animal to pilocarpine treatment on the morning of estrus. Another possibility to consider is that progesterone itself is key. The morning of estrus is unique in this respect, relative to other cycle stages and ovariectomized rats. During the night between proestrus and estrus, progesterone peaks (at approximately 12:00 a.m., when the light/dark

cycle begins at 6:00 a.m.; Freeman, 1975) and then rapidly declines, reaching low levels in the early morning (Freeman, 1975). Although serum levels of progesterone have declined substantially by the morning of estrus, brain levels of this hormone may still remain elevated (Corpechot et al., 1997). Bioactive metabolites of progesterone are likely to be elevated in the brain also, including allopregnanolone. Indeed, evidence to date suggests that the highest levels of brain allopregnanolone in the intact cycling rat are reached at estrus (Paul and Purdy, 1992). Allopregnanolone may be critical because it has been shown to enhance activity at the $GABA_A$ receptor (Kokate et al., 1994; Majewska et al., 1986), and is anticonvulsant in several animal models of epilepsy (Frye and Scalise, 2000; Rhodes and Frye, 2004). Thus, elevated brain allopregnanolone levels on the morning of estrus may be responsible for the low incidence of status that we observed at that time.

Interestingly, previous studies of neuronal excitability in female rats with low levels of progesterone would not necessarily have predicted that estrus was a time of relative protection from status. On the contrary, an increase in excitability was noted following a rapid decline in progesterone (‘‘progesterone withdrawal’’; Reddy et al., 2001). However, in the experiments of Reddy et al. (2001), allopregnanolone levels were clearly decreased when seizure susceptibility was high. Analogous findings were made in another model of progesterone withdrawal that used ovariectomy to reduce progesterone, which would reduce allopregnanolone levels (Moran and Smith, 1998).

The results of the present study are more difficult to reconcile with demonstration of increased excitability in hippocampal slices on the morning of estrus (Scharfman et al., 2003). An explanation for the difference is that increased excitability was studied in vitro, and under these conditions, a compound such as allopregnanolone could be rapidly eluted from the tissue, because the preparation is perfused continually with fresh buffer and excess is continually removed. In contrast, under in vivo conditions, brain allopregnanolone levels may not decline so rapidly. In rats, the 5α-reduced metabolites of progesterone, such as allopregnanolone, accumulate in the brain after progesterone administration (Karavolas et al., 1976), consistent with the hypothesis that local intracerebral concentrations of allopregnanolone and its immediate metabolic precursor (5α-dihydroprogesterone) may remain elevated after circulating progesterone levels have declined.

Incidence of status is influenced by age

Previous studies have demonstrated significant effects of age and body weight on chemoconvulsant sensitivity in female rats (Kliouéva et al., 2001; Pericic et al., 1996). Our results indicate that there is indeed a major interaction between age and reproductive state. Thus, while in young adult animals the differences between stages of the cycle were profound, in older animals, these differences tended to disappear. A reasonable interpretation of these observations is that the incidence of status in response to a fixed dose of pilocarpine may increase with age, superceding the effects of the reproductive hormones.

Why would age be so important? It has been well documented that epilepsy increases with age in man (Hauser et al., 1993), and sensitivity to chemoconvulsants appears to increase with age in female rats (Kliouéva et al., 2001; Pericic et al., 1996). Our results therefore support the previous reports in the literature. One obvious potential factor, that previous studies did address, was body weight, because as rats age, they continue to grow, and tend to accumulate fat (Thomas et al., 2002). However, our results suggest that, specifically with respect to the incidence of status, body weight is not a significant factor.

Age-related changes in hormone levels could be involved. Declining allopregnanolone production is one possibility, since serum and brain allopregnanolone levels have been reported to decline with age in female rats (Genazzani et al., 1988). However, Genazzani et al. (1988)

defined ‘‘old’’ as 16 months, an age much greater than used in the present study. There was no evidence in rats housed under our experimental conditions that progesterone declined over the age range used in the present studies (Fig. 1). However, serum hormone levels were only examined at one time point (in the morning), which might not detect potentially important age-related changes in hormone levels occurring at other times in the day. Moreover, serum levels may not reflect brain neurosteroid concentrations (Corpechot et al., 1997). The influence of the adrenal gland may be important to consider, given that the adrenal gland produces neuroactive steroids that can increase excitability, such as corticosterone (albeit not at all concentrations), and corticosterone production appears to increase throughout adult life of the female rat (de Almeida et al., 1998; Sencar-Cupovic and Milkovic, 1976).

Mortality after status

In contrast to previous studies suggesting that reproductive hormones are protective after pilocarpine-induced status (Valente et al., 2002), the results presented here did not show that mortality was significantly different across experimental groups. This may be due to the administration of diazepam in the present study at 1 h after the onset of status, which decreases the severity of status. Valente et al. (2002) did not use an anticonvulsant after the onset of status epilepticus, which would be expected to increase mortality.

Other studies have shown that estradiol is protective after seizures, but the ‘‘protection’’ has focused on seizure-induced neuronal damage rather than mortality (Azcoitia et al., 1998; Hoffman et al., 2003; Veliskova et al., 2000), and the experimental groups usually included ovariectomized rats and hormone-replaced ovariectomized rats, which makes comparison to the current study difficult.

Progression to status epilepticus

Ovariectomized rats had a shorter latency to the first stage 5 seizure, as well as a shorter latency to status. This was only observed after taking into account their increased latency to the first abnormal behavior in response to pilocarpine administration. Presumably, this initial period was slow because the injection of pilocarpine in the peritoneal cavity was influenced by the increased amount of fat in that compartment in ovariectomized rats. Pilocarpine absorption may have been delayed as a result. Consistent with this interpretation, Valente et al. (2002) also found that there was a shorter latency to status in ovariectomized rats relative to intact estrous rats. Their ovariectomized rats were examined only 4 days after surgery, and therefore the ovariectomized animals had not yet gained much weight. Thus, in their studies, simply measuring the latency to status from the time of pilocarpine administration was sufficient to show a distinctly faster latency in ovariectomized rats. Taken together, the studies strongly suggest that ovariectomized rats have a rapid progression to status epilepticus.

An important caveat is that ovariectomized rats, because they were significantly heavier than their intact counterparts, might have received a higher effective pilocarpine dose in relation to lean body mass—resulting in an initially delayed, but ultimately more rapid rise in circulating pilocarpine concentrations. We are inclined to believe, however, that this is not the explanation, for one important reason. If circulating pilocarpine concentrations had risen more rapidly in ovariectomized rats because these animals received a higher effective dose of drug, it is reasonable to suppose that this would have also resulted in a higher frequency of status epilepticus relative to intact rats or sham-operated rats. This was not the case, except for the group of animals that were tested on the morning of estrus.

Why do ovariectomized rats progress more rapidly to status epilepticus? This is difficult to explain because the precise mechanisms underlying progression to pilocarpine-induced status are not well understood at the present time. It is assumed that pilocarpine first activates certain

brain areas with dense muscarinic receptors. Sufficient activation then is thought to activate target structures in limbic regions, which ultimately cause reverberatory seizure activity in non-cholinergic pathways (i.e., glutamatergic circuits). Status is likely to begin at the time when mechanisms for the termination of individual seizures are inhibited, exhausted, or malfunction. Given this conceptualization, a concerted lack of estradiol and progesterone could facilitate the process leading to status in several ways. One mechanism would be the decrease in GABAergic inhibition caused by a loss of estradiol and progesterone, which would lead to enhanced seizure propagation given that GABAergic inhibition serves as a brake on many, if not all, relevant glutamatergic pathways. There are several studies that have shown that both estradiol and progesterone maintain GABAergic function: (1) estradiol increases synthesis of the enzyme responsible for GABA biosynthesis, glutamic acid decarboxylase (GAD), in the hippocampus (Weiland, 1992), (2) estradiol maintains many processes and proteins that are important to GABAergic function, such as K+/Cl− cotransporters (Nakamura et al., 2005), and (3) progesterone, via allopregnanolone, normally enhances activity at $GABA_A$ receptors (Kokate et al., 1994). In a study of seizures initiated by cyclosporin-A (Tominaga et al., 2001), estradiol replacement to ovariectomized rats reversed the effects observed after ovariectomy, consistent with the hypothesis that estradiol may play a critical role in maintaining GABAergic inhibitory tone.

Although the above arguments provide a reasonable explanation for disinhibition in the ovariectomized rat, they do not fully explain the data. This enigma remains: if ovariectomized rats have deficits in GABAergic inhibition, why was their incidence of status unremarkable? One possibility is that ovariectomy may result in *concurrent* diminution of excitatory as well as inhibitory function. Thus, an ovariectomized rat not only has deficits in inhibitory function, but the loss of ovarian hormones also decreases spine synapses (MacLusky et al., 2005; Woolley and McEwen, 1992), thought to mediate much of the glutamatergic afferent input in limbic circuits. By weakening recurrent excitatory circuitry, seizures may not readily become continuous. Another potentially important factor is the decline in muscarinic M1 receptors after ovariectomy (Vaucher et al., 2002), which would limit actions of pilocarpine, because pilocarpine-induced seizures appear to be mediated by M1 receptor activation (Hamilton et al., 1999; Maslanski et al., 1994). Thus, the initial stages of the progression to status may occur rapidly in ovariectomized rats due to disinhibition. However, pilocarpine may be less effective at inducing status in ovariectomized rats because the reverberatory circuits involved in continuous seizures, and the muscarinic receptors necessary to respond to pilocarpine, may both be diminished in the absence of the ovarian hormones.

Another question is why rats tested on diestrus 2, a time when estradiol and progesterone are low, do not have a similar rapid progression to status as ovariectomized rats? At first glance, both have low levels of ovarian hormones at the time of pilocarpine administration. The most likely explanation is that *acute* reduction in reproductive steroids on diestrus 2 is quite distinct in its effects, relative to chronic reduction in the ovariectomized rat. The importance of the *chronic* loss of estradiol is supported by studies showing that GAD levels decline progressively as the time after ovariectomy increases beyond several days (Nakamura et al., 2005). Thus, the ovariectomized rat is likely to have less GAD than the diestrus 2 rat, with a greater reduction in GABAergic function as a result. Indeed, this may explain a major difference in the studies of Valente et al. (2002) and the present study: Valente et al. did not find a distinct incidence of status in ovariectomized rats and rats tested on estrus, yet we did. The reason? Valente et al. tested their animals just 4 days after ovariectomy, whereas our ovariectomized rats were tested at least 15 days after ovariectomy. Our animals may have had a considerably lower level of GAD than the animals used by Valente et al. (2002), given the data from Nakamura et al. (2005), and this would be likely to facilitate status.

Status epilepticus in ovariectomized rats

The results of this study demonstrate, as indicated above, that ovariectomized rats are distinct from estrous rats in their incidence of status. These findings are consistent with the results of previous studies that have also suggested that animals at estrus are less susceptible to chemoconvulsant-induced seizures (Frye and Bayon, 1998; Tan and Tan, 2001). This does not reflect a generalized protective effect of the ovarian steroids, because animals that were tested at stages other than estrus did not show differences in the incidence of status from ovariectomized rats.

The similarities and differences to the studies of ovariectomized and estrous rats of Valente et al. (2002), some of which are indicated above, are enlightening. As suggested above, a major factor could be the delay after ovariectomy. In contrast, it is unlikely that other methodological differences are as critical. Thus, Valente et al. (2002) actually used a lower dose of pilocarpine (320 mg/ kg) than that used here (350 mg/kg). Although Valente et al. (2002) did not specify the ages of their animals, the body weight range cited (200–250 g) lies within the range of the youngest animals examined in the present experiments (65–75 days).

The length of time after ovariectomy before convulsants are tested is important for several reasons. Ovariectomy does not immediately terminate responses to the hormones present at the time of surgery (Clark et al., 1981); there are potentially lasting effects of the surgery itself; and, moreover, it may be many days after ovariectomy before sex steroid concentrations fall to stable low levels. In addition, as mentioned above, the time after ovariectomy could also influence seizure susceptibility because GABA levels decline during the week after ovariectomy (Nakamura et al., 2005), and the brain and periphery adjust in other ways to the dramatic loss of steroids. Stress is also known to alter the expression in the brain of a number of factors involved in the regulation of neuronal activity (Avishai-Eliner et al., 2002; Cavus and Duman, 2003). In the study by Valente et al. (2002), the relative protective effect of estrus against pilocarpine-induced status epilepticus could have been subsumed by factors related to the surgical stress that the animals experienced only 4 days previously.

Conclusions

These data show that the incidence of status in female rats following pilocarpine injection, and the progression to pilocarpine-induced status, are influenced by reproductive state as well as age. The hormonal milieu present specifically on the morning of estrus appears to confer a protective effect against pilocarpine-induced status. This effect is most powerful in young adult animals (~ 65–130 days of age). Given the anticonvulsant nature of allopregnanolone and the likelihood that the levels of this neurosteroid are elevated in the brain on the morning of estrus, we suggest that susceptibility to status in the estrous female may be reduced as a result of allopregnanolone-mediated enhancement of GABAergic inhibitory neurotransmission.

In contrast to the susceptibility to status, the progression to status seems to be most affected by chronic steroid deprivation, i.e., in ovariectomized rats. The reason for this may lie in the chronic absence of both estradiol and progesterone, which leads to diminished GABAergic inhibitory function, decreased muscarinic receptors, as well as a loss of spine synapses in limbic structures that are involved in status epilepticus.

Additional experiments will be required to establish whether the proposed mechanisms are correct. Nevertheless, this study demonstrates the utility of a controlled comparison of female rats under distinct endocrine conditions to evaluate the effects of the chemoconvulsant pilocarpine. The striking effect of estrus in reducing the incidence of status, the pronounced effect of age (but not weight), and the unique effect of chronic vs. acute reduction of estrogen

and progesterone underscore the utility in using the pilocarpine model to allow greater insight into the influence of estrogen and progesterone on seizures, seizure progression, and status.

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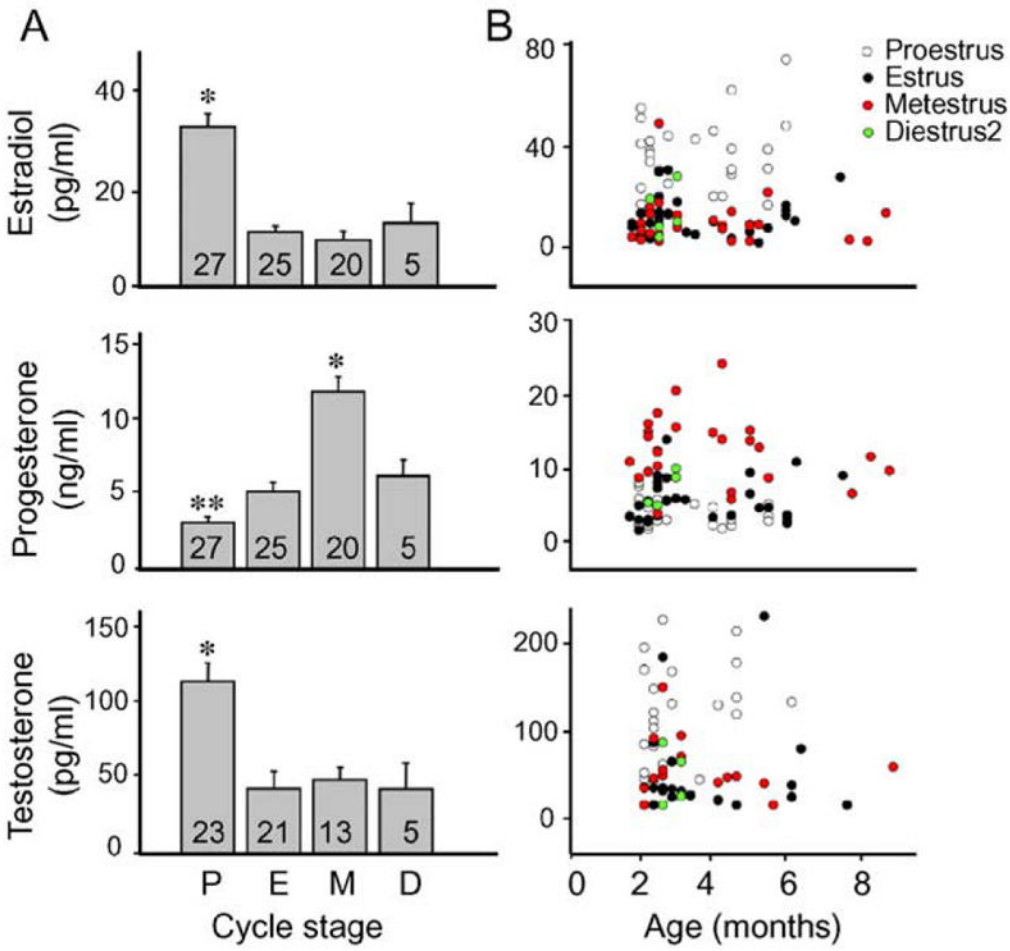

Fig. 1.

Radioimmunoassay of serum hormone levels at different cycle stages and their lack of correlation with age. (A) Mean ± SEM are shown for animals euthanized on the morning of proestrus (P), estrus (E), metestrus (M), or the second day of diestrus (D). TOP: serum estradiol; CENTER: serum progesterone; BOTTOM: serum testosterone. None of these animals received pilocarpine. The sample size is denoted by the numeral at the base of each vertical bar. Statistical analysis: one-way ANOVA; estradiol, $F = 28.5$; df 3,86; $P < 0.0001$; progesterone, $F = 29.7$; df 3,83; $P < 0.0001$; testosterone, $F = 9.1$; df 3,63; $P < 0.0001$. The single asterisk indicates significantly higher mean value relative to all other cycle stages; the double asterisk reflects a significantly lower mean value relative to other cycle stages (Bonferroni–Dunn test, $P < 0.05$). (B) For the data presented in panel A, individual values are plotted as a function of the age of the animal at the time of blood collection. There was no statistically significant correlation between age and either estradiol ($r = 0.006$; $P = 0.956$); progesterone ($r = 0.020$; $P = 0.857$); or testosterone ($r = 0.028$; $P = 0.819$) concentrations. The color of each symbol indicates the day of the estrous cycle when animals were euthanized for trunk blood collection and subsequent RIA analysis of hormone levels (proestrus—clear; estrus—black; metestrus—red; diestrus 2—green).

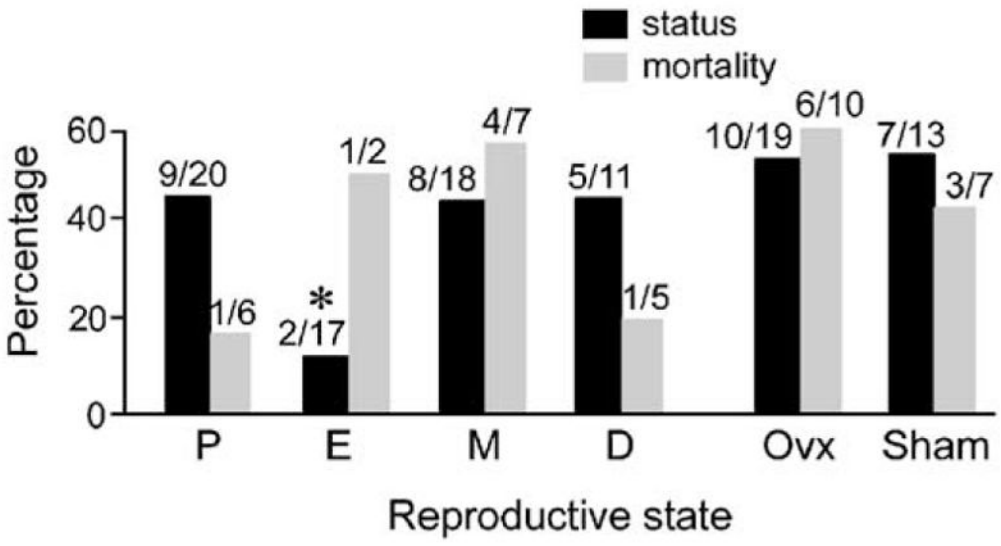

Fig. 2.

Incidence of status epilepticus after pilocarpine administration and subsequent mortality. Black bars indicate the percentage of animals that reached status epilepticus for each experimental group (proestrus, P; estrus, E; metestrus, M; the second day of diestrus, D; ovariectomized rats, Ovx; sham-ovariectomized rats, Sham). Values used to calculate percentages are provided above each bar. Asterisk indicates statistical significance of the incidence of status at estrus relative to proestrus and Ovx rats (Fisher's Exact Test, $P < 0.05$). Gray bars reflect mortality, as defined by the percentage of animals that died of respiratory arrest during status out of the total number of animals that had status. There was no statistical difference in mortality (χ^2 test, $P = 0.472$).

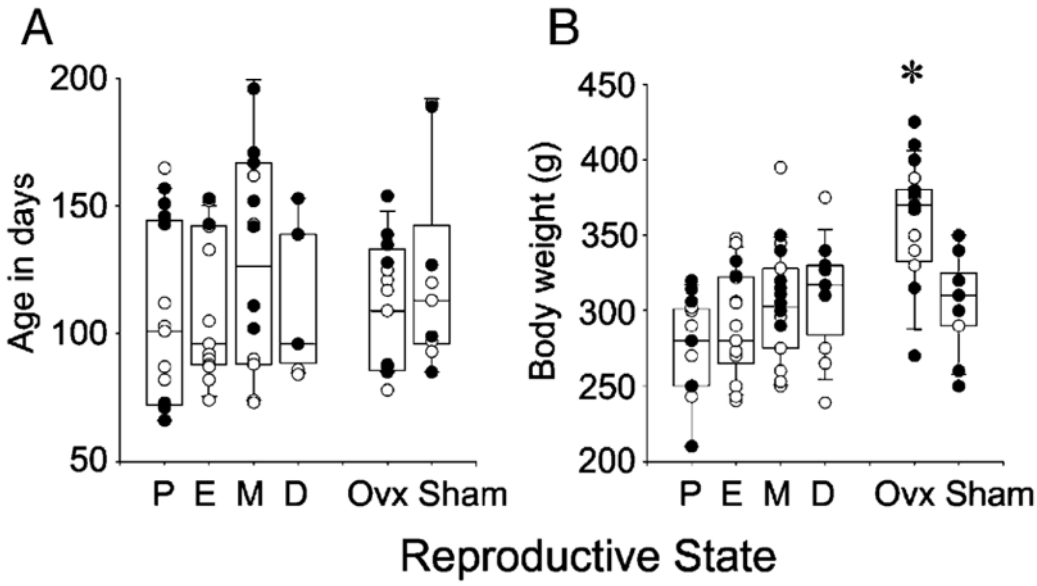

Fig. 3.

Age and body weight of experimental subjects treated with pilocarpine. (A) The age of each animal tested with pilocarpine is shown as a function of their reproductive state (proestrus, P; estrus, E; metestrus, M; the second day of diestrus, D; ovariectomized, Ovx; sham-ovariectomized, Sham). Each animal is indicated by an individual symbol. Animals who had status are shown by dark circles and animals who did not reach status are denoted by open circles. The length of each box reflects the 25th and 75th percentiles. Horizontal lines in the boxes indicate the median. Bars extending outside the box indicate the largest and smallest values that are not outliers. Outliers are values that are 1.5–3 box-lengths from the 25th or 75th percentiles. (B) The body weights of the animals are shown for each reproductive state. Ovx rats had a statistically greater mean body weight (asterisk; Bonferroni–Dunn test vs. sham-operated animals and intact females at different stages of the cycle, $P < 0.05$).

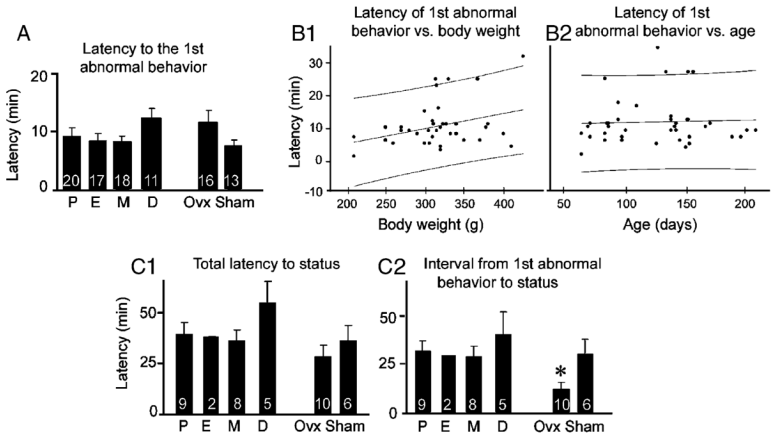

Fig. 4.

Latency to status epilepticus and the contribution from body weight. (A) The latency to the first abnormal behavior observed after pilocarpine administration is shown. All animals that were administered pilocarpine are included. The sample size is indicated at the base of each bar. Although the mean latencies appeared to be longer for the diestrous and Ovx groups, these differences were not statistically significant (one-way ANOVA; $F = 2.06$; df 5,90; $P = 0.078$). (B) Latency to the first abnormal behavior is plotted as a function of body weight (B1) or age (B2) for animals that eventually reached status epilepticus. The latency correlated with body weight significantly ($r = 0.336$; $P = 0.0.032$), but there was no significant correlation with age ($r = 0.042$; $P = 0.793$). Lines indicate the best fit least-squares regression and 95% confidence limits of the data. (C) The latency to status is illustrated, measured either from the time of pilocarpine administration (total latency, C1) or from the time of the first abnormal behavior (C2). The same experimental animals used in panel B are used for this graph. As shown in C2, Ovx rats had a significantly shorter latency to status than sham-operated animals (one-way ANOVA; $F = 2.83$; df 5,48; $P = 0.026$; Ovx vs. Sham, Bonferroni–Dunn test, $P < 0.05$).

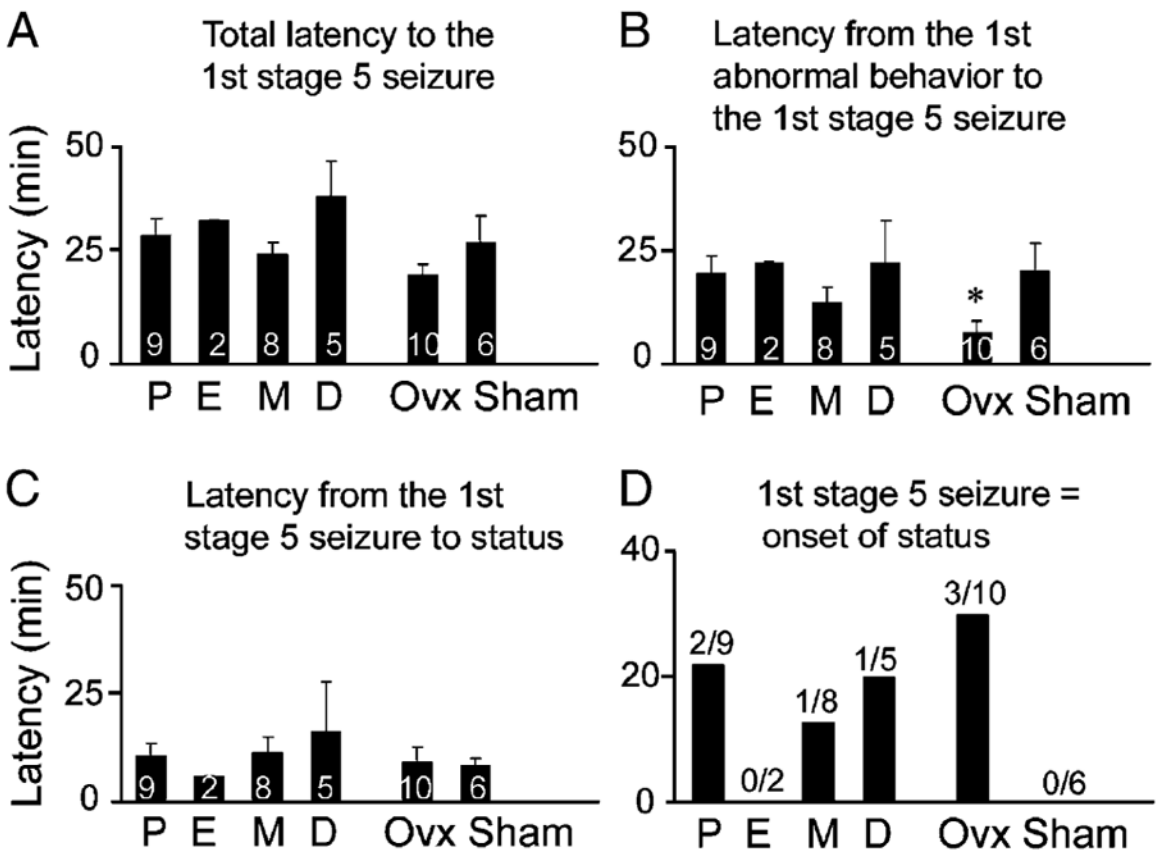

Fig. 5.

Latency to stage 5 seizures induced by pilocarpine in rats that had status epilepticus. (A) The latency to the first stage 5 seizure is shown for all animals that had status epilepticus. This measurement was made from the time of pilocarpine administration to the first stage 5 seizure. Numbers at the base of each bar indicate sample size (one-way ANOVA; $F = 1.323$; df 5,34; $P = 0.278$). (B) The latency to the first stage 5 seizure is shown again, but in this case the measurement was made from the time of the first abnormal behavior to the first stage 5 seizure. The ovariectomized rats had a shorter latency to the first stage 5 seizure relative to other experimental groups (one-way ANOVA; $F = 3.98$; df 5,34; $P = 0.006$; Asterisk: Ovx vs. Sham, Bonferroni–Dunn test, $P < 0.05$). (C) The latency from the first stage 5 seizure to the onset of status is illustrated. There were no significant differences across experimental groups (one-way ANOVA; $F = 0.770$; df 5,34; $P = 0.578$). (D) The percentage of animals that had a stage 5 seizure as the onset of status (i.e., the first stage 5 seizure did not terminate) is shown. The numbers of animals represented by the percentages are shown above each bar. Differences between the groups were not statistically significant (χ^2 test, $P = 0.701$).

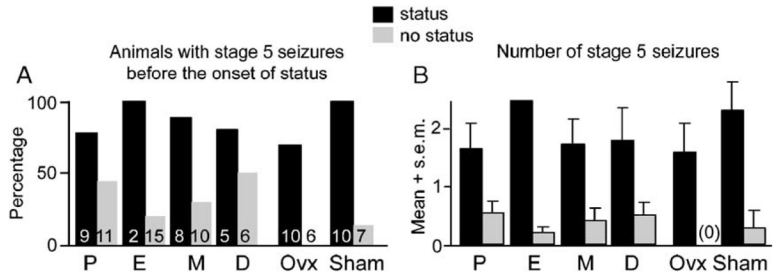

Fig. 6.

Incidence of stage 5 seizures after pilocarpine administration. (A) For animals that had status (black bars) and those that did not (gray bars), the percentage of animals that demonstrated at least 1 stage 5 seizure is shown. At the base of each bar is the sample size. Thus, of 9 animals that were administered pilocarpine on proestrus and had status, 7/9 (88%) had at least 1 stage 5 seizure prior to the onset of status. Of the 11 proestrous rats that did not have status, 5/11 (45%) had at least 1 stage 5 seizure. There were no significant reproductive state-dependent differences in these percentages for either the animals that had status (χ^2 test, $P = 0.527$) or animals that did not have status (χ^2 test, $P = 0.215$). However, overall, a higher percentage of the animals that had status demonstrated at least 1 stage 5 seizure relative to the animals that never reached status (status, 35/75 or 45% vs. non-status, 15/70 or 27%; Fisher's Exact Test, $P = 0.019$). (B) The mean number of stage 5 seizures for each experimental group is shown. The sample sizes are the same as in panel A. There were no significant differences in the mean for those animals that had status (one-way ANOVA; $F = 0.293$; *df* 5,33; $P = 0.913$). Animals that did not have status also did not show an influence of reproductive state (one-way ANOVA; $F = 0.985$; *df* 5,49; $P = 0.437$). However, animals that had status had a significantly higher mean number of stage 5 seizures than animals without status (status, 1.82 ± 0.20, $n = 40$; non-status, 0.33 ± 0.078, $n = 55$; *t* test assuming unequal variance, $P < 0.0001$).

Table 1

Seizures in animals treated with pilocarpine but without status epilepticus

	Number of animals with >1 stage 3–4 seizure	Number of animals with ≥1 stage 5 seizure	Latency to 1st abnormal behavior	Latency to 1st stage 5 seizure	Interval from 1st abnormal behavior 1st stage 5 seizure
Proestrus					
%	36	45	8.64 ± 0.97	32.60 ± 4.19	23.40 ± 3.66
n	4/11	5/11	11	5	5
Estrus					
%	27	20	7.60 ± 0.62	38.00 ± 8.33	33.00 ± 7.64
n	4/15	3/15	15	3	3
Metestrus					
%	30	30	5.60 ± 0.67	29.00 ± 2.64	21.67 ± 3.18
n	3/10	3/10	10	3	3
Diestrus 2					
%	33	50	6.33 ± 0.71	42.67 ± 12.02	37.33 ± 12.25
n	2/6	3/6	6	3	3
Ovariectomized					
%	14	14	8.71 ± 1.16	29.0	21.0
n	1/7	1/7	7	1	1
Sham-ovariectomized					
%	0	0	9.33 ± 1.38	(none)	(none)
n	0/6	0/6	6		

Latency values are presented as means ± SEM of the indicated number of observations. No significant differences were observed between animals at each of the different reproductive states in terms of latency to first abnormal behavior, latency to 1st stage 5 seizure, or the interval between the first abnormal behavior and the first stage 5 (one-way ANOVA; $P > 0.1$ in each case). No significant differences were observed between groups in the frequencies of either stage 3–4 (χ^2 test, $P = 0.45$) or stage 5 (χ^2 test, $P = 0.15$) seizures.