DISSERTATION

STATUS EPILEPTICUS, RECURRENT SEIZURES, HIPPOCAMPAL DAMAGE AND THE ESTROUS CYCLE IN A MODEL OF TEMPORAL LOBE EPILEPSY

Submitted by

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ABSTRACT OF DISSERTATION

STATUS EPILEPTICUS, RECURRENT SEIZURES, HIPPOCAMPAL DAMAGE AND THE ESTROUS CYCLE IN A MODEL OF TEMPORAL LOBE EPILEPSY

Temporal lobe epilepsy is the most common form of epilepsy and is associated with hippocampal sclerosis and spontaneous recurrent seizures. These pathologies generally develop after a latent period from a precipitating brain injury, which often results in status epilepticus (SE). SE is a neurological emergency that is not only associated with an increased risk of developing temporal lobe epilepsy, but also significant mortality (Chin et al., 2004; Vignatelli et al., 2005; Logroscino et al., 2005; Koubeissi and Alshekhlee, 2007). Sex and hormones have been reported to influence SE and mortality in both clinical and experimental settings, although results have not been consistent.

Temporal lobe epilepsy is also associated with an increase in reproductive disorders. Polycystic ovary syndrome and hypogonadotropic hypogonadism are the two most common reproductive disorders in people with epilepsy and are often the result of altered pulsatile release of luteinizing hormone (LH). Gonadotropin-releasing hormone (GnRH) controls LH release; therefore, reproductive abnormalities associated with epilepsy could hypothetically involve hypothalamic disturbances, particularly to the GnRH network, resulting in altered secretion of GnRH. A multiplicity of neuronal and endocrine interactions

presumably results in the complex and reciprocal relationship between epilepsy and the reproductive system. The aim of this dissertation was to 1) to examine the effects of SE and/or temporal lobe epilepsy on the GnRH network using a GnRH-eGFP mouse and 2) utilize recordings of electroencephalogram (EEG) activity to systematically quantify sex and hormone influences on SE and the subsequent recurrent seizures.

Utilizing a GnRH-eGFP transgenic mouse, I studied the acute effects of pilocarpine-induced SE, the chronic effects of temporal lobe epilepsy and spontaneous seizures on hippocampal damage and the number of GnRH-positive neurons. Adult female mice were given systemic injections of pilocarpine to induce SE, and then diazepam was given 90 min after the first seizure. Control mice received all drugs except pilocarpine. The mice were euthanized either 1 week (acute SE) or 3 months (chronic temporal lobe epilepsy) after pilocarpine-induced SE. Hippocampal damage was evident at both time points. There was a significant reduction in the number of hippocampal pyramidal neurons in the CA1 region at 1 week (wk) and a reduction in the CA1 and CA3 regions at 3 months (mo). After SE, the estrous cycle was disrupted in all mice. Neither the acute (i.e., 1 wk post-SE) nor the chronic treatment group (i.e., 3 mo post-SE) had a significant change in total or regional numbers of GnRH-immunopositive neurons. These data do not support the hypothesis that SE and/or temporal lobe epilepsy results in a reduction in the number of GnRH neurons.

For the second aim, I utilized pilocarpine to induce SE in males, ovariectomized females (ovx), and naturally cycling female rats that were implanted with EEG recording electrodes to test the hypothesis that sex and estrous cycle stage influence EEG parameters of SE, the resulting recurrent seizures and neuronal loss in the hippocampus. Controls animals were given all drugs except pilocarpine. Rats were euthanized 24 h or 3 wk after pilocarpine-induced SE. I report no significant differences in the electrographic frequency of spikes during seizures before the onset of SE, latency from first pilocarpine-induced seizure to continuous ictal spiking or duration of SE between treatment groups. There was also no significant difference between groups in the latency until first recurrent seizure, or the average daily number of seizures. The average frequency of the electrographic spikes was 11.4 ± 0.3 Hz and was not significantly different between groups. The average duration of electrographic seizures was 28.7 ± 0.7 s and was not significantly different between groups. Hippocampal damage was quantified using cresyl violet and Fluoro-Jade staining. Based on cresyl violet staining, proestrus-treated rats had significantly less hippocampal neuronal loss (6% ± 0.03) than diestrus- (32% ± 0.05) and metestrus-treated rats (25% ± 0.05) 24 h after pilocarpine-induced SE. Males (12% ± 0.01), ovx (0.13% ± 0.05) and estrus-treated rats (0.15 ± 0.06) all had similar neuronal loss that was not significantly different from the other groups. Fluoro-Jade staining was significant from controls but there were no statistical differences between treatment groups 24 hr after pilocarpine-induced SE. The average number of Fluoro-Jade-positive neurons in each region of the

hippocampus was 6.8 ± 0.5 in the dorsal CA1, 5.6 ± 0.6 in the dorsal CA3, 4.4 ± 0.2 in the upper-blade of the dentate gyrus, 7.0 ± 0.2 in the hilus, 11.1 ± 0.3 in the ventral CA1, 5.2 ± 0.1 in the ventral CA3. In the 3 wk group, there was significant neuronal loss in all areas of the hippocampus. There were no significant differences between any female treatment groups (21-40% neuronal loss) while males had significantly less neuronal loss in dorsal CA1 (than ovx, estrus-and diestrus-treated rats) and ventral CA1 (than ovx and estrus-treated rats) with an overall reduction of 11% ±0.04. Fluoro-Jade staining was also significantly different from controls in all regions of the hippocampus 3 wk after pilocarpine-induced SE, but was not significantly different between treatment groups. The average number of Fluoro-Jade neurons in each region of the hippocampus was 14.5 ±0.6 in the dorsal CA1, 10.0 ± 0.6 in the dorsal CA3, 0.5 ± 0.1 in the upper-blade of the dentate gyrus, 7.6 ± 0.3 in the hilus, 16.2 ± 0.8 in the ventral CA1 and 9.0 ± 0.3 in the ventral CA3. Neuronal damage was progressive and significantly different at 24 h versus 3 wk after pilocarpine-induced SE. Neurons in the hippocampus were significantly decreased in each region of the hippocampus (average of 30% loss, overall). There were also significantly more degenerating neurons (Fluoro-Jade staining) at 3 wk than at 24 hr (average increase of 139%). After SE, the estrus cycle was disrupted in all rats, with the days spent in the following cycle stages changed by the indicated amount: metestrus (+ 24%), abnormal cytology (+13%), estrus (-18%), proestrus (+19%), while the amount of time in diestrus did not change (25%). Electrographic seizures were also analyzed in intact females according to the

stage of the estrous cycle. The normalized, average number of electrographic seizures was not significantly different across the days of the estrous cycle. The average frequency of spikes during electrographic seizures was not significantly different. The average duration of electrographic seizures was not significantly different. When analyzed individually, 15.8% of the female rats had a significant increase in the seizure spike frequency and duration, indicating that a certain portion of rats (similar to human data) experience cycle-influenced seizures (catamenial epilepsy). These data do not support the hypothesis that sex/cycle stage influences SE, or the progression to temporal lobe epilepsy. However, this model of SE/temporal lobe epilepsy will be useful to further study temporal lobe epilepsy-associated reproductive alterations.

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Chapter 1: Introduction

1. Temporal Lobe epilepsy

1.1 Human Condition

Temporal lobe epilepsy occurs in 1% of the world's population and is the most common type of epilepsy in humans (Engel, 1989; Shin and McNamara, 1994; Shneker and Fountain, 2003). Temporal lobe epilepsy is associated with spontaneous recurrent and intractable seizures following a latent period from the initial injury or insult, which often results in a prolonged period of seizures (SE) (French et al., 1993; Engel, 1989). Status epilepticus and the chronic seizure activity that occurs with temporal lobe epilepsy both cause damage to the temporal lobes. This includes gliosis and neuronal loss in the hippocampus (mostly in the hilus, CA1 and CA3 regions) and amygdala, and mossy fiber sprouting in the dentate gyrus. These resulting pathologies also have been associated with altered synaptic properties in temporal lobe structures (Tauck and Nadler, 1985; Tuunanen et al., 1996; Buckmaster and Dudek, 1997; Kobayashi and Buckmaster, 2003); including increased recurrent excitatory circuits in the hippocampus (Tauck and Nadler, 1985), loss of GABAergic cells in the amygdala (Tuunanen et al., 1996), and reduced inhibition of dentate granule cells (Buckmaster and Dudek, 1997; Kobayashi and Buckmaster, 2003). These

and other changes in temporal structures are hypothesized to lead to a decrease in seizure threshold and an increased likelihood to generate epileptiform activity.

1.2. Animal Models

Epilepsy is generally divided into genetic versus acquired forms, where the latter involves the development of spontaneous seizures after neuronal injury. Many animal models are available for both of these general types of epilepsy, but most animal-based research is focused on injury-induced epilepsy, particularly temporal lobe epilepsy. Animal models for this disorder are probably best grouped as (1) the "kindling" model and (2) those models that result from chemoconvulsant-induced SE. All of the animal models have their strengths and weaknesses regarding how well they simulate the human epileptic condition (Engel, 1989), but they may be useful for answering questions about how chronic seizures lead to alterations in the neuronal networks responsible for hormone secretion.

Rodent models of epilepsy have been extensively, and almost exclusively, produced in rats (i.e. Turski et al., 1983; Hellier et al., 1998; Rao et al., 2006). Rats display hippocampal sclerosis, mossy fiber sprouting and recurrent seizures after both pilocarpine- and kainic acid-induced SE. The recent emergence of various transgenic mouse lines that allow for the examination of particular genes in the pathogenesis of epilepsy, *in vivo* function, and potential therapeutic treatments has produced a desire to develop models of epilepsy in mice. However, the development of mouse models of epilepsy has been hampered by the resistance of key mouse strains, particularly the C57BL/6 mouse, which is

extensively used as the background strain for gene targeting experiments, is resistant to kainic acid (Ferraro et al., 1995; Schauwecker and Steward, 1997; Shibley and Smith, 2002; Borges et al., 2003). While certain mouse strains (129/SvEMS and FVB/N) are resistant to kainic acid-induced SE, switching to the chemoconvulsant pilocarpine can successfully induce SE that produces hippocampal sclerosis, recurrent seizures and mossy fiber sprouting even in resistant strains (C57BL/6 and CD-1 mice) (Shibley and Smith, 2002). However, even the same strain of mouse supplied by different vendors (Jackson vs. Charles River) can display differences in mortality rates with the same does of pilocarpine (Borges et al., 2003). This illustrates the importance of verifying key components (i.e. recurrent seizures, hippocampal sclerosis) of the epilepsy model, regardless of chemoconvulsant, when establishing a model of epilepsy in new strains of mice.

2. Reproductive disorders associated with epilepsy

2.1. Males

Men with epilepsy have a higher incidence of sexual dysfunction relative to the general population. Clinical studies have reported that hyposexuality and reduced potency occur in up to 71% of men with epilepsy (Isojarvi et al., 2004; Morrell and Montouris, 2004). However, more recent, controlled studies have reported sexual dysfunction in only 20% of men with localization-related epilepsy (Herzog et al., 2005). Sexual dysfunction is a complex disorder that can include a wide range of symptoms, such as impotency, erectile dysfunction and reduced

libido (Fenwick et al., 1985, Toone et al., 1983; Toone et al., 1989; Smaldone et al., 2004). In addition, men with temporal lobe epilepsy often have abnormal semen analyses, including reduced motility, reduced sperm count, and abnormal sperm morphology. These abnormalities have been attributed to antiepileptic drug use (Isojarvi et al., 2004; Taneja et al., 1994), but also to epilepsy itself (Taneja et al., 1994). A major underlying cause of sexual dysfunction in men is hypogonadotropic hypogonadism, which is associated with altered gonadotropin release and low-amplitude LH pulses (Crowley et al., 1985). Thus, sexual dysfunction in men with epilepsy may be due to alterations in the secretion of reproductive hormones, which are controlled by the hypothalamus and pituitary.

2.2. Females

Women with epilepsy have a higher incidence of reproductive disorders (33-35% versus 8-14% in the general population (Herzog et al., 2003)). The most common reproductive disorders in women with temporal lobe epilepsy include premature ovarian failure, functional hyperprolactinaemia, polycystic ovary syndrome, and hypogonadotropic hypogonadism. Polycystic ovary syndrome is a form of hyperandrogenic chronic anovulation and affects 4-6% of women in the general population, but 10-25% of women with temporal lobe epilepsy are affected (Herzog et al., 1986). The clinical characteristics of the syndrome include hirsutism, oligomenorrhea, infertility, and in some cases, cystic ovaries. This condition generates abnormal hormone levels that provide positive feedback at the level of the hypothalamus, creating a self-sustaining cycle of dysfunction. Hypogonadotropic hypogonadism is the second-most common

reproductive disorder, which is present in approximately 12% of women with temporal lobe epilepsy versus 1.5% in the general population (Bauer et al., 2002), and causes loss or infrequent menstrual cycles and infertility (Herzog et al., 1986). The cause of the comorbidity is unknown, but the hypothalamic-pituitary-gonadal axis is a potential target.

3. Hypothalamic-pituitary-gonadal axis, GnRH and epilepsy

Reproductive endocrine disorders and infertility often result from disruption of the hypothalamic-pituitary-gonadal axis, and this neuroendocrine system could be adversely affected in people with epilepsy. The reproductive axis consists of the hypothalamus, the pituitary, and the gonads (Figure 1). A central component of this axis is a population of 800-2000 neurons that synthesize the decapeptide, gonadotropin-releasing hormone (GnRH) (Silverman et al., 1994) and form a loose network in and around the basal hypothalamus. Activation of GnRH neurons located in the diagonal band of Broca, organum vasculosum of the lamina terminalis and preoptic area result in the coordinated secretion of GnRH into the hypophysial-portal vasculature (Silverman, 1994). The pulsatile release of GnRH from the median eminence regulates the production and release of the two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary, which in turn controls gonadal steroidogenesis and gametogenesis, respectively (Levine et al., 1982; Everett, 1994) (see figure 1). The amplitude and frequency of the GnRH pulse is responsible for the differential production of LH and FSH; high frequency pulses favor LH release

and low frequency pulses favor FSH release (Wildt et al, 1981). The pulsatile release of GnRH and thus the synchronized activity of the GnRH neuronal network are essential for normal reproduction. Therefore, if seizures and/or epilepsy disrupt this neuroendocrine system, reproductive function could be altered.

3.1. Reproductive function

Normal reproductive function is the result of proper frequency modulation of the pulse generator. LH pulses in the peripheral blood closely match the release of GnRH into the portal vascular system (Levine et al., 1982; Clarke et al., 1982). Measuring concentrations of LH in the peripheral blood is currently the best, although indirect, method for assessing the hypothalamic release of GnRH.

In men, the average LH pulse interval is approximately 92 minutes and FSH is 85 minutes (Veldhuis et al., 1986). LH stimulates the testes to produce testosterone, which in turn provides negative feedback at the level of the hypothalamus and pituitary (Sheckter et al., 1989). FSH and adequate intratesticular testosterone are required for spermatogenises. The correct amplitude and frequency of GnRH release is required for proper development of sperm and secretion of testosterone and hence, normal reproductive function in males (Stuenkel, 1991).

In women, the regulation of GnRH pulses is more complex. During the follicular phase, progesterone is responsible for negative feedback on the GnRH neurons, keeping the pulse frequency low. This stimulates the production of

FSH, which is responsible for follicular development. As the follicle develops, it secretes estradiol, which eventually reaches a threshold level and initiates positive feedback at the level of the anterior hypothalamus. This induces a preovulatory surge of GnRH, with increased frequency and amplitude that stimulates the LH surge and induces ovulation within 24hr (Schwartz, 2000).

In addition to steroid hormone feedback, other influences such as stress (Tilbrook et al., 2000), photoperiod (Gerlach and Aurich, 2000), and nutrition (Wade and Jones, 2004) play a role in modulating pulses of the GnRH system in both males and females.

3.2. The GnRH Pulse

The amplitude and frequency of the GnRH pulse is crucial for reproduction. How the GnRH network synchronizes to produce surge activity as well as the pulsatile release of GnRH remains elusive. This is due to the fact that the GnRH network forms a loose continuum in the basal forebrain rather than a discrete nucleus, which has made the study of this network difficult. The term "GnRH-pulse generator" has been coined and refers to the mechanism by which the GnRH network is synchronized to produce surge and pulse activity. The exact nature and location of the generator remains controversial. Several mechanisms of synchronization have been proposed including both an intrinsic pulse generator and an extrinsic pulse generator.

3.2.1 The intrinsic pulse. The first proposed hypothesis for synchronization is that GnRH neurons are intrinsically pulsatile, and that direct connections can synchronize the neuronal network (Nunemaker et al., 2001,

Wetsel et al., 1992; Martinez de la Escalera et al., 1992; Kuehl-Kovarik et al., 2002). The bulk of this data has been gathered from culture systems where GnRH-eGFP-positive neurons display intrinsic bursting patterns with periods of quiescence (Kuehl-Kovarik et al., 2002) and isolated immortalized GT1 cells can also release GnRH in a pulsatile manner with an interpulse interval similar to *in vivo* data (Martinez de la Escalera et al., 1992). Additionally, GT1-7 cells in culture can become synchronized, and several hypotheses for this mechanism have been tested (Wetsel et al., 1992; Suter et al., 2000; Witkin et al., 1995). Gap junctions have been proposed as a possible mechanism for this because GT1-7 cells in culture are dye-coupled (Wetsel et al., 1992) and have connexin-26, a gap-junction protein. However, neither connexin-32 or -43 proteins have been detected (Matesic et al., 1993) and *in vivo* studies have shown that the rate of coupling is low (1 out of 92), which suggests that gap junctions are not likely the sole mechanism for synchronization (Suter et al., 2000). Intercellular bridges have also been proposed to synchronize GnRH neurons (Witkin et al., 1995). Electron microscopic reconstruction of GnRH neurons revealed that some were connected to each other through syncytia, which would electrically couple the neurons. While this could potentially explain the basis of synchronization, the actual number of neurons that are connected are small (3-15% in monkeys, 2-7% in rats), so again, it is unlikely that this contributes significantly to network interactions (Witkin et al., 1995). In addition, both of these culture systems (GT1 and GnRH-eGFP) may differ from the endogenous, *in vivo* system. Reasons for this include that GT1 cells have been transformed and GNRH-eGFP positive

neuron cultures are made from immature animals which may not reflect the adult properties. Hence, neurons in both of these culture systems may foster alternative strategies for hormone release that do not occur in the natural system. These data provide evidence that GnRH neurons have the capacity to be intrinsically pulsatile and may synchronize hormonal release through direct contact.

3.2.2. The extrinsic pulse. A second proposed mechanism for synchronization is the extrinsic GnRH pulse generator. The exact nature and location of the generator remains controversial, but it is hypothesized that it regulates and coordinates the pulses of the GnRH neurons. There is evidence to suggest that the pulse generator resides in the mediobasal hypothalamus (Knobil, 1980; Mori et al., 1991). Multi-unit activity (MUA) recorded in this area corresponds to the release of GnRH into the portal vascular system (Knobil, 1980). The electrical activity associated with GnRH release is presumed to be the result of activity of the GnRH pulse generator. The MUA consistently precedes each LH pulse, even if the pulse frequency is experimentally altered (Mori et al., 1991). The main criticism for this mechanism coordinating the network is that the mediobasal hypothalamus is a region that has very few GnRH neurons. However, it has been demonstrated that GnRH can be released in an episodic fashion in experimental preparations that contain GnRH neuron terminals in the absence of GnRH neurons (Rasmussen 1993) and the mediobasal hypothalamus may coordinate GnRH release using a similar mechanism.

In addition to the mediobasal hypothalamus, it has been proposed that other areas of the brain that project to hypothalamic regions that contain GnRH neurons including the cerebral cortex, limbic regions (olfactory, hippocampal, septal and amygdala) and the brainstem (Palkovits, 2000; Brown et al., 1994; Jennes, 1987) can modulate GnRH release through local circuitry and the release of neurotransmitters in the vicinity of GnRH neurons. In addition to these afferents, there is also a high degree of intrahypothalamic neuronal connections (Palkovits, 2000). These projections could either regulate GnRH pulse activity directly or indirectly by influencing the activity of interneurons that synapse on GnRH neurons.

Many neurotransmitters have been proposed to regulate GnRH release. The two post prominent neurotransmitters in the brain, GABA and glutamate, have been the most extensively studied. GABAergic synapses and receptors have been identified on GnRH neurons (Leranth et al., 1985, Pape et al., 2001) and GABA has been shown to play a role in both surge and pulse release of LH (Jarry et al., 1988, 1991). Glutamate transporters and NMDAR subunits have been identified on GnRH neurons (Hrabovszky, 2004; Gore et al. 1996, Yin et al., 2007), metabotropic glutamate receptor agonists can activate (Sortino et al., 1996, Kuehl-Kovarik et al., 2002; Chu and Moenter, 2005; Arias et al., 1993) and antagonists can suppress GnRH release (Arslan et al., 1988). There is also strong evidence to suggest a permissive role of noradrenaline in the LH surge (Herbison, 1997). In addition, there is also evidence to support a role of nitric oxide (Calka, 2006), GnRH (Martinez de la Escalera et al., 1992; Feleder et al.,

1996) dopamine (Leblanc et al., 1976; Pehrson et al., 1983; Gore and Terasawa 2001), NPY (Sullivan and Moenter, 2004), kisspeptin (Gottsch et al., 2004; Dungan et al., 2007; Pielecka-fortuna et al., 2007), and serotonin (Kim et al., 2006; Wada et al., 2006). These data indicate that the regulation of the GnRH neuronal network is a complex integration of multiple signals that influence the local circuitry of the GnRH network and cumulate as the central signal for the regulation of fertility.

Seizures have the ability to disrupt the pulse-generator-mechanism. If any mechanism that is responsible for the synchronization of this network is altered, either through direct damage to the GnRH neurons, damage of the projections that regulate the GnRH neurons, or damage to areas in the brain that transmit aberrant electrical activity during seizures through intact projections to GnRH neurons, it is likely that it would disrupt the normal function and synchronization of the network. These alterations could lead to reproductive disorders. Proper frequency modulation of the pulse generator is essential for normal reproductive function. Abnormalities in the pulse generator, resulting in abnormal release of GnRH, are associated in pathological states with endocrine disorders (Filicori et al., 1986; Taylor et al., 1997).

4. Hypothalamic dysfunction and temporal lobe epilepsy

Evidence supporting the hypothesis that seizures and/or epilepsy disrupt hypothalamic function derives from data showing acute, post-ictal changes in hypothalamic peptide secretion. For example, after generalized tonic/clonic

seizures, prolactin is elevated immediately, peaks around 15-20 min and may remain elevated for up to 60 min (Trimble, 1978; Pritchard et al., 1983). Elevations in serum LH (in males and females) and FSH (in women) are also observed for up to 60 min (Dana-Haeri et al.,1983). In addition to the acute changes associated with seizure activity, men and women with temporal lobe epilepsy have chronic changes in LH pulse frequency (Herzog et al., 1990; Drislane et al., 1994; Bilo et al., 1991). While abnormal pulse frequency does not always culminate in reproductive disorders, and pulse frequency may not be altered in every patient with epilepsy (Bilo et al., 1991), it is likely that the disruption in the tightly regulated LH pulse frequency could lead to the development of endocrine disorders and infertility in certain patient populations.

5. Rodent models of chronic epilepsy and reproductive dysfunction

Rodent models have demonstrated the role of seizure activity in reproductive dysfunction and disruption of the GnRH network. Kindling in the basolateral amygdala in female rats resulted in arrested ovarian cyclicity with persistent vaginal cornification, which was associated with polyfollicular ovaries and cystic follicles, high serum estradiol and increased pituitary weight (Edwards et al., 1999). Focal application of kainic acid in the amygdala of rats also decreased GnRH fibers in the ventromedial hypothalamus, a region important in the regulation of reproductive function (Friedman et al., 2002). The density of GnRH immunoreactive fibers was also reduced 60-90 days after pilocarpine-induced SE in rats (Amado et al., 1993). The reduction of GnRH containing

fibers suggests that either a change in the expression of the GnRH peptide or loss of GnRH neurons may underlie the endocrine dysfunction associated with temporal lobe epilepsy. These data provide further support for the hypothesis that epilepsy and the resulting seizure activity disrupts the normal function of the hypothalamus, which may lead to chronic reproductive disorders.

6. Pathogenic mechanisms for epilepsy and GnRH dysfunction

Pathophysiological mechanisms in temporal-limbic structures (i.e. the amygdala and hippocampus) associated with epilepsy may result in the alteration of efferent activity to the hypothalamus or loss of these inputs, which would alter the GnRH system. Therefore, the dysregulation of GnRH pulsatility may be due to a loss of afferents or the propagation of epileptiform activity to GnRH neurons.

6.1. Connections to the hypothalamic GnRH network

The projection of temporal structures to hypothalamic regions may provide a substrate for seizures and/or epilepsy to disrupt the GnRH neuronal network. The amygdala and hippocampus have efferent projections to the hypothalamus, in addition to other areas (Renaud and Hopkins, 1977; Price, 2003). Axonal tracing studies have demonstrated that both of these structures have efferents that directly project to the medial septum, diagonal band of Broca and preoptic areas, regions containing GnRH neurons (Simerly and Swanson, 1986; Gaykema et al., 1991). In animal studies, stimulation and/or lesion of the hippocampus and amygdala can influence the activity of neurons in the hypothalamus and modulate gonadotropin release (Velasco and Taleisnik, 1971;

Kawakami and Terasawa, 1972; Brown-Grant and Raisman, 1972; Carrer et al., 1978). These connections, as well as their influence on the hypothalamic-pituitary-gonadal axis, are illustrated in Figure 1. While the exact nature of these projections and their influence on the hypothalamic-pituitary-gonadal axis has received little systematic investigation, the hippocampus and amygdala via their efferent pathways can modulate hypothalamic peptide secretion. Thus, temporal-limbic structures could directly influence the output of the GnRH neuronal network through either mono- and/or poly-synaptic pathways.

6.2. Role of temporal-limbic afferent activity

6.2.1. Loss of efferent activity. The amygdala and hippocampus undergo structural changes in temporal lobe epilepsy, which is reproduced in a general manner after experimental SE. This includes gliosis and neuronal loss in the hippocampus (mostly in the hilus, CA1 and CA3 regions) and amygdala (Tauck and Nadler, 1985; Tuunanen et al., 1996). Neuronal loss can also occur in both mono- and poly-synaptic connected regions such as the cortex and thalamus (Hopkins et al., 2000; Pitkanen et al., 2002; Nairismagi et al., 2004). These changes often result in synaptic reorganization, which can include a loss of efferents, as is seen in the hippocampus when CA3 pyramidal neuron degeneration leads to deafferentation of CA1 pyramidal neurons (Shetty, 2002). It is therefore reasonable to hypothesize that synaptic reorganization occurs in the hypothalamus due to its connections with limbic regions, and that deafferentation may also occur at the level of the GnRH neuron, which would alter the normal regulation of the GnRH network.

6.2.2. Abnormal efferent activity. As a result of synaptic reorganization, limbic structures such as the amygdala and hippocampus experience increased recurrent excitatory circuits (Tauck and Nadler, 1985), loss of GABAergic neurons (Tuunanen et al., 1996), and reduced synaptic inhibition (Buckmaster and Dudek, 1997; Kobayashi and Buckmaster, 2003). These and other changes in temporal structures are hypothesized to lead to a decrease in seizure threshold and an increased likelihood to generate epileptiform activity. Ictal activity from these structures may propagate to hypothalamic areas through efferent connections, similar to other subcortical structures (e.g., the thalamus) (Norden and Blumenfeld, 2002). In addition, the pathophysiology of epilepsy is not confined to temporal lobe structures (Bertram and Scott, 2000), and it is therefore possible that similar alterations in excitatory and inhibitory circuits occur in hypothalamic regions. Abnormal electrical activity, either through propagation of ictal events from temporal lobe structures, or through hypothesized synaptic reorganization of the hypothalamus, could be responsible for disruption in GnRH pulses.

6.3. Role of global neurotransmitter dysfunction

An alternative explanation for reproductive dysfunction is that the pathological changes due to epilepsy may result in global neurotransmitter dysfunction in the brain. Neurochemical alterations in the opioidergic, catecholaminergic and GABAergic systems have been implicated in SE and temporal lobe epilepsy (Simon et al., 1984; Hong et al., 1988; Tasker and Dudek, 1991; Glass and Dragunow, 1995). These neurotransmitters also modulate the

GnRH neuronal network. In the GnRH system, opioid peptides inhibit while catecholamines stimulate GnRH release (Negro-Vilar et al., 1979; Ojeda et al., 1982; Gore and Terasawa, 2001). The role of GABA is controversial and proposed to be either inhibitory or excitatory to GnRH neurons (DeFazio et al., 2002; Han et al., 2004). Thus, modifications in these neurotransmitter systems due to seizures and/or epilepsy could ultimately influence the coordinated activity of the GnRH neuronal network and disrupt normal reproductive function.

7. Reproductive disorders: antiepileptic drugs vs. epilepsy

7.1 The effect of antiepileptic drugs

In addition to the effects of seizures and epilepsy on the reproductive axis, antiepileptic drugs may affect reproduction, although the evidence remains controversial. Patients used in these studies have epilepsy, and it is therefore difficult to determine whether reproductive disorders are the result of epilepsy or the drugs used to treat it. Although clinical studies are obviously essential, this approach alone does not permit the controlled experimental manipulations that can be performed in animal models, thus allowing for a more direct analysis. Several common antiepileptic drugs (e.g. phenytoin and carbamazapine) induce cyctochrome P450, which stimulates the production of sex hormone-binding globulin and increases the metabolism of adrenal and sex steroid hormones, thus reducing the amount of biologically active hormones in the circulation (Stoffel-Wagner et al., 1998; Morrell et al., 2001; Macphee et al., 1988). Valproate is a common antiepileptic drug that does not induce hepatic enzymes, but has been

implicated in increased incidence of reproductive disorders, especially polycystic ovary syndrome. Valproate use is associated with increased levels of androgens, which can lead to frequent anovulatory cycles and other polycystic ovary syndrome-like phenotypes (Morrell, 2003). Antiepileptic drugs have various differential effects on endocrine function in both males and females (Isojarvi et al., 2005), have the ability to alter peripheral levels of hormones, and may result in reproductive disorders.

7.1.1. Animal studies. Animal models have the potential to resolve the problems with clinical data. Using rats, a polycystic ovary syndrome-like phenotype was induced with high doses of valproate. While increased ovarian weight and ovarian cysts (only at 200 mg/kg dose) were observed, there were no significant changes in estrogen or progesterone levels, and the rats showed reduced testosterone levels, which is unlike polycystic ovary syndrome (TaubLll et al., 1999). It has also been suggested that valproate alters levels of GABA in hypothalamic regions, which is an important neurotransmitter for GnRH neurons (Illig et al., 2000). However, a model using rhesus monkeys, which have similar menstrual cycles to humans, demonstrated that long-term (12-15 months) valproate treatment did not cause alterations in the menstrual cycle or hormone level, or cause cystic ovaries, which are the main characteristics of polycystic ovary syndrome (Ferin et al., 2003). This study used a physiological and therapeutic dose of valproate over a time period that patients would normally be exposed to the drug. Therefore, a controversy exists as to whether valproate

directly induces polycystic ovary syndrome and highlights the need for animal models which closely mimic the multifactorial nature of the human disorder.

7.2. The effect of epilepsy

Independent of the effects of antiepileptic drugs, epilepsy and the associated seizures appear to have an important role in causing reproductive disturbances. Acute seizures and chronic temporal lobe epilepsy have been associated with the development of reproductive disorders. Evidence for the direct role of seizures in reproductive disorders is provided by studies that show the control of seizure activity, either through successful surgery or use of antiepileptic drugs, reduces the incidence of reproductive disorders (Herzog et al., 1986; Bauer et al., 2000), while women who have uncontrolled seizures and are not using antiepileptic drugs have an increase in those disorders (Herzog et al., 1986; Bilo et al., 1991; Meo et al., 1993).

Clinical studies report that certain reproductive disorders are associated with the laterality of the seizure. In women, left-sided discharges have been associated with increased LH pulsatility and polycystic ovary syndrome (Herzog et al., 2003), while left-sided discharges were associated with a decrease in LH pulsatility in men (Herzog et al., 1990). In contrast, women with right-sided discharges had decreased LH pulsatility and hypogonadotropic hypogonadism (Herzog et al., 1993; Herzog et al., 2003), while men had increased LH pulsatility (Herzog et al., 1990). The association of the laterality of the seizure foci and the development of certain reproductive disorders suggests that seizures and the

pathologies of epilepsy directly influence the development of certain reproductive endocrine disorders.

8. Role of sex steroids in epilepsy

An additional factor to complicate this issue is that endocrine disorders also have an effect on temporal lobe epilepsy. People with endocrine disorders are exposed to abnormal fluctuations of hormones. In women, polycystic ovary syndrome has been shown to have a direct impact on temporal lobe epilepsy. Seizures are exacerbated during periods of anovulation and low progesterone (Backstrom, 1976; Herzog et al., 1997). In men, hormonal fluctuations due to reproductive disorders and/or antiepileptic drugs may also increase seizure frequency and cause premature aging of the reproductive system (Herzog, 1991). Therefore, the increase in endocrine disorders associated with temporal lobe epilepsy has an impact on both reproduction and on epilepsy itself. This complex series of relationships illustrates the difficulties in studying all of the facets of temporal lobe epilepsy as they relate to the many types of reproductive abnormalities. This also demonstrates the importance of continuing research in these areas due to their impact on the health and quality of life of people with epilepsy.

8.1 Catamenial epilepsy

Catamenial epilepsy is a common disorder in females with epilepsy that exemplifies the role of steroid hormones in seizure exacerbation. Alterations in seizure frequency in relation to the menstrual cycle were noted as early as

Hippocrates and later by Gowers in 1881 (Rogawski, 2003). The increase in seizure frequency was related to fluctuations in plasma estrogen and progesterone across the menstrual cycle (Backstrom, 1976). Between 20 and 70% of women report an increase of seizure frequency or severity that coincides on or around menses (Klein and Herzog, 1998). The discrepancies in the reported percentages are attributed to the different clinical definitions used for recognizing increases in seizure frequency; some studies have required a six-fold increase around the menses to be considered catamenial epilepsy (Duncan et al., 1993). A conservative, two-fold increase in seizure activity is generally accepted, and includes approximately 39% of women (Herzog, 2004).

Three different classes of catamenial epilepsy have been described (Herzog et al., 1997). These classes account for the different times in the menstrual cycle when an increase in seizure frequency or severity is observed. The first class, C1, refers to a perimenstrual seizure exacerbation that coincides with a withdrawal of progesterone when estrogen levels are high. This is the most common type of catemenial epilepsy, accounting for 70% of women with the disorder. The C2 class refers to periovulation exacerbation (approximately 1-2 weeks prior to menstruation) that coincides with an estrogen surge. The C3 class is when the exacerbation period includes the entire second half of the menstrual cycle due to an inadequate luteal phase.

The mechanisms responsible for the increase in seizure frequency are unknown, but several hypotheses have been developed. The three most common hypotheses are described here. The first accounts for the overall ratio

of estrogen to progesterone. The second hypothesis cites that the estrogen surge is responsible for the increase in seizure frequency. The last reports that the withdrawal of progesterone is responsible (Reddy, 2004). There is evidence to support each theory, as these three situations all occur in at least one of the classes of catamenial epilepsy. This suggests that fluctuating hormone levels influence the expression of seizures.

8.2. Steroid hormones

Steroid hormones have well defined roles in reproduction via classic nuclear receptors. These actions are mediated by long-latency genomic and post-transcriptional mechanisms (McEwen et al., 1979; Coleman and Smith, 2001). There is also evidence that they produce non-classical actions via direct, membrane-mediated events on the central nervous system. In 1962, Woolley and Timiras described the role of female sex hormones on seizure threshold; estradiol had a generally excitatory action on neurons, while progesterone had inhibitory actions. These findings have been corroborated in many additional studies (Kawakami et al., 1970; Buterbaugh, 1986; Marcus and Watson, 1966). Since the level of hormones circulating in the bloodstream fluctuates over the menstrual cycle, and have abnormal fluctuations in endocrine disorders, they may play an important role in seizure threshold in women with temporal lobe epilepsy.

Estrogen is considered proconvulsive based on studies demonstrating that it facilitates the induction of seizures and/or SE in several rodent models (Logothetis and Harner 1960; Buterbaugh 1987; Hom and Buterbaugh, 1986;

Buterbaugh and Hudson, 1991; Edwards et al., 1999; Frye and Rhodes, 2005). Several mechanisms for the excitatory action of estrogen have been identified including rapid effects on the neuronal plasma membrane by increasing the cell's response to glutamate (Smith, 1989; Wong and Moss, 1992; Foy et al., 1999) and long term effects by increasing the number or density of dendritic spines on the cell (Woolley et al., 1997; Murphy et al., 1998). Since estrogen has excitatory actions in the CNS, it has been proposed that the high estrogen levels in the perimenstrual phase in the menstrual cycle is the underlying cause of catamenial epilepsy.

Progesterone has been consistently reported to slow kindling and reduce the onset of behavioral seizures in animal models (Edwards et al., 1999; Frye, 1995; Frye and Bayon, 1998, 1999; Frye and Scalise, 2000; Frye and Muscatiello, 2001; Frye et al., 2000, 2002). Evidence suggests that progesterone receptors are not mediating these antiseizure effects because progesterone's actions are fast (minutes), not blocked by RU486 (Mohammad et al., 1998) 2) and are present in PRKO mice (Reddy et al., 2004). Increasing evidence has suggested that progesterone's anticonvulsant actions are mediated by its conversion to allopregnanolone (Belelli et al., 1989; Kokate et al., 1999; Frye and Bayon, 1998), which has modulatory actions on the GABAA receptor. During the estrus and menstrual cycles, progesterone levels fluctuate within the concentration range that has been shown to potentiate the action of GABA at the GABAA receptor (Finn and Gee 1994).

Testosterone is associated with both increased and decreased seizure susceptibility. This biphasic response has been attributed to the ability of testosterone to be readily metabolized into androgens and estrogen. Androgen metabolites of testosterone have demonstrated anticonvulsant properties (Kaminski et al., 2005), while the estrogen metabolite has demonstrated to be a proconvulsant (Edwards et al., 1999b; Reddy 2004).

Ultimately, there is evidence to support the role of both progesterone and estrogen in catamenial epilepsy. However, most of the data are presented in animal models. While these rodent models seem to answer basic mechanistic questions, the issue becomes how translational is each animal model? There are currently no models of chronic epilepsy displaying increased seizure frequency or severity that coincides with an increase in estrogen, or the withdrawal of progesterone.

8.3. Animal models of catamenial epilepsy

To date, a rodent model has not been established to investigate recurrent seizure frequency and severity as it relates to ovarian cycle. This has largely been due to the observation that most female rats stop cycling normally after chemically- and electrically-induced SE (Amado et al., 1987; Amado and Cavalheiro, 1998; Bhanot and Wilkinson 1982; Edwards et al., 1999, 2000). Instead, studies have been designed to examine how the level(s) of a hormone(s) affects seizure susceptibility (Bujas et al., 1997; Finn and Gee 1994; Kokka et al., 1992; Medina et al., 2001; Pericic and Bujas 1997; Pericic et al., 1986; Pericic et al., 1985; Pericic et al., 1996; Schwartz-Giblin et al., 1989;

Thomas and Yang, 1991; Mejias-Aponte et al., 2002; Reddy et al., 2001). Seizure susceptibility has also been reported to correlate with various stages of the estrous cycle in naturally cycling rodent seizure models, although results have not been consistent. Two possible explanations for this can be the observation that not every cycle stage is examined in each study, and that there are often differences in the time of day the experiments take place. Some studies have indicated that female rodents are more susceptible to seizures during proestrus than estrus (Scharfman et al., 2005, Tan & Tan, 2001) and this has been supported using ovariectomized females treated with hormones to produce levels similar to proestrus and estrus (Edwards et al., 1999b). However, other studies have reported a reduced susceptibility at proestrus (Rhodes and Frye, 2004; Pesce et al., 2000). In studies that have not included females in proestrus, females in estrus have been shown to be more sensitive than females in metestrus or males (Finn & Gee, 1994) or diestrus (Maguire et al., 2005). Males have also been shown to be more sensitive than females (without taking stage into account) and ovariectomized rats more susceptible to status than females in estrus (Tan & Tan, 2001). While overall these data indicate that hormonal state affects the susceptibility to seizure induction, there is a need to develop an animal model that more closely resembles the human condition (i.e. recurrent seizures) to resolve the discrepancies.

It is important to consider the basis for the discrepancies, the most obvious being that not every cycle stage is examined in these studies. It is difficult to draw conclusions regarding how sex and estrous cycle affect

susceptibility using a hormonal snapshot of only a select subset of animals (ovx rats might have similar threshold etc as di/met rats). Another possible explanation for the discrepancies includes the observation that there are often differences in the time of day the experiments take place. Time of day is crucial because the rodent estrous cycle is only approximately 4 days, over which time estrogen and progesterone cycle in a manner that is similar to the pattern observed in women, although on an accelerated time-scale, so that the relative levels of each hormone can be extremely different in the morning versus the afternoon. In addition, these studies have been generally based on behavioral observations. Also, the most frequently reported observation is differences in "latency" defined as the time from the interperitoneal injection of chemoconvulsant to the onset of the first motor seizure (Mejias-Aponte et al., 2002; Scharfman et el., 2005, Tan & Tan 2001; Rhodes and Frye 2004; Pesce et al., 2000; Finn & Gee, 1994). This phenomenon is illustrated in a study where different, and sometimes opposite sex differences to induction of SE were obtained after intravenous versus interperitoneal administration of various chemoconvulsants (Pericic and Bujas, 1997). While these studies have provided evidence of the role of sex, hormones, and estrous cycle on the susceptibility to SE, significant questions remain regarding whether these results are model specific.

Figure 1. Hypothalamic–pituitary–gonadal axis and projections of the temporal lobe.

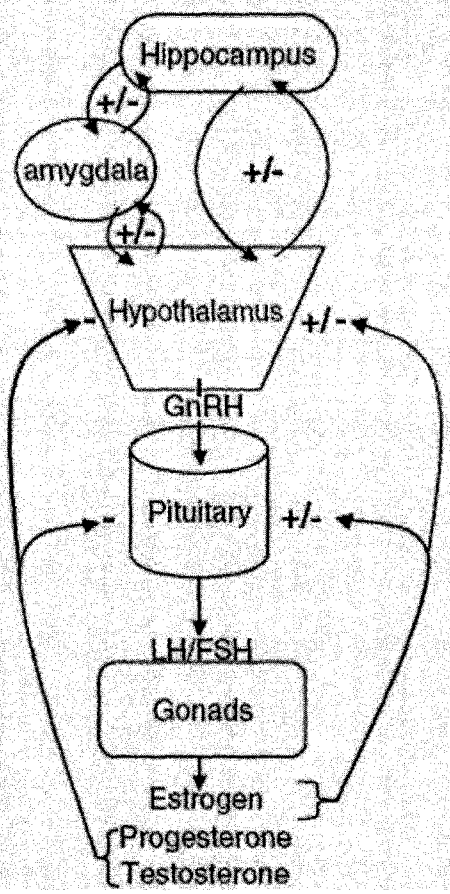

Fig. 1. Hypothalamic–pituitary–gonadal axis and projections of the temporal lobe. The hypothalamic–pituitary–gonadal axis consists of the hypothalamus, the pituitary, and the gonads. The hypothalamus releases gonadotropin-releasing hormone (GnRH) and stimulates the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. In men, LH stimulates the testes to produce testosterone, which, in turn, provides negative feedback at the level of the hypothalamus and pituitary. In women, the differential production of FSH and LH determines the phase of the menstrual cycle and stimulates the production of progesterone and estrogen. Progesterone has a negative feedback effect on the hypothalamus and pituitary, whereas estrogen has both positive and negative feedback effects, dependent on the phase of the menstrual cycle. In addition to the feedback mechanisms of hormones, the hippocampus and amygdala have connections to the hypothalamus and each other. These connections have either a positive or negative influence on each other and the hypothalamic–pituitary–gonadal axis.

Chapter 2: Methods

Animals and Treatment

Adult GnRH-eGFP transgenic female mice (greater than 90 days of age) were obtained from an established breeding colony of GnRH-eGFP transgenic mice located at Colorado State University and the University of Utah. Rats (60 days of age) were obtained from Charles River. Animals were housed under a 12-h light-dark cycle with food and water ad libidum.

Implantation of tethered recording electrodes

Rats were injected with atropine (0.8 ml sc) penicillin (0.2mL, SC), and dexamethasone (0.2 mL, SC) then anesthetized with 2% isofluorane subcutaneously. The surgical sites were clipped and prepped with betadine scrub and solution. A 0.5 inch incision was made medially on the animal's head to expose the skull. Three holes were drilled into the skull into which wire electrodes were placed so that they made contact with the dura. The unit, including the wire electrodes was covered with dental cement to keep it in place and cover the holes in the skull. The skin was sutured shut with non-dissolvable suture around the skull cap.

Ovariectomy

At this time, a portion of the female rats were also ovariectomized. Briefly, the medial dorsal sides of the rat were shaved and bilateral incisions (approx. 0.5 in)

made on each side of the animal's flanks. The ovary was removed from the abdominal cavity using forceps and sutures were used to tie the ovary at the junction with the uterus. The ovary was then cut from the uterus and the uterus was placed back inside of the abdominal cavity. The body wall was closed with dissolvable suture and the skin was closed with wound clips. This procedure was repeated on the opposite side to remove the second ovary.

Induction of SE

Study 1: GnRH-eGFP mice. To counteract peripheral cholinergic effects, mice were injected with methylscopolamine-bromide (1 mg/kg i.p.) 30 min prior to a single injection (280 mg/kg, i.p.) of pilocarpine hydrochloride to induce SE (Turski et al., 1983). Mice were monitored for behavioral-related seizure activity for 2 hrs and seizure activity was scored based on the following scale: stage 3, tail extension paired with forelimb or hind limb clonus; stage 4, rearing with full forelimb clonus; stage 5, rearing and falling (Racine, 1972). SE was defined as a minimum of three, stage 3-5 seizures events during the 2 hrs of monitoring. Diazepam (5 mg/kg, i.p.) was given 90 minutes after the first seizure.

Mice that experienced SE and control mice were randomly assigned to either a 1 wk or a 3 mo treatment group. Both experimental groups were paired with control mice that received methylscopolamine bromide and saline instead of pilocarpine and diazepam at intervals matching the treatment group. Control and pilocarpine treated mice were video-monitored for 24 consecutive hours per week.

Study 2: Rats. Six experimental groups were used: proestrus, estrus, diestrus, metestrus, ovx females and males. To be included in the study, intact female rats had to exhibit three normal, 4-day cycles after surgery. Cycle stage was determined between 7:30 am and 8:30 am on the day of pilocarpine treatment. Rats were randomly assigned to either the acute (24 h) or chronic (3 w) group before treatment. The rats were then placed in a recording chamber equipped with commutators and cables (Plastics One, Roanoke, VA) and the implanted unit was attached to a cable to record EEG activity. Signals are amplified using EEG100C amplifiers, digitized using an MP150 digital-analog converter, and acquired with AcqKnowledge acquisition software (BioPac Systems Inc., Santa Barbara, CA). The rats were also continuously video monitored using eight color cameras linked to a multiplexer to allow for eight animals to be recorded on one DVD. Twenty-four hour recordings were made by setting up three DVD recorders (DMR-ES20, Panasonic) and recording for 8 hr epochs on each recorder.

To induce SE, methylscopolamine (1 mg/kg, i.p.) was given 30 min prior to the pilocarpine injection (450 mg/kg, i.p.) to reduce the peripheral effects of pilocarpine. Pilocarpine was administered at approximately 9 am. Rats without any electrographic seizures were given additional injections of pilocarpine (150 mg/kg) every 30 min until the rat entered SE (maximum of 4 additional doses). In addition to EEG monitoring, behavioral seizures were also scored and recorded based on a modified Racine scale. However, the EEG traces were used to mark the beginning of SE which was defined as the start of continuous ictal spiking

that had a frequency greater than 4 Hz. This time point correlated with the previously used behavioral classification of at least three, stage 3 or higher seizures required for marking the onset of SE. Ninety min after the first stage 3 or higher seizure, diazepam (5 mg/kg, i.p.) was given every 30 min until either EEG spiking activity was < 1 Hz, or they received the maximum of 4 injections. Immediately after the first diazepam injection, the rats were given 3 ml of lactated Ringers and pieces of fresh fruit and food softened with Gatorade, which was replenished daily until the rat was eating and drinking normally. Control rats for each experimental group (n=3 for each, 18 for each treatment group time, 36 overall) were given all drugs except pilocarpine and their diet was also supplemented with Gatorade and fresh fruit for three days. Fisher's exact test was used to determine statistical significance between groups of the incidence of SE and mortality. ANOVA comparisons were used to measure EEG parameters (number/frequency/duration) between groups.

Analysis of the EEG data. The following parameters were used to analyze EEG data: the latency to first seizure after pilocarpine injection, the severity of the status (amplitude/electrographic frequency of spikes/duration), the duration and frequency of individual electrographic events, drug response (number of doses required for termination SE), mortality due to SE (defined as death within 24h after treatment) and mortality associated with complications from SE (rats that died later than 24 hr after treatment). Seizures were defined as spiking activity that lasted longer than 10 sec with a frequency > 4 Hz. Statistical significance was determined by performing one-way ANOVAs (Prism,

Graphpad) of each measured parameter (number/ electrographic frequency of spikes /duration) between treatment groups.

Histology

For histological analyses, rats and mice were anesthetized with an overdose of sodium pentobarbital and perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. After perfusion, brains were removed and post-fixed overnight in 4% buffered parformaldehyde at 4°C and then cryoprotected in 30% sucrose. Ovaries were removed and placed in 10% buffered formalin. Mouse brains were processed using immunocytochemistry against GnRH (hypothalamic areas) and cresyl violet (hippocampal regions). Rat brains were processed using cresyl violet (1/2 of hippocampal sections) and Fluoro-Jade (1/2 of hippocampal sections).

1. Immunocytochemistry for GnRH. For the mouse brains, sections from the diagonal band of Broca through the hippocampus were cut at 35 μm using a freezing microtome. The free floating sections from the diagonal band of Broca to the medial preoptic area were then rinsed in PBS and incubated in 4% normal goat serum and 0.1% Triton X-100 for 1 h, followed by several washes in PBS. Immunostaining for GnRH neurons was performed by incubating the sections with the GnRH antibody (1:7500 Affinity BioReagents) at 4°C for 48 h and then visualized by biotinalated goat anti-rabbit antibody (1:200, Vector laboratories). All sections from both treatment groups were processed at

the same time to reduce variance. Labeled sections were then rinsed with PBS and mounted onto slides.

2. Cresyl Violet Staining. For tissue sections from both rats and mice, neuronal damage was assessed by cresyl violet staining. Serial sections (in mice) and ½ of the serial sections (in rats) of the hippocampus (35 μm) were mounted onto slides and dried overnight. For cresyl violet staining, the sections were dehydrated through a graded ethanol series, defatted with chloroform/ether and then rehydrated. Next, sections were stained with 0.5% cresyl violet acetate, rinsed in distilled water, dehydrated, differentiated and coverslipped with Permount (Fisher).

3. Fluoro-Jade staining. The remaining ½ of the rat hippocampal sections were mounted onto slides and dried overnight. For Fluoro-Jade staining, the slides were immersed in potassium permanganate (0.06%) for 15 min, rinsed in dH20 for 1 min, then immersed in Fluoro-Jade (0.001%, Chemicon) for 30 min, rinsed in dH2O and dried in the dark for several hours before submersion in xylene and coverslipped with DPX (Electron Microscopy Sciences, Inc.).

4. Ovaries. The ovaries were processed and stained by ARUP Laboratories, Salt Lake City, UT. Briefly, paired ovaries were processed through a series of graded ethanols, xylene, then paraffin-embedded. Sections were then cut at 5 μm through the ovary. The tissue was mounted and stained with hematoxylin and eosin (H&E). Four-sets of representative sections of the ovary

were examined per mouse. The number of follicles and abnormal structures were counted.

5. Quantitative Analysis. The total number of immunocytochemically labeled neurons was determined from serially reconstructed brains (medial septum/diagonal band of Broca, preoptic area and hypothalamus) using Neurolucida software (MicroBrightField, VT). All slides were coded before analysis to prevent experimenter bias. Counting was done bilaterally on serial coronal sections (35 µm). To investigate the possibility that selective cell loss occurs, regional analysis was also performed on the following regions: medial septum/diagonal band of Broca, and rostral and caudal preoptic areas.

6. Neuronal counts in the hippocampus. To assess neuronal damage in the hippocampus, cresyl violet-stained neurons were analyzed based on averaging the number of neurons in 4 samples of two 50 µm X 50 µm boxes at 40X placed randomly in the neuronal cell layer in each area (dorsal CA1 and CA3, ventral CA1 and CA3, and the upper and lower blade of the dentate gyrus). Fluoro-jade-positive neurons were analyzed based on averaging the number of positive neurons in 4 samples of 4, 50 µm X 50 µm boxes at 10X placed randomly in neuronal cell layers with positive staining (dorsal CA1 and CA3, ventral CA1 and CA3, the upper blade of the dentate gyrus and the hilus).

Determination of the stages of the estrous cycle

Estrous cycle stage was determined by vaginal cytology. 10µm of sterile saline was gently perfused and extracted from the vagina using a pipetman. Fluid was then placed on a glass slide, stained with CV and examined using a 10X objective. Proestrus was characterized by nucleated epithelial cells, estrus by cornified epithelial cells, metestrus by a combination of leukocytes, cornified and nucleated epithelial cells, diestrus by leukocytes and nucleated epithelials.

Chapter 3: Pilocarpine-induced status epilepticus and subsequent spontaneous seizures do not alter the number of GnRH-positive neurons in a mouse model of temporal lobe epilepsy

Introduction:

Temporal lobe epilepsy is the most common type of epilepsy in humans and occurs in approximately 1% of the world's population (Engel, 1989; Shin and McNamara, 1994; Shneker and Fountain, 2003). Temporal lobe epilepsy is associated with spontaneous recurrent seizures following a latent period from the precipitating injury or insult, which often results in SE (a period of prolonged seizure activity lasting greater than 30 min) (French et al., 1993; Engel, 1989). Status epilepticus and the chronic seizure activity both cause damage to the temporal lobes. This includes, but is not limited to, gliosis and neuronal loss in the hippocampus (mostly in the hilus, CA1 and CA3 regions) and amygdala, and mossy fiber sprouting in the dentate gyrus. Temporal lobe epilepsy is also associated with an increased incidence of reproductive disorders, especially in women (Herzog et al., 1986; Nappi et al., 1994; Morrell 1999). This comorbidity may be caused by antiepileptic drugs, seizures and/or epilepsy, or pathological changes in the reproductive axis.

The reproductive axis consists of the hypothalamus, the pituitary, and the gonads. A central component of this axis is a network of 800-2000 neurons that synthesize the decapeptide, gonadotropin-releasing hormone (GnRH) (Silverman et al., 1994) which are located in and around the basal hypothalamus. Activation of these neurons results in the coordinated secretion of GnRH into the hypophysial-portal vasculature (Silverman, 1994). The amplitude and frequency of GnRH release into the median eminence differentially regulates the production and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH)

from the anterior pituitary, which in turn controls gonadal steroidogenesis and gametogenesis, respectively (Levine and Ramirez, 1982; Everett, 1994). High frequency pulses favor LH release and low frequency pulses favor FSH release (Wildt et al, 1981). The synchronized activity of the GnRH neuronal network and the pulsatile release of GnRH are essential for normal reproduction. The two most common reproductive disorders in women with epilepsy are polycystic ovary syndrome and hypogonadotropic hypogonadism, which are often the result of altered pulsatile release of luteinizing hormone (LH) (Herzog et al., 1986; Drislane et al., 1994; Taylor et al., 1997; Marshal et al., 2001). Therefore, the reproductive abnormalities associated with seizures and/or epilepsy hypothetically involve hypothalamic disturbances resulting in altered secretion of GnRH.

Evidence supporting the hypothesis that seizures and/or epilepsy disrupt hypothalamic function derives from both clinical and animal model data demonstrating alterations in hypothalamic peptide secretion. Clinically, acute, post-ictal elevations in serum levels of prolactin, LH (in males and females) and FSH (in women) are observed after generalized tonic/clonic seizures (Trimble 1978; Pritchard et al., 1983; Dana-Haeri et al., 1983), as well as chronic changes in LH pulse frequency (Herzog et al., 1990; Drislane et al., 1994; Bilo et al., 1991). Similar pathologies are also observed in animal models of temporal lobe epilepsy including arrested ovarian cyclicity, elevated serum levels of prolactin, estradiol, LH and FSH and decreased progesterone (Amado et al., 1987; Amado and Cavalheiro,1998; Bhanot and Wilkinson 1982; Edwards et al., 1999;

Edwards et al., 2000). These changes in serum gonadotropin levels provide evidence that seizures disrupt hypothalamic function at the level of the GnRH neuronal network.

Alternatively, the hypothetical disruption of the GnRH system associated with temporal lobe epilepsy may be due to alterations in the GnRH network itself. More specifically, the observed reproductive dysfunction may be a direct result of reduced expression of the GnRH peptide. Focal application of kainic acid in the amygdala decreased GnRH fiber staining in the ventromedial hypothalamus, a region important in the regulation of reproductive function (Friedman et al., 2002). The density of GnRH immunoreactive fibers was also reduced 60-90 days after pilocarpine-induced SE (Amado et al., 1993). However, neither of these studies quantified the number GnRH-positive neurons. The reduction of GnRH containing fibers in epileptic brains suggests that either a change in the expression of the GnRH peptide or loss of GnRH neurons may underlie the endocrine dysfunction associated with temporal lobe epilepsy.

In this study, we will utilize the pilocarpine model of temporal lobe epilepsy in the GnRH-eGFP transgenic mouse line (Suter et al., 2000) to examine the acute effects of SE and the chronic effects of temporal lobe epilepsy on the GnRH system. While there has been some debate over mouse strain susceptibility to kainic acid (Ferraro et al., 1995; Schauwecker and Steward, 1997; Shibley and Smith, 2002; Borges et al., 2003), pilocarpine is a chemoconvulsant that produces neuropathology in mice similar to human patients with temporal lobe epilepsy (Borges et al., 2003; D'Antuono et al., 2002;

Shibley and Smith, 2002; Cavalheiro et al., 1996; Turski et al., 1983). We validated our model of epilepsy by quantifying hippocampal neuronal loss and detecting recurrent seizures. The estrous cycle was monitored in a sub-set of mice. The number of GnRH-positive neurons was quantified to examine the hypothesis that pilocarpine-induced SE and/or the resulting epilepsy causes a reduction in the number of GnRH-positive neurons. A loss of GnRH neurons could theoretically lead to alterations in the pulsatile release of GnRH, which would in turn disrupt LH and FSH secretion, potentially resulting in reproductive disorders.

Results

Status epilepticus (SE)

A total of 117 mice were treated with pilocarpine. The first seizure was observed on average 30 ± 25 min after the pilocarpine injection. Fifty-nine mice died during pilocarpine treatment (50.4%), as a result from either SE and/or the toxic effects of pilocarpine. Twenty-seven mice did not experience SE as a result of pilocarpine treatment and were not included in the study (23%). The average number of P3-P5 seizures during the observation period was 6.1 (with a range of 3-11). The mice were then randomly assigned to either an acute (1 wk) or chronic (3 mo) group.

Status Epilepticus (1 wk group)

Pilocarpine-induced SE resulted in spontaneous seizures within 1 wk in approximately half of the GnRH-eGFP mice (n=9/17). None of the saline-treated control mice were observed to have seizures during or after treatment.

Pilocarpine-induced SE caused bilateral neuronal loss in the hippocampus. This loss was apparent on gross visualization (Fig 3.1) and was significant in the CA1 region (t-test, $p< 0.05$). The CA3 and upper blade of the dentate gyrus of the hippocampus had neuronal loss that was not significant (t-test, $p=0.08$ and 0.12, respectively) and there was no reduction in the lower blade of the dentate gyrus (t-test, $p=0.7$). Prominent gliosis was present throughout the hippocampus. These histopathological alterations are similar to those normally associated with temporal lobe epilepsy.

The estrous cycle was followed in seven mice that experienced SE and survived for the week following SE. Overall, the most striking result was the absence in all mice of a consecutive, 4 day cycle after pilocarpine-induced SE. There was a significant reduction in the number of proestrus days after 1 wk (t-test, $p=0.03$). There was also an increase in abnormal cytology. Mice that received pilocarpine, but did not enter into SE, returned to a normal cycle in an average of 3.6 days ($n=12$). The ovaries from pilocarpine-treated mice after one week did not show any apparent differences in follicle number or abnormal structures.

GnRH neurons and fibers were present after pilocarpine-induced SE (Fig 3.3). GnRH-positive neurons were fusiform in shape and were either bipolar or unipolar. GnRH-positive cell bodies and fibers were scattered throughout the basal forebrain and were most dense in the septum-diagonal band of Broca, and had scattered distribution through rostral and caudal preoptic areas. GnRH-positive fibers were punctate and were visible in all sections with GnRH-positive

cells bodies. Fiber staining was most robust in the OVLT and median eminence, the two main regions that GnRH neurons send their axons. The number of GnRH-positive neurons was not significantly altered 1 wk after pilocarpine-induced SE (t-tests, overall count: p=0.91; Ovlt regions: p=0.6; Rostral preoptic areas p= 0.9; caudal preoptic areas 0.86) (Fig 3.4).

Chronic Epilepsy (3 mo group)

Pilocarpine-induced SE resulted in spontaneous seizures in all of the GnRH-eGFP mice (11/11). Recurrent seizure activity did not depend on the severity of SE (linear regression, p=0.3). None of the saline-treated control mice were observed to have seizures during or after treatment.

Pilocarpine-induced SE and the resulting spontaneous seizures caused bilateral neuronal loss in the hippocampus. This loss was apparent on gross visualization (Fig 3.5) and was significant in the CA1 (t-test, $p< 0.05$), and CA3 (t-test, $p< 0.05$) regions with non-significant reductions in the upper blade of the dentate gyrus (t-test, p= 0.25) and lower blade of the dentate gyrus (t-test, p= 0.14) (Fig 3.6).

Two mice that experienced SE and survived had the estrous cycle followed for 3 mo. The changes in the estrous cycle were similar to those that occurred after 1 wk, with the most striking result again being the absence of a consecutive, 4 day cycle after pilocarpine-induced SE. There was an overall reduction in the number of proestrus days after three months and an increase in abnormal cytology. The ovaries from pilocarpine- treated animals had a number of abnormalities including hemorrhagic follicles and cyst-like formations.

GnRH neurons and fibers were present 3 mo after pilocarpine-induced SE that caused loss of neurons in the hippocampus and also lead to chronic spontaneous recurrent seizures (Fig 3.7). The appearance and distribution of GnRH-positive neurons was the same as in the 1 wk group (see above). We did not detect a significant reduction in the total (t-test, p=0.2) or regional number (ovlt p= 0.3; rpoa p=0.3; cpoa p=0.6) of GnRH-positive neurons (Fig 3.8).

Discussion

In this study, pilocarpine was successful in inducing SE in the GnRH-eGFP mouse. This insult, the resulting seizures, and development of temporal lobe epilepsy caused bilateral neuronal loss in the hippocampus. This supports previous studies showing that pilocarpine-induced seizures in murine strains with background C57BL/6 results in cell loss in the hippocampus (Schauwecker, 2002; Shibley and Smith, 2002). In this study, the pyramidal neuron loss was significant in the CA1 region of hippocampus in the 1 wk (acute) treatment group, with variable neuronal loss in the CA3 region that was non-significant. However, in the 3 mo (chronic) group, the neuronal loss was significant in both the CA1 and CA3, which lends further support to the hypothesis that spontaneous seizures and epilepsy leads to progressive neuronal cell loss (Meldrum 1997, Hellier et al., 1998, Roch et al., 2002). While the hippocampus is not the only structure that is damaged in both human and animal models of temporal lobe epilepsy, it is a reliable indicator for both abnormal electrical discharges and

seizures (Margerison and Corsellis, 1966; Mathern et al., 2002; Buckmaster and Dudek 1997; Roch et al., 2002) and is a key hallmark in temporal lobe epilepsy.

This confirms previous findings in other models of temporal lobe epilepsy where repeated seizures/SE result in altered ovarian cyclicity (Amado et al., 1987; Amado and Cavalheiro,1998; Bhanot and Wilkinson 1982; Edwards et al., 1999; Edwards et al., 2000). In this model, we observed that the mice stopped cycling normally immediately after pilocarpine-induced SE (n=11). After 1 week, we report that none of the mice had a consecutive, 4 day cycle, and there was an overall decrease in the time spent in proestrus after 1 wk ($p=0.03$) and an increased observation of abnormal cytology (only leukocytes). This change is not mediated by pilocarpine because mice that received pilocarpine, but did not enter into SE, resumed cycling. After 3 months, there was still no observed consecutive 4 day cycle (n=2). The same trend was observed with a decreased time spent in proestrus ($p=0.04$) and an increase in abnormal cytology. These abnormal cycles are reflected in the ovarian histology, where mice that experienced pilocarpine-induced SE had an increased number of abnormalities including hemorrhagic follicles and cyst-like formations at 3 mo. Animal models have provided evidence that seizure activity alters hypothalamic peptide secretion and this may lead to chronic changes in reproductive (Amado et al., 1987; Amado and Cavalheiro,1998; Bhanot and Wilkinson 1982; Edwards et al., 1999; Edwards et al., 2000). It is likely that similar fluctuations in hormones are responsible for the acyclicity in the estrous cycle seen in this model.

However, even with altered estrous cycles, we report that there is not a detectable reduction in GnRH-positive neurons in the acute (1 wk) or chronic (3 mo) treatment groups. This is despite a robust SE, significant neuronal loss in the hippocampus, presence of spontaneous seizures and alterations in the estrous cycle. There was also no qualitative change in GnRH-positive fibers between control and pilocarpine treated mice. While this is in contrast to Amado et al., 1993 and Friedman et al., 2002, discrepancies in the data may simply reflect methodological differences, both in the model of epilepsy used and GnRH antibody. This may indicate that alterations in the GnRH network only occur after specific insults. Alternatively, changes in reproduction may be due to a reduced number of activated GnRH neurons during LH surge activity rather than a loss of GnRH neurons, which has been observed in the age-associated reduction in fertility and LH surge (Lloyd et al., 1994). There is also evidence to suggest that there is functional redundancy in the GnRH network so that only between 12-34% of the population is required for normal reproductive function (Herbison et al., 2007). This would indicate that a gross reduction in GnRH-positive neurons and fibers would be necessary before it would cumulate as a disruption in the estrous cycle.

In summary, our results suggest that the observed changes in reproduction after pilocarpine-induced SE and the resulting epilepsy are not due to a reduction in GnRH-positive neurons, and that they might alternately be due to either altered synaptic input to these neurons, altered regulation of the network, or changes in the HPG axis other than the hypothalamus. This mouse

model, which exhibits neuronal loss in the hippocampus, recurrent seizures and abnormal estrous cycles, may prove particularly useful to explore the interaction between epilepsy and the endocrine axis.

Figure 3.1. Photomicrographs of cresyl violet- stained sections of the hippocampus from saline-treated (A-C) and pilocarpine-treated (D-F) mice 1 wk after status epilepticus.

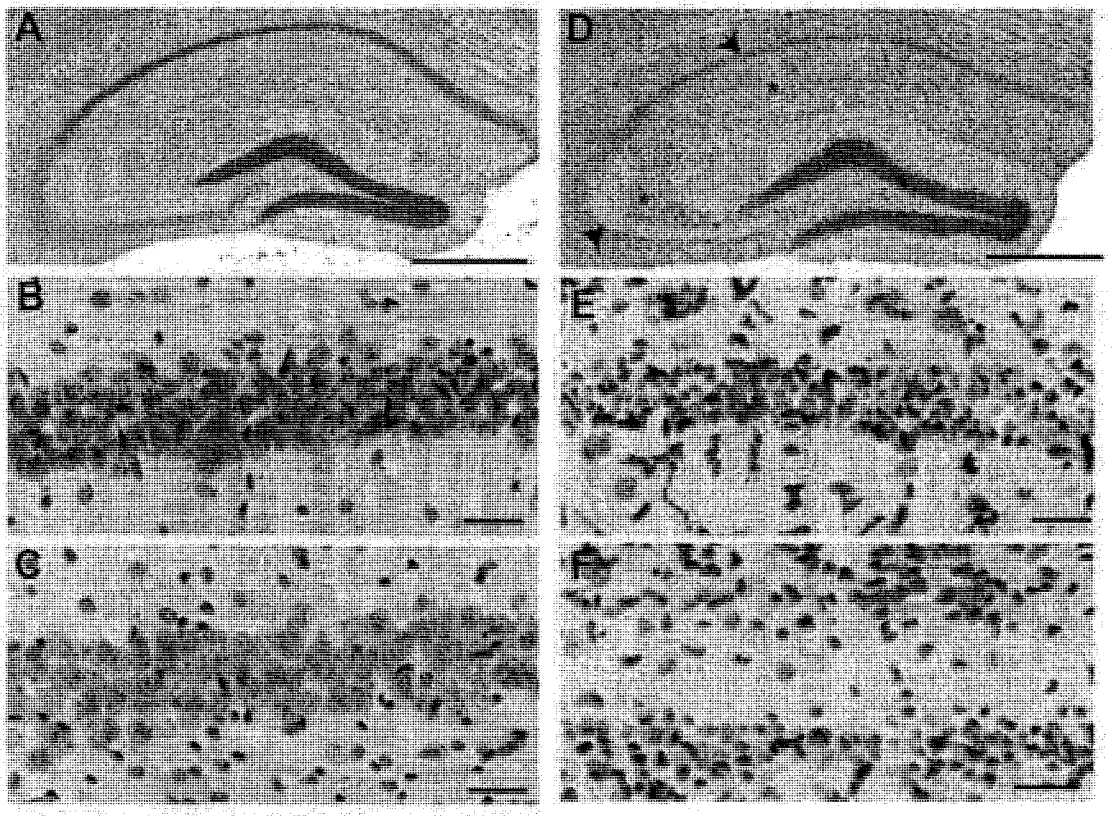

Figure 1. Photomicrographs of cresyl -violet- stained sections of the hippocampus from saline-treated (A-C) and pilocarpine-treated (D-F) mice 1 wk after status epilepticus. Compared to the saline-treated controls (A), pyramidal cell loss (arrowheads) and gliosis (*) is apparent in the pilocarpine-treated mouse (D) at low magnification. B-F represent a higher magnification of the CA1 (B,E) and CA3 (C,F) pyramidal layers. Note the prominent loss of pyramidal cells in the CA1 and CA3 regions of the hippocampus from pilocarpine treated mice as compared to saline-treated mice. Scale Bars: A and D 250 µm; B, C, E and F 25µm.

Figure 3.2. Quantification of the number of cresyl violet-stained pyramidal neurons in the hippocampus from saline-treated (control, open bar) and pilocarpine-treated (pilocarpine, black bar) mice 1 wk after pilocarpine-induced status epilepticus.

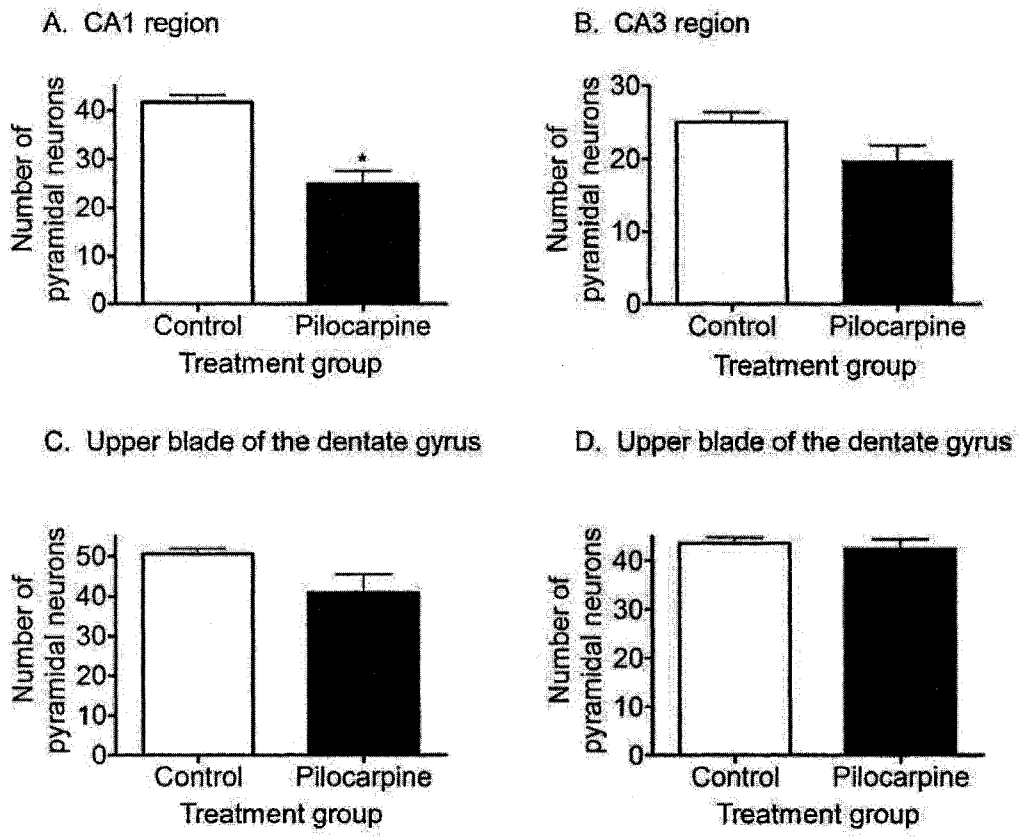

Figure 3.2. Quantification of the number of cresyl violet-stained pyramidal neurons in the hippocampus from saline-treated (control, open bar) and pilocarpine-treated (pilocarpine, black bar) mice 1 wk after pilocarpine-induced status epilepticus. There was a significant loss of CA1 pyramidal neurons (A) one week post-status epilepticus. However, the number of pyramidal neurons in the pilocarpine-treated mice was not significantly different from the control for the CA3 region (B), as well as the upper (C) and lower blade (D) of the dentate gyrus. * $p<0.05$, t-test. Error bars indicate S.E.M.

Figure 3.3. Photomicrographs of hypothalamic sections immunostained for GnRH from saline-treated (A, C, E) and pilocarpine-treated (B, D, F) mice 1 wk after treatment.

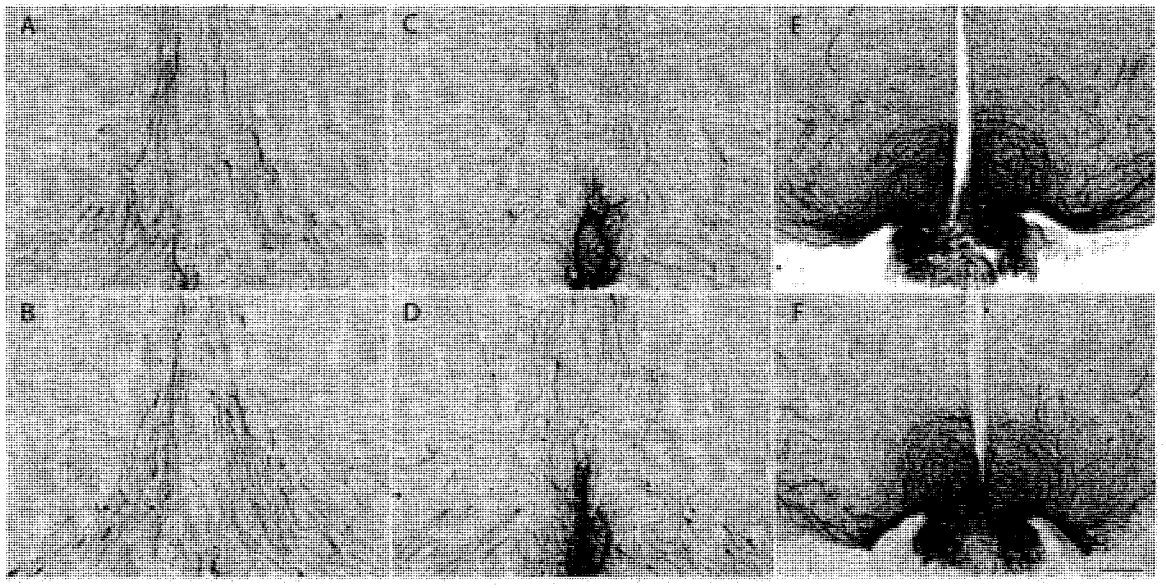

Figure 3.3. Photomicrographs of hypothalamic sections immunostained for GnRH from saline-treated (A, C, E) and pilocarpine-treated (B, D, F) mice 1 wk after treatment. GnRH-positive neurons and fibers were present in the diagonal band of Broca (A, B) and organum vasculosum of the lamina terminalis (C, D) regions from both saline- and pilocarpine-treated mice. Robust fiber staining was also present in the median eminence (E, F) from both treatment groups. Scale bar 50 μm.

Figure 3.4. Quantification of the number of GnRH-immunopositive neurons in hypothalamic regions from saline- (control, open bar) and pilocarpine- (pilocarpine, black bar) treated mice 1 wk after pilocarpine-induced status epilepticus.

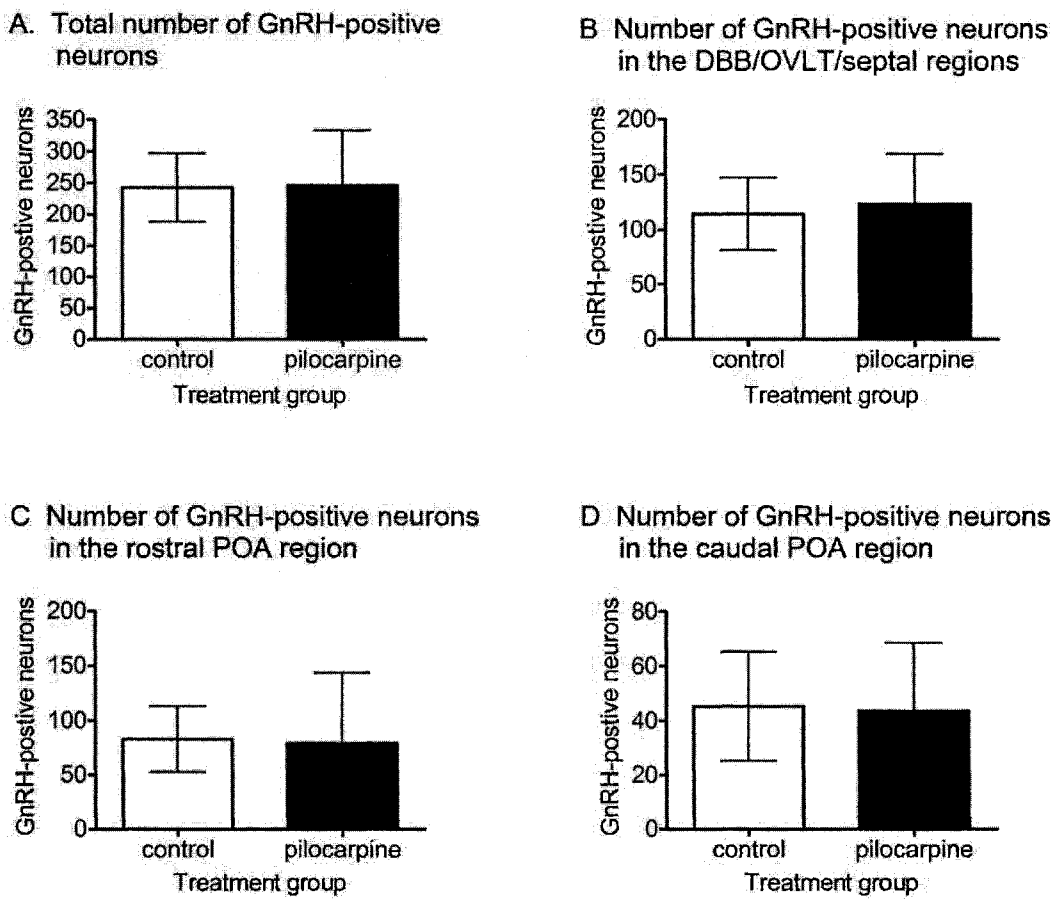

Figure 3.4. Quantification of the number of GnRH-immunopositive neurons in hypothalamic regions from saline- (control, open bar) and pilocarpine- (pilocarpine, black bar) treated mice 1 wk after pilocarpine-induced status epilepticus. Bar graph summarizing the average total number of GnRH-immunopositive neurons is shown in (A) with the regional distribution shown in B-D. No significant differences were observed for either the total number or regional distribution of GnRH-immunopositive neurons between the saline- and pilocarpine-treated mice. $p>0.05$, t-test. Error bars indicate S.E.M.

Figure 3.5. Photomicrographs of cresyl violet-stained sections of the hippocampus from saline-treated (A-C) and pilocarpine-treated (D-F) mice 3 mo after status epilepticus.

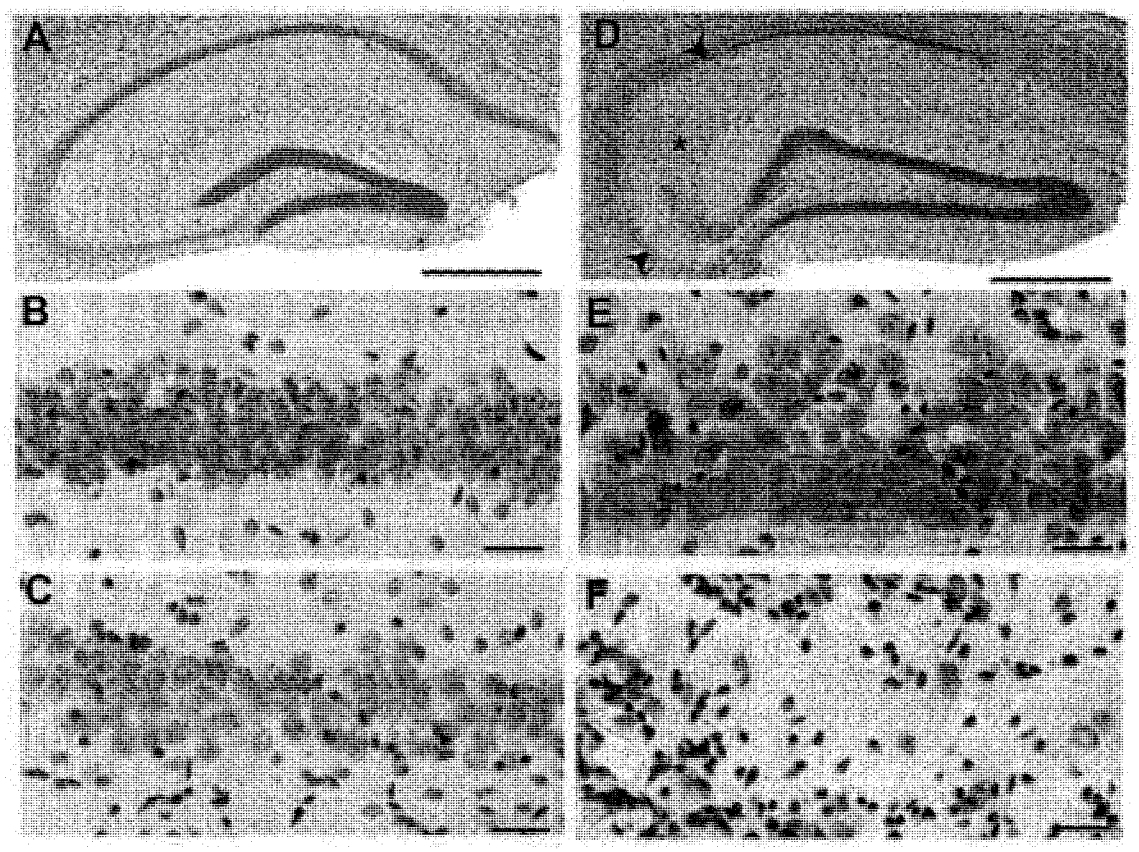

Figure 3.5. Photomicrographs of cresyl violet-stained sections of the hippocampus from saline-treated (A-C) and pilocarpine-treated (D-F) mice 3 mo after status epilepticus. Compared to the saline-treated control (A), pyramidal cell loss (arrowheads) and gliosis (*) is apparent in the pilocarpine-treated mouse (D) on low magnification. Higher magnification of the CA1 (B, E) and CA3 (C, F) pyramidal layers. Note the prominent loss of pyramidal cells in the CA1 and CA3 regions of the hippocampus from pilocarpine-treated mice as compared to saline-treated mice. Scale Bars: A and D 250 µm; B, C, E and F 25 µm.

Figure 3.6. Quantification of the number of cresyl violet-stained pyramidal neurons in the hippocampus from saline- (control, open bar) and pilocarpine-(pilocarpine, black bar) treated mice 3 mo after pilocarpine-induced status epilepticus.

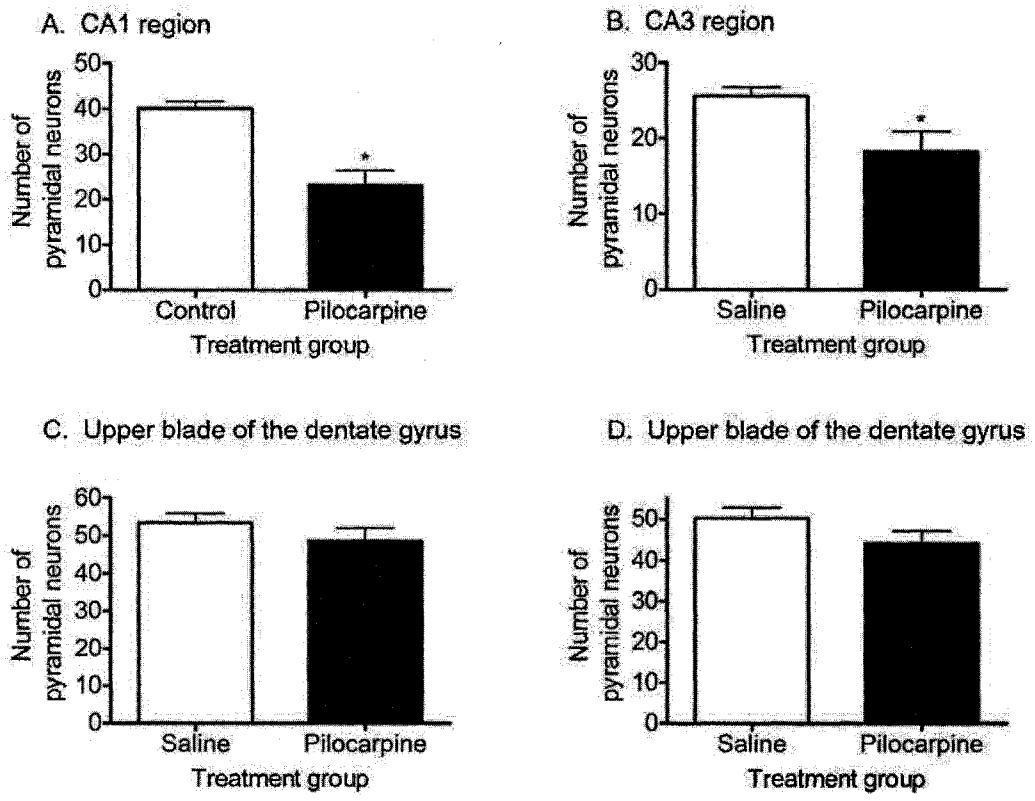

Figure 3.6. Quantification of the number of cresyl violet-stained pyramidal neurons in the hippocampus from saline- (control, open bar) and pilocarpine-(pilocarpine, black bar) treated mice 3 mo after pilocarpine-induced status epilepticus. At 3 mo after pilocarpine-induced status epilepticus, there was a significant loss of pyramidal cells in both the CA1 (A) and CA3 (B) regions, and no loss in the upper and lower blade of the dentate gyrus (D, E). * $p<0.05$, t-test. Error bars indicate S.E.M.

Figure 3.7. Photomicrographs of hypothalamic regions immunostained for GnRH from saline-treated (A, C, E) and pilocarpine-treated (B, D, F) mice 3 mo after treatment.

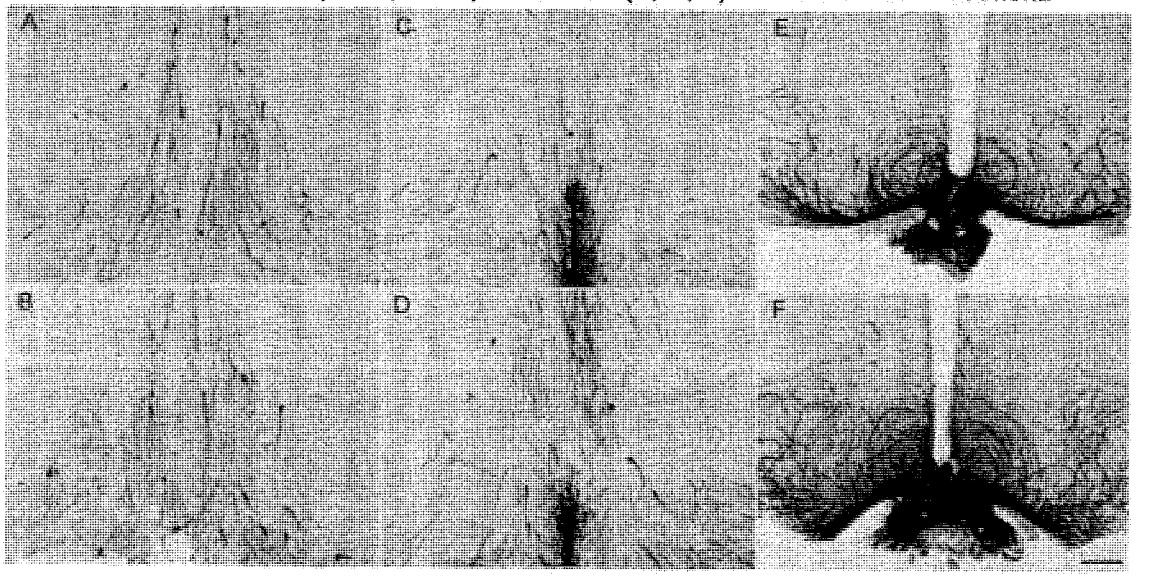

Figure 3.7. Photomicrographs of hypothalamic regions immunostained for GnRH from saline-treated (A, C, E) and pilocarpine-treated (B, D, F) mice 3 mo after treatment. GnRH-immunopositive neurons and fibers were present in the diagonal band of Broca (A, B) and organum vasculosum of the lamina terminalis (C, D) regions in both saline- and pilocarpine-treated mice. Robust fiber staining was also present in the median eminence (E, F) of both treatment groups. Scale bar 50 μm.

Figure 3.8. Quantification of the number of GnRH-positive neurons in hypothalamic regions from control (open bar) and pilocarpine-treated (black bar) mice 3 mo after pilocarpine-induced status epilepticus.

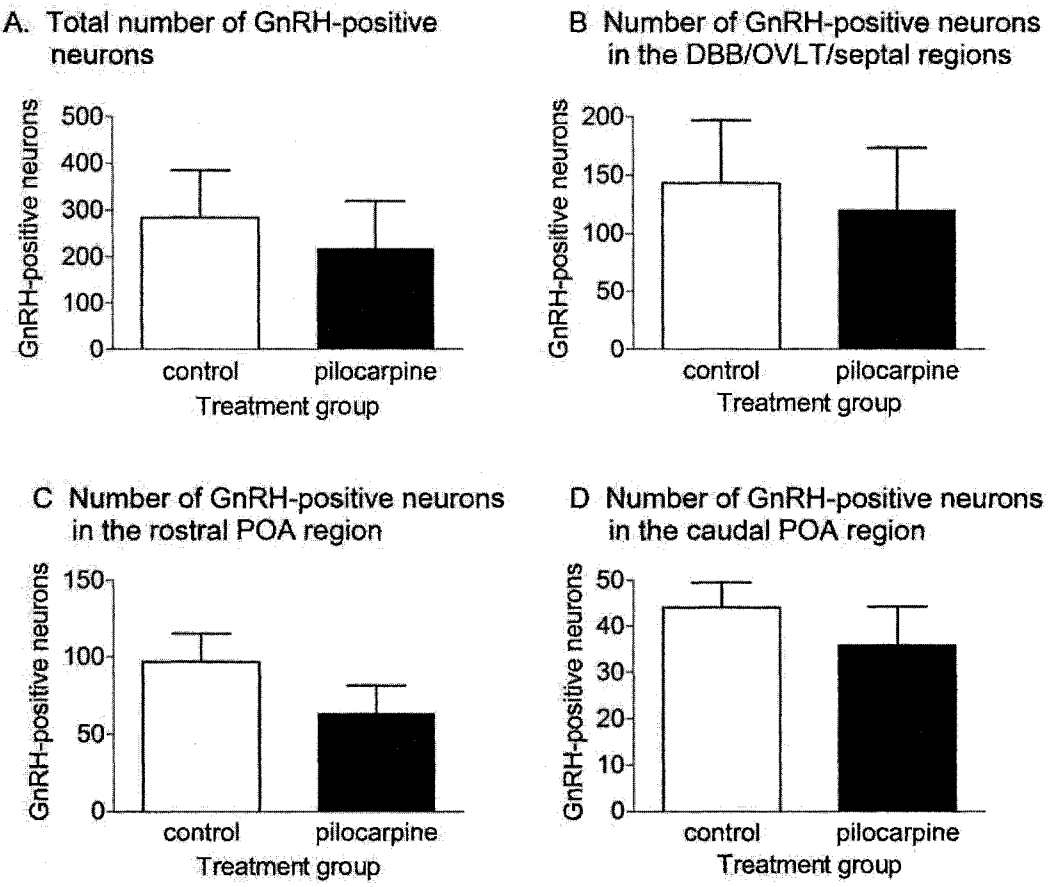

Figure 3.8. Quantification of the number of GnRH-immunopositive neurons in hypothalamic regions in saline- (control, open bar) and pilocarpine-treated (pilocarpine, black bar) mice 3 mo after pilocarpine-induced status epilepticus. There was no significant difference in the averaged total number (A) and regional distribution (B-D) of GnRH-immunopositive neurons between the saline- and pilocarpine treated mice at 3 mo after pilocarpine-induced status epilepticus. $P>0.05$, (t-test). Error bars indicate S.E.M.

Chapter 4: The effect of pilocarpine-induced status epilepticus on epileptogenesis, hippocampal damage and reproductive function in intact and gonadectomized rats

Introduction

Status epilepticus (SE) is defined as continuous seizure activity that lasts longer than 30 min without recovery of consciousness between seizures and is a common neurological emergency that results in mortality in up to 43% of cases, depending on age and etiology. In addition to the risk of mortality, SE is associated with an increase risk of developing temporal lobe epilepsy (TLE) (Chin et al., 2004; Vignatelli et al., 2005; Logroscino et al., 2005). Chemoconvulsant-induction of SE is one of the most common methods to generate animal models of epilepsy (Dudek et al., 2002). SE and the chronic seizure activity that occurs with temporal lobe epilepsy both cause damage to the temporal lobes (French et al., 1993; Engel, 1989). Neuronal loss and gliosis in the hippocampus is the most frequent pathological marker in patients with TLE and is a hallmark for animal models of TLE, although damage is not limited to the hippocampus (Margerison and Corsellis, 1966; Mathern et al., 2002; Buckmaster and Dudek 1997; Roch et al., 2002).

Sex- and hormone-dependent differences in the incidence of and mortality associated with SE have been demonstrated in both clinical situations (DeLorenzo et al., 1999; Towne et al., 1994; DeLorenzo et al., 1992; Koubeissi and Alshekhlee, 2007; Logroscino et al., 1997; Hesdorffer et al., 1998; Knake et al., 2001) and in various rodent models of SE (Mejias-Aponte et al., 2002; Scharfman et el., 2005, Tan & Tan 2001; Rhodes and Frye 2004; Pesce et al., 2000; Finn & Gee, 1994; Edwards; Maguire et al., 2006), although contradictory

results have been reported in both cases. These differences have been attributed to steroid hormones; estrogen is classically considered proconvulsive, (Logothetis and Harner 1960; Buterbaugh 1987; Horn and Buterbaugh 1986; Buterbaugh and Hudson 1991; Edwards et al., 1999; Logothetis et al. 1959), progesterone is anticonvulsive (Edwards et al., 1999; Frye and Bayon, 1999; Frye and Scalise, 2000; Herzog, 1986), and testosterone's effect has been reported as both. This biphasic effect has been attributed to the ability of testosterone to be readily metabolized into both androgen (anticonvulsive) and estrogen (proconvulsive) metabolites (Kaminski et al., 2005; Edwards et al., 1999; Reddy 2004 Harden and Maclusky 2005, Herzog, 1999). Hormones and sex have the ability to modulate neuronal excitability and may underlie the observed differences in susceptibility to SE.

In addition to modulating neuronal excitability, hormones have been reported to be neuroprotective (Reibel et al., 2000; Veliskova et al., 2000; Hoffman et al., 2003). Pretreatment with estrogen before the induction of SE attenuates damage in the hippocampus (Reibel et al., 2000; Veliskova et al., 2000; Hoffman et al., 2003) while progesterone pretreatment was not detected to have a neuroprotective effect (Hoffman et al., 2003). If hormones can prevent and/or lessen neuronal damage, they may be able to alter epileptogenesis.

Clinically, catamenial epilepsy is a condition that exemplifies the relationship between gonadal hormones and seizures where seizure exacerbations occur at times in the menstrual cycle where there is relatively high estrogen with low or recently declined progesterone (Herzog et al., 1997; Herzog,

2007). To date, a rodent model has not been established to investigate recurrent seizure frequency and severity as it relates to the estrous cycle. This has largely been due to the observation that most female rats stop cycling normally after SE (Amado and Cavalheiro, 1987; Amado et al., 1987; Bhanot and Wilkinson, 1982; Edwards et al., 2000). The development of animal models to study this condition is a crucial step towards understanding the mechanisms responsible for the seizure exacerbation and to develop therapies for women with catamenial epilepsy.

This study aimed to test the hypothesis that sex and hormones influence susceptibility to pilocarpine-induced SE and recurrent seizure frequency. To test differences in susceptibility, the frequency of electrographic spikes and duration of SE was quantified. To test differences in recurrent seizures, the latency to, number, frequency of electrographic spikes and duration of electrographic seizures were quantified using continuous EEG recordings. The stage of the estrous cycle was determined daily to investigate whether SE and/or recurrent seizures altered the 4 day cycle. The number, spike frequency and duration of recurrent seizures were also analyzed to determine whether these parameters change as a function of the cycle stage. Histology of the hippocampus including Fluoro-jade and cresyl violet staining were utilized to quantify differences in the evolution of hippocampal damage.

Results:

Status epilepticus

The total number of rats that survived treatment was 155, which were grouped into diestrus (n=30), proestrus (n= 24), estrus (n=23), metestrus (n=24), and ovariectomized females (ovx) (n=28), and males (n=26). An average of 68.9% of rats entered SE and 36.5% died as a result of this injury within 24 hr after pilocarpine treatment. A small percentage of rats died after 24 hr (5.6%). Fisher's exact test revealed that males were more likely to die from SE than females treated in proestrus (p=0.04). There were no other significant differences between groups either in the number of rats to enter SE or those that died as a result (Table 1). The average weight of all of the treated rats was 276.2 g ± 4.5, with male rats having a significantly greater average weight than all other treatment groups.

During SE, no significant differences were detected between groups in any of the measured parameters (Fig 4.1). The average dose of pilocarpine needed to induce SE in all groups was 625 ± 38.5 mg/kg, i.p. The average number of seizures before the onset of status was 4.3 ± 0.2 and the average frequency of electrographic spikes of these seizures was 11.0 ± 0.4 Hz. The average time from the first seizure to the onset of SE was 14.1 ±1.2 min. The average duration of status was 397.8 ± 29.1 min. Once the frequency of spikes were less than 1 Hz, (marking the end of SE), the average number of seizures observed within 24 hr was 4.37 ± 1.1.

Recurrent seizures

The average latent period until the first recurrent seizure after pilocarpine-induced SE was 4.9 ± 0.2 days and did not differ significantly between treatment groups. The average number of seizures observed in all groups over 3 weeks was 48.8 (range of 1-172), with an average of 3.3 ± 0.3 seizures daily and was not significantly different between groups (Fig 4.2). The average number of clusters (defined as more than 3 seizures in 1 hr or 2 seizures within 10 min) was 0.07 ± 0.02 (i.e. 7% of seizures occurred in clusters) and was not significantly different between groups (data not shown).

Hippocampal data

The hippocampi of rats that were examined 24 h after SE displayed varying degrees of pyramidal cell loss and gliosis (Fig 4.3). The dorsal CA3 and ventral CA1 regions had more consistent damage than other areas of the hippocampus. Diestrus- and metestrus-treated rats had the most neuronal loss in the hippocampus which was reduced from controls by an average of 32% and 25%, respectively. Diestrus-treated rats had significant neuronal loss in all hippocampal regions compared to controls and metestrus-treated rats had significant neuronal loss compared to controls in all regions except the upper-blade of the dentate gyrus and ventral CA3 (also significantly less neurons than ovx and male rats in the dorsal CA1). Proestrus-treated rats had the least amount of neuronal loss (6% reduction overall), which was not significant from controls in any region, but was significantly less than the neuronal loss in metestrus- (dorsal CA1, lower-blade of the dentate gyrus) and diestrus-treated

(dorsal CA1 and CA3, and the upper-blade of the dentate gyrus) rats. Estrus-treated and male rats both had an average of 15% reduction in neurons in the hippocampus which was not significantly different than the loss of ovx rats, which was 13%.

Fluoro-Jade staining revealed positively-stained neurons in all treatment groups that were significantly different from controls (which did not show any positive cells) but not significant between groups at 24 h (Fig 4.4). The average number of Fluoro-Jade neurons in the sampled area of each region of the hippocampus was 6.8 ± 0.5 in the dorsal CA1, 5.6 ± 0.6 in the dorsal CA3, 4.4 ± 0.2 in the upper-blade of the dentate gyrus, 7.0 ± 0.2 in the hilus, 11.1 ± 0.3 in the ventral CA1, 5.2 ± 0.1 in the ventral CA3.

Three weeks after pilocarpine-induced SE, the hippocampi exhibited neuronal cell loss and extensive gliosis. The neuronal damage was most consistent in the dorsal CA3 (Fig 4.5), upper-blade of the dentate gyrus, and ventral CA1 (Fig 4.6) and is quantified in Fig 4.7. Estrus-treated and ovx rats had the largest average reduction from controls with 40% and 37%. Metestrus-, diestrus- and proestrus-treated rats had similar average reductions of 21%, 32%, and 23%, which were all significant from the controls but not significant from each other. Males had the least amount of neuronal loss at 11% of controls (and was significant from estrus, diestrus and ovx treated rats in the dorsal CA1 and estrus and ovx treated rats in the ventral CA1). Neuronal damage in the hippocampus was progressive and visible in low power photomicrographs (Figs 4.5 and 4.6). Neuronal loss was significantly greater after 3 wk compared to 24

hr in most rats. Estrus-, proestrus-, and ovx-treated rats had the greatest percentage loss after 3 wk compared to 24 h with 44%, 35% and 42%, respectively. Metestrus- and diestrus-treated, and male rats had 15%, 21%, and 16% reductions.

Fluoro-Jade staining revealed positively- stained neurons in all treatment groups that were significant from controls (which did not show any positive cells) but not significantly different between groups at 3 wk (Fig 4.8). The average number of Fluoro-Jade neurons in each region of the hippocampus was 14.5 ±0.6 in the dorsal CA1, 10.0 ± 0.6 in the dorsal CA3, 0.5 ± 0.1 in the upper-blade of the dentate gyrus, 7.6 ± 0.3 in the hilus, 16.2 ± 0.8 in the ventral CA1 and 9.0 ± 0.3 in the ventral CA3. There was a significant increase in Fluoro-jade positive-staining at 3 weeks compared to 24hrs in the dorsal and ventral CA1 and CA3 of the hippocampus, a significant decrease in positive staining in the upper-blade of the dentate gyrus and no change detected in the hilus. These changes are illustrated in Fig 4.9. The overall average increase in Fluoro-Jade positive neurons according to treatment group was estrus (+144%), metestrus (+137%), Diestrus (+195%), proestrus (+124%), ovx (+145%) and males (+91%).

Estrous cycle data

After SE, the estrous cycle was significantly altered. The changes observed were relatively uniform (were not significantly different). Rats had an increase in the amount of time spent in metestrus (+24%), a decreased amount of time in estrus (-18%) and proestrus (-19%), while the amount of time in

diestrus did not change (25%). Rats also displayed abnormal vaginal cytology (+13%) where there were only leukocytes in the cytology (Fig 4.10).

Electrographic seizures were also analyzed in intact females according to estrous cycle stage (Fig 4.11). There were no significant differences in the normalized average number of seizures, frequency of electrographic spikes, or duration of seizures that occurred on the different days of the estrous cycle. However, individual seizure analysis revealed anecdotal evidence that three intact females (15.8%) had EEG parameters of recurrent seizures that correlated with particular stages of the estrous cycle stage. One estrus-treated rat had a statistically significant increase in the frequency of electrographic spikes during seizures at metestrus when compared to estrus. A diestrus-treated rat had statistically significant increase in the duration of seizures at metestrus compared to diestrus. A metestrus-treated rat had a statistically significant increase in seizure duration in diestrus compared to metestrus.

Discussion

Status Epilepticus. The primary purpose of this study was to determine whether there are differences in the susceptibility to pilocarpine-induced SE between males and females at different times of their estrous cycle. The present data demonstrate that in this model, sex (i.e. male vs female) and the stage of the estrous cycle at the time of treatment had no marked influence on the incidence or duration of SE. This is potentially relevant to catamenial epilepsy

because it is viewed that changes in seizure susceptibility as a function of stages of the estrous cycle reflect the hormonal changes associated stages of the cycle.

In previous studies, reported differences in seizure susceptibility were based on behavioral observations, the most frequent being "latency" to a seizure, which is defined as the time from the intraperitoneal injection of a chemoconvulsant to the onset of the first motor seizure (Mejias-Aponte et al., 2002; Scharfman et el., 2005, Tan & Tan 2001; Rhodes and Frye 2004; Pesce et al., 2000; Finn & Gee, 1994). This measure may actually only reflect the variability in administration of the drug (i.e. injection into the colon or fat pad). We attempted to address this issue in our methods by 1) using repeated injection of pilocarpine until the rat had seizures, insuring that enough chemoconvulsant was available and 2) measuring the time from the first seizure (which presumably marks the time when pilocarpine has reached sufficient levels in the brain to induce seizures) until the onset of SE. However, our results indicate that weight was a factor in mortality, and since the weight of male rats was significantly higher than all other treatment groups, we conclude that the increased mortality in males is potentially due to variability in dose/administration of the pilocarpine. Discrepancies between previous studies may also be attributed to the different methods used to induce SE (pilocarpine, pentylenetetrazol, GABA antagonists, etc.), and that differences in induction may actually reflect sex differences in receptors for the particular method of induction/chemoconvulsant that was used.

In fact, GABA, muscarinic, and NMDA receptors have all been shown to be directly modulated by sex and hormones (Romeo et al., 2005; Connell et al.,

2007; Cyr et al., 2001; Foy et al., 1999; Wilson, 1996; Olsen et al., 1988; Cardoso et al., 2004), and these receptors are the main targets of commonly used chemoconvulsants (De Deyn et al., 1990; Turski et al., 1987; Coyle, 1987; Schneider et al., 2006). However, the use of EEG recordings in our study demonstrates that the electrographic parameters that were measured *during* SE were not significantly different between groups. A similar electrographic insult would presumably lead to the development of similar neuronal loss, gliosis, and recurrent seizures, which are the hallmarks of temporal lobe epilepsy.

Spontaneous Recurrent Seizures. Another clinically relevant question to examine was how sex or stage of the estrous cycle at the induction of SE may influence the progression to recurrent seizures and the associated hippocampal damage. We found that sex and stage of the estrous cycle did not affect the latency to onset of recurrent seizures, the average number or frequency of electrographic spikes, and duration of recurrent seizures 3 wk after pilocarpine-induced SE.

Hippocampal Neuronal Damage. Previous studies using the pilocarpine model (e.g., Dudek et al., 2002) have provided evidence that the CA1 area is the most vulnerable area of the hippocampus to pilocarpine-induced SE, yet damage occurs throughout the hippocampus and in other structures of the brain. We assessed the hippocampal damage in the dorsal and ventral CA1 and CA3 pyramidal layers and the dentate gyrus granule cell layer across all treatment groups. After pilocarpine-induced SE, there was more consistent damage in the dorsal CA3 and ventral CA1 regions than other areas in the hippocampus,

independent of treatment group. Typically, cresyl violet staining is used to analyze neuronal loss a week or two after the seizure-induced injury, but since it is known that severe SE causes substantial swelling and potentially immediate necrosis of individual CA1 pyramidal cells; we assessed hippocampal damage with cresyl violet staining after 24 hr. Thus, the cresyl violet staining and Fluoro-Jade staining represent two different approaches with strengths and weaknesses for analyzing this question. With cresyl violet staining, possible damage was seen at different stages of the estrous cycle in different areas of the hippocampus. Female rats that enter SE during metestrus and diestrus had more hippocampal damage than other groups, and female rats treated during proestrus had significantly less damage than these groups in all (sampled) areas of the dorsal hippocampus. Since relative levels of estrogen are high in the morning of proestrus and low in the morning of both metestrus and diestrus, our data lends further support to the hypothesis that estrogen is neuroprotective in seizure models (Reibel et al., 2000; Veliskova et al., 2000; Hoffman et al., 2003), at least in the short-term. However, ovx female and male rats showed a trend for less damage, which might indicate that estrogen and/or its receptors are not the only factors mediating neuronal protection.

On the other hand, the Fluoro-Jade staining revealed neuronal damage in all areas and at all stages of the estrous cycle, with the CA1 area being the most sensitive and the other areas (i.e., hilus upper blade of the dentate gyrus, dorsal and ventral CA3) being roughly similar. However, the failure to detect a significant difference between treatment groups with Fluoro-Jade staining does

cast doubt on the reliability of the cresyl violet data that suggests neuronal loss is a function of treatment group. Again, each approach has its limitations. With cresyl violet, it is extremely difficult to detect small losses of neurons reliably because of variance in the large number of cells counted. With Fluoro-Jade, when the total number of cells in each section is low, there is a relative lack of statistical power. It is difficult to determine which of these issues is more important, but the most conservative interpretation is that neuronal loss occurs throughout the hippocampus, particularly in dorsal and ventral CA1 (see Dudek et al., 2002), but no obvious change was seen as a function of the stage of the estrous cycle.

Histopathological analyses undertaken at 3 wk after SE yielded similar results to those performed at 24 h in regard to the general areas of the hippocampus that were most damaged and in regard to the apparent lack of effect of stage of estrous cycle on neuronal death. The rationale was that cresyl violet staining would be more likely to reveal major neuronal loss at 3 wk, as neurons would ultimately degenerate and disappear over the 3 wk period compared to the 24 h period. On the other hand, Fluoro-Jade staining could persist for as long as a few weeks and other activity that might occur with seizures corresponding to increased epileptogenesis as a function of cycle would also potentially lead to increased Fluoro-Jade staining. The key result is again that damage was found in all of the different areas analyzed in the hippocampus, particularly in the CA1 area both dorsal and ventral. Somewhat surprisingly the upper blade of the dentate gyrus also showed neuronal loss. Nonetheless, there

was again no clear estrous cycle stage-dependent loss of hippocampal neurons. These data, when taken in conjunction with the observation that no clear differences occurred in the parameters of SE and there were no clear differences seen in the histopathology at 24 h or 3 wk, suggest that the hippocampus of males and females, and females as a function as stage of estrous cycle are not clearly different in their susceptibility to SE.

Interestingly, males had significantly less neuronal damage in both the dorsal and ventral CA1 after 3 wk compared to the other treatment groups. These regions (dorsal and ventral CA1), and the lower blade of the dentate gyrus were regions of the hippocampus that did not experience a significant neuronal loss after 3 wk (count was similar at 3 wk vs 24 hr). This may suggest that males are less susceptible to neuronal damage in these areas at 3 wk, which could be mediated either by testosterone and/or its metabolites, or some other unknown mechanism. Another possibility is that the male rats had a trend for increased mortality, and it is possible that the surviving rats experienced less of an insult, and as a result, had less damage.

Positive Fluoro-Jade staining was present at both 24 hr and 3 wk after pilocarpine-induced SE. This indicates that the brain (hippocampus) starts to undergo cell death almost immediately and continues to die up to 3 wk after an insult. The increase in positive neurons at the 3 wk time point would indicate that either 1) the process of neuronal death caused by the initial injury (SE) has not yet reached a maximum at 24 hr or 2) recurrent seizure activity induces additional neuronal loss. While additional time points would be necessary to

resolve which mechanisms are responsible for the increase in Fluoro-Jade staining, these data again support the hypothesis that epilepsy has temporal progression of neuronal loss (French et al., 1993; Mathern et al., 1995; Hellier et al., 1998).

Cyclicity. A key question, particularly as it relates to catamenial epilepsy and reproductive problems of women with epilepsy, is whether the estrous cycle is altered after SE. The data from this study support previous findings that pilocarpine-induced SE results in altered ovarian cyclicity (Amado and Cavalheiro 1987, Amado et al., 1987, Bhanot and Wilkinson 1982; Edwards et al., 2000). Although the time spent in diestrus was essentially unchanged, metestrus doubled in duration which led to a reduction in estrus and proestrus of roughly 75 %. This may suggest that there are anovulatory estrous cycles and inadequate luteal phases similar to those observed in other rat models and clinically in women with temporal lobe epilepsy (Amado and Cavalheiro 1998; Herzog). Currently, it is unknown how epilepsy and seizures cause reproductive abnormalities. However, it is plausible that seizures arising in temporal structures such as the hippocampus and amygdala might disrupt normal hypothalamic function via the extensive interconnections between the hypothalamus and limbic system (Fawley et al., 2006).

These experiments also addressed the issue of catamenial epilepsy by analyzing the spontaneous recurrent seizures as they related to the estrous cycle in intact female rats. In this case, the data were grouped together independent of when they were treated during the estrous cycle in order to increase statistical

power primarily because we found no obvious differences during our examination of susceptibility to SE as a function of estrous cycle. Furthermore, our data show that when seizure frequency was analyzed across this time period, normalizing for differences in the duration of the different stages of estrous, there was no change in seizure frequency. Thus, although the time in metestrus was double in duration, the seizure frequency was comparable to the other cycle stages. Similarly, the shorter stages of the estrous cycle did not alter the seizure frequency (i.e., normalized for time in that period). One potential caveat is that we only studied seizure frequency in the first 3 wk after SE. Given that the latent period was up to a week, and the seizure rate is low, we may not have seen a difference in seizure frequency as a function of stage of estrous cycle because we had too few seizures.

Another possibility, however, is that hormonal changes occur on an faster time scale than our sampling may account for (hourly or multi-hourly basis within those stages of the estrous cycle), and we cannot be sure that a detailed analysis of hormone levels would not reveal a correlation between part of the estrous cycle and seizure frequency. Another issue was that seizure frequency was assessed in weeks 2 and 3, when the seizure rate is relatively low. It is conceivable that animals later in the process of epileptogenesis, with higher seizure rates, might have seizure exacerbations and/or clusters that relate to a specific part of the estrous cycle or even hormonal levels. Thus, although the estrous cycle was altered, there was no clear effect on seizure frequency when considered across the 4 day cycle. In spite of our concern of the low seizure

frequency, the total number of animals in this series was 19, which would partially mitigate the problem of low seizure frequency. Another potential issue is that some (15.8%) animals appeared to show seizures that coincided with particular parts of the cycle, and it is conceivable that since only roughly 30 percent of women have catamenial epilepsy, and that this patient group is not homogenous (i.e., at least three types of catamenial epilepsy) (Herzog et al., 1997), that a larger sample size with a more coordinated analysis of hormone levels could reveal a relationship between hormone state and the frequency of seizures.

Summary

In summary, although estrous cycle and sex have been hypothesized to influence mortality associated with pilocarpine-induced SE and extent of pyramidal neuron loss in the hippocampus, electrographic SE is not significantly different between groups. Therefore, sex and stage of the estrous cycle are not predictors of long-term hippocampal damage or recurrent seizures. This would lend support to the hypothesis that once SE occurs, a cascade of neuropathological alterations including neuronal damage occurs in the brain that leads to recurrent seizures and temporal lobe epilepsy, regardless of sex or stage of the estrous cycle at the time of SE. Intervening with anti-epileptic drug therapies as soon as possible after the onset of SE may be crucial for not only reducing brain damage and controlling seizures, but also to potentially prevent the reproductive abnormalities associated with seizures and temporal lobe

epilepsy. This study has established a rodent model of SE/temporal lobe epilepsy-induced reproductive alterations to explore these problems. This model may be useful in further studies to examine 1) how to rescue and/or prevent reproductive abnormalities after SE, 2) how anti-epileptic drug therapy may alter the course of these abnormalities and epilepsy, and 3) the potential mechanisms of catamenial epilepsy.

Table 1. Summary of pilocarpine-induced status epilepticus.

	Diestrus	Proestrus	Estrus	Metestrus	OVX	Male
Total N	30	24	23	24	28	26
SE	22 (73%)	15 (63%)	16 (70%)	15 (63%)	20 (71%)	22 (85%)
Died w/i 24 h	9 (41%)	2 (13%)	6 (38%)	4 (27%)	9 (45%)	11 (50%)
Died post 24h	2 (10%)	1 (7%)	0	1 (7%)	2 (10%)	0

Table 1. Summary of pilocarpine-induced status epilepticus. The overall number of rats treated in each group, the number (%) that entered status epilepticus and the mortality within 24 h of pilocarpine as well as the mortality that occured after 24 h. Male rats had significantly greater mortality (50%) than proestrus-treated rats (13%) (fisher's exact test $p=0.04$). $P>0.05$ for all other comparisons.

Figure 4.1. Electrographic analyses of pilocarpine-induced status epilepticus.

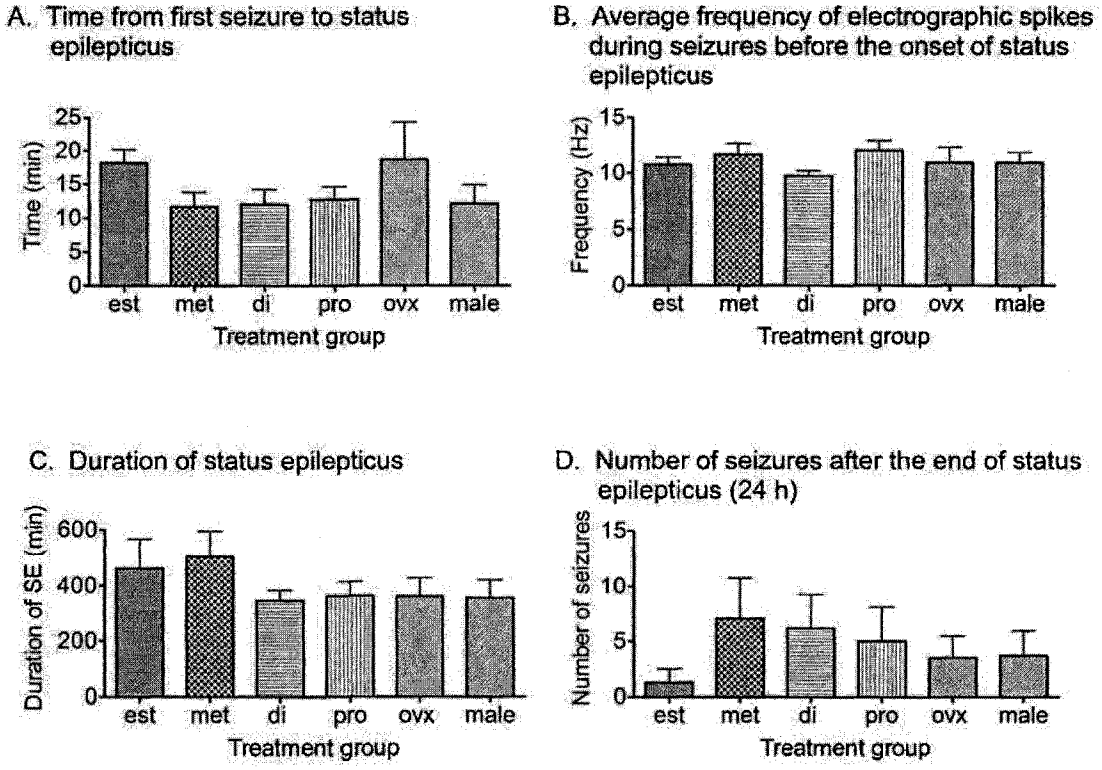

Figure 4.1. Electrographic analyses of pilocarpine-induced status epilepticus. A) Quantification of the average time from the first electrographic seizure to the onset of status epilepticus (defined as the start of continuous ictal spiking with a frequency > 4 Hz) across the various treatment groups. B) A summary of the average spike frequency for seizures that occurred before the onset of status epilepticus. C) A comparison of the average duration of status epilepticus (with the start defined as electrographic spiking > 4 Hz and the end as electrographic spiking < 1 Hz) between the different groups. D) Quantification of the average number of seizures observed after the end of status epilepticus but within 24 h of pilocarpine treatment. One-way ANOVA, $p>0.05$. Data reported as mean ± S.E.M. Abbreviations: est, estrous; met, metestrous; di, diestrous; pro, proestrous; ovx ovariectomized female.

Figure 4.2. Analyses of the recurrent seizure parameters.

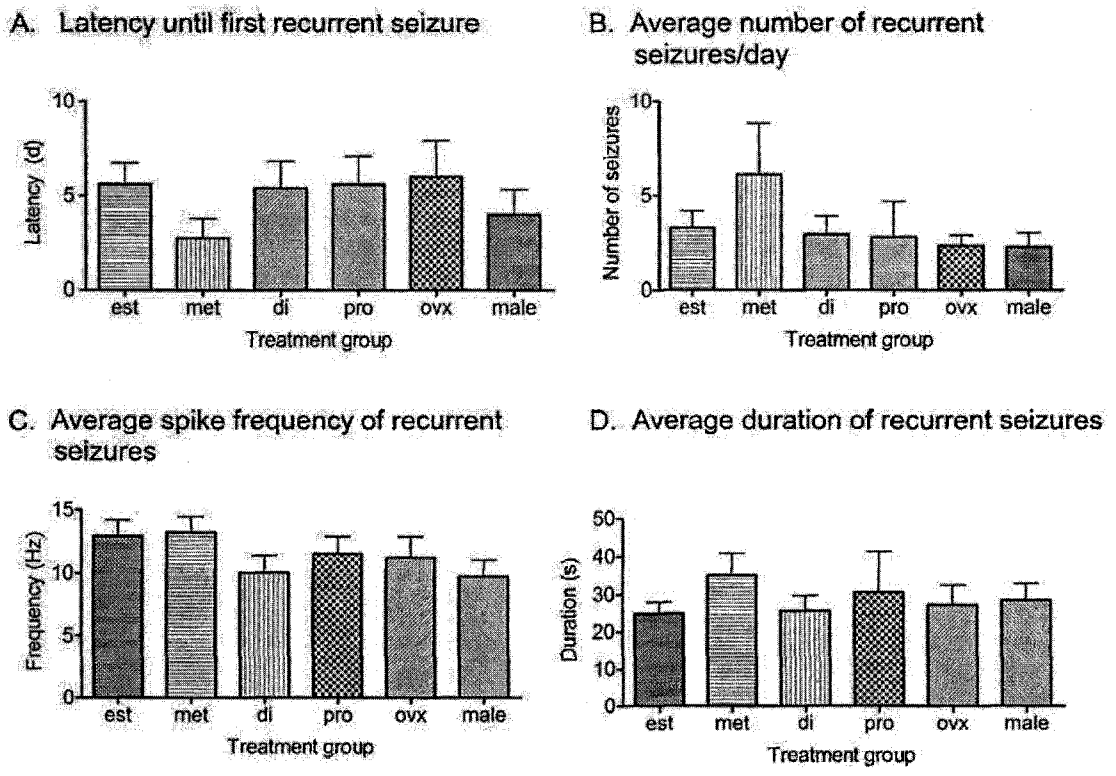

Figure 4.2. Analyses of the recurrent seizure parameters. A) Quantification of the average latency to the first recurrent electrographic seizure. This time was not significantly different between treatment groups. B) Quantification of the average number of recurrent seizures per day after the latent period (based on 21 days of monitoring). The seizure rate was not significantly different between groups. C) Quantification of the average spike frequency of recurrent seizures. The average spike frequency was not significantly different between groups. D) The average duration of the recurrent seizures was not significantly different between groups. One-way ANOVA, $p > 0.05$. Data reported as mean ± S.E.M. Abbreviations: est, estrus; met, metestrus; di, diestrus; pro, proestrus; ovx ovariectomized female.

Figure 4.3. Quantification of the number of cresyl violet-stained neurons in the hippocampus 24 h after pilocarpine-induced status epilepticus.

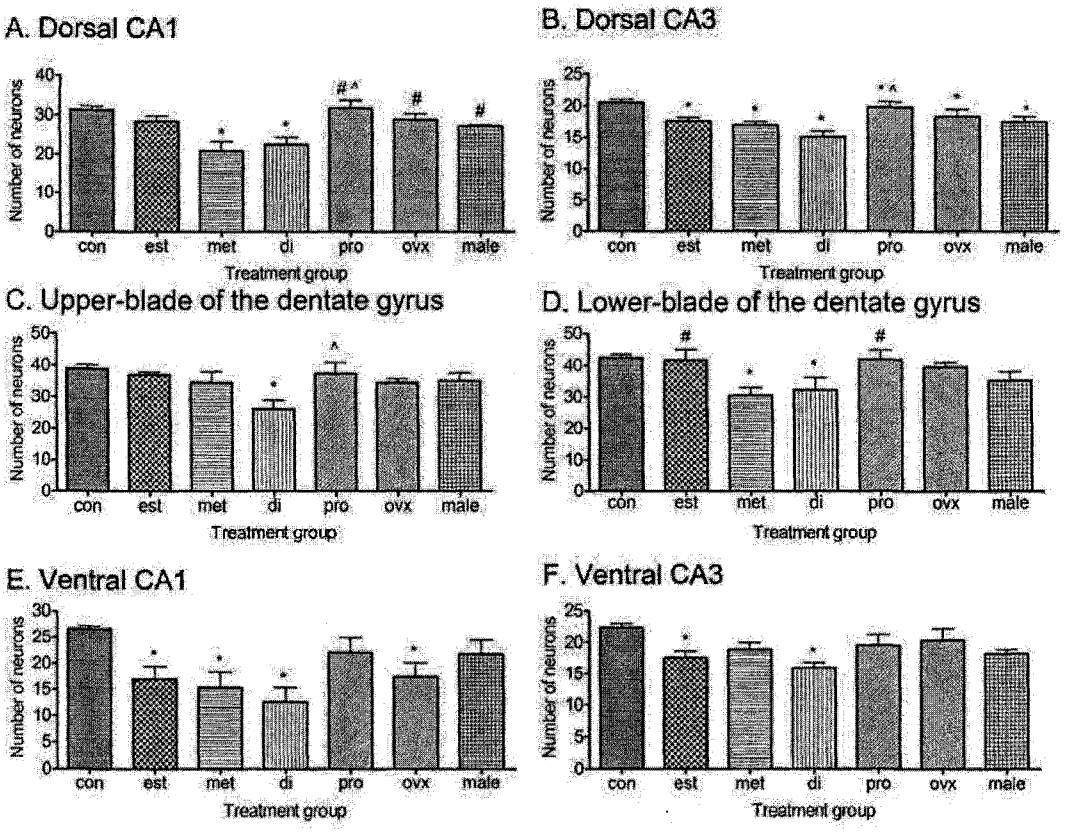

Figure 4.3. Quantification of the number of cresyl violet-stained neurons in the hippocampus 24 h after pilocarpine-induced status epilepticus. Six different regions in the hippocampus were analyzed: dorsal CA1 (A) CA3 (B), the upper and lower blade of the dentate gyrus (C, D), and the ventral CA1 and CA3 (E, F). There was a variable amount of neuronal loss that was significant from controls and between groups in the indicated regions. One-way ANOVA, significant differences between groups indicated (* $p<0.05$ from controls; # $p<0.05$ from metestrus-treated rats; ^$p<0.05$ diestrus-treated rats). Error bars indicate S.E.M. Abbreviations: con, control; est, estrus; met, metestrus; di, diestrus; pro, proestrus; ovx ovariectomized female.

Figure 4.4. Quantification of the number of Fluoro-Jade-positive neurons in the hippocampus 24 h after pilocarpine-induced status epilepticus.

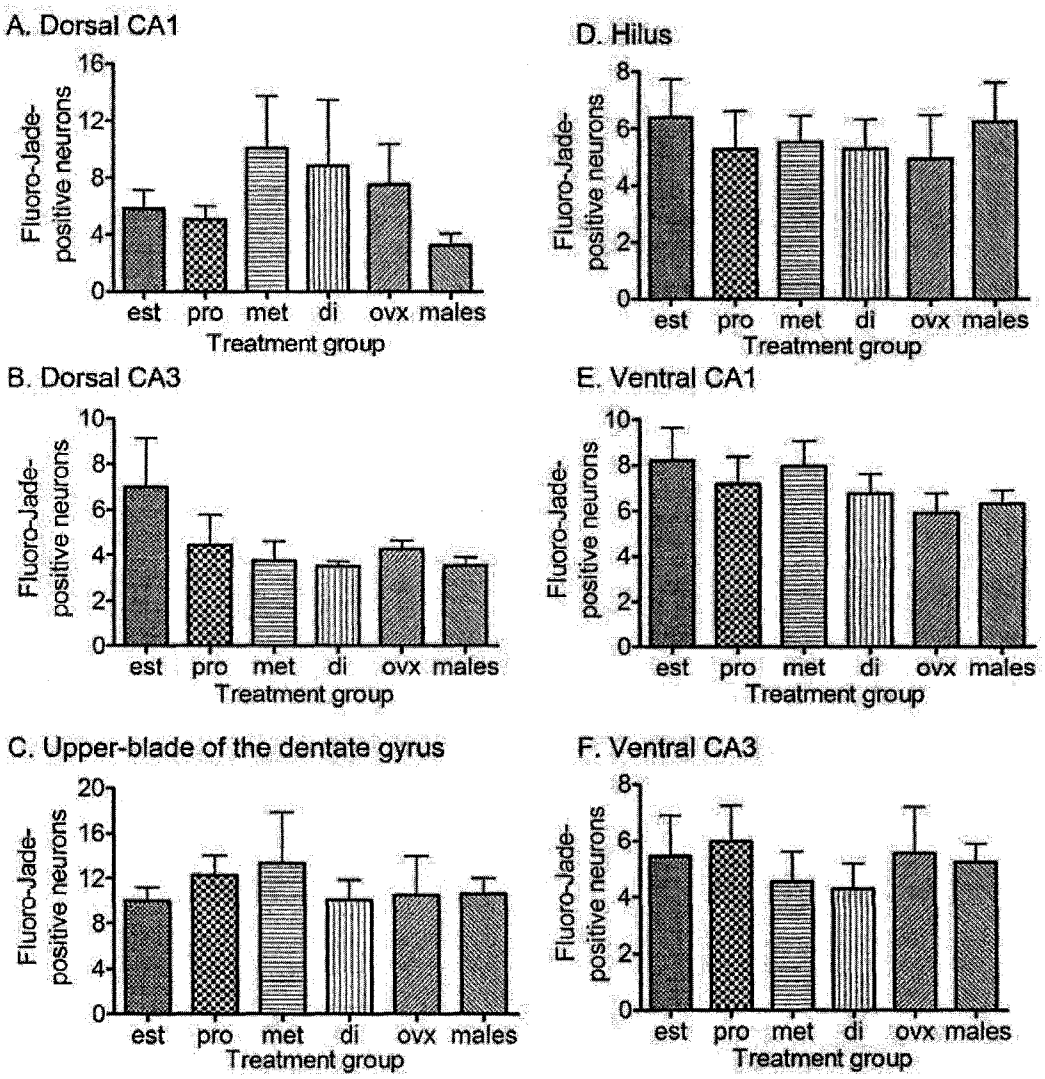

Figure 4.4. Quantification of the number of Fluoro-Jade-positive neurons in the hippocampus 24 h after pilocarpine-induced status epilepticus. Six different regions in the hippocampus were analyzed: dorsal CA1 (A) CA3 (B), the upper blade of the dentate gyrus (C), hilus (D), and the ventral CA1 (E) and CA3 (F). Control rats had no positive-stained neurons and were therefore not included on the graph ($p<0.05$ for all groups). However, there was no significant difference in the number of Fluoro-Jade-positive neurons between the treatment groups. One-way ANOVA, $p> 0.05$. Data reported as mean ± S.E.M. Abbreviations: est, estrus; met, metestrus; di, diestrus; pro, proestrus; ovx ovariectomized female.

Figure 4.5. Photomicrographs of cresyl violet-stained sections of the dorsal hippocampus 24 h and 3 wk after pilocarpine-induced status epilepticus.

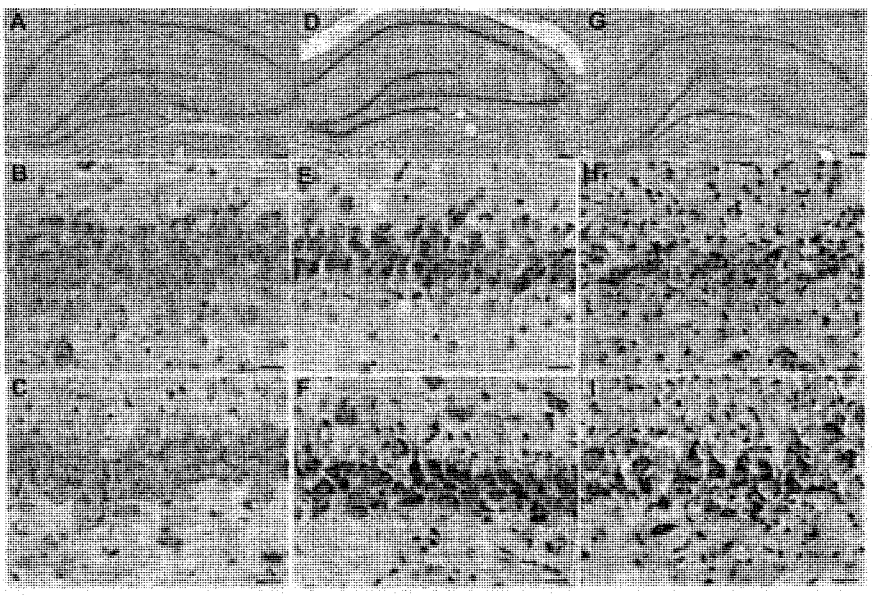

Figure 4.5. Photomicrographs of cresyl violet-stained sections of the dorsal hippocampus 24 h and 3 wk after pilocarpine-induced status epilepticus. Low magnification of the hippocampus from saline-treated (3 wk controls, A) and pilocarpine-treated rats, 24 h (D) and 3 wk (G) after status epilepticus. B-I represent a higher magnification of the CA1 (B, E, H) and CA3 (C, F, I) pyramidal layers. Note the loss of pyramidal neurons in the dorsal CA1 and CA3 regions at both 24 h (E, F) and 3 wk (H, I) after pilocarpine-induced status epilepticus compared to controls (B,C). Scale Bars: A-G 200 µm; B-I 20 µm.

Figure 4.6. Photomicrographs of cresyl violet-stained sections of the ventral hippocampus 24 h and 3 wk after pilocarpine-induced status epilepticus.

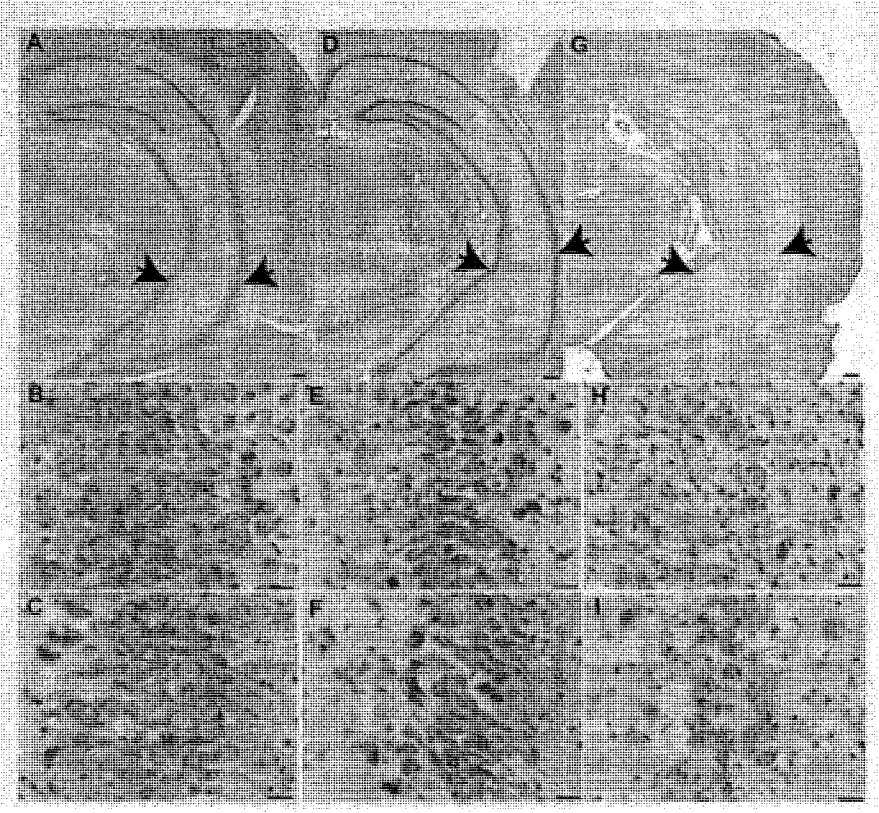

Figure 4.6. Photomicrographs of cresyl violet-stained sections of the ventral hippocampus 24 h and 3 wk after pilocarpine-induced status epilepticus. Low magnification of the hippocampus from saline-treated (3 wk controls, A) and pilocarpine-treated rats, 24 h (D) and 3 wk (G) after status epilepticus. B, C, E, F, H, and I are higher magnifications of the areas highlighted by arrowheads. Note the pyramidal neuron loss in the ventral CA1 and CA3 of rats 24 h (E, F) and 3 w (H, I) after pilocarpine-induced status epilepticus compared to controls (B,C). Scale Bars: A-G 500 μm; B-I 20 μm.

Figure 4.7. Quantification of cresyl violet-stained neurons in the hippocampus 3 wk after pilocarpine-induced status epilepticus.

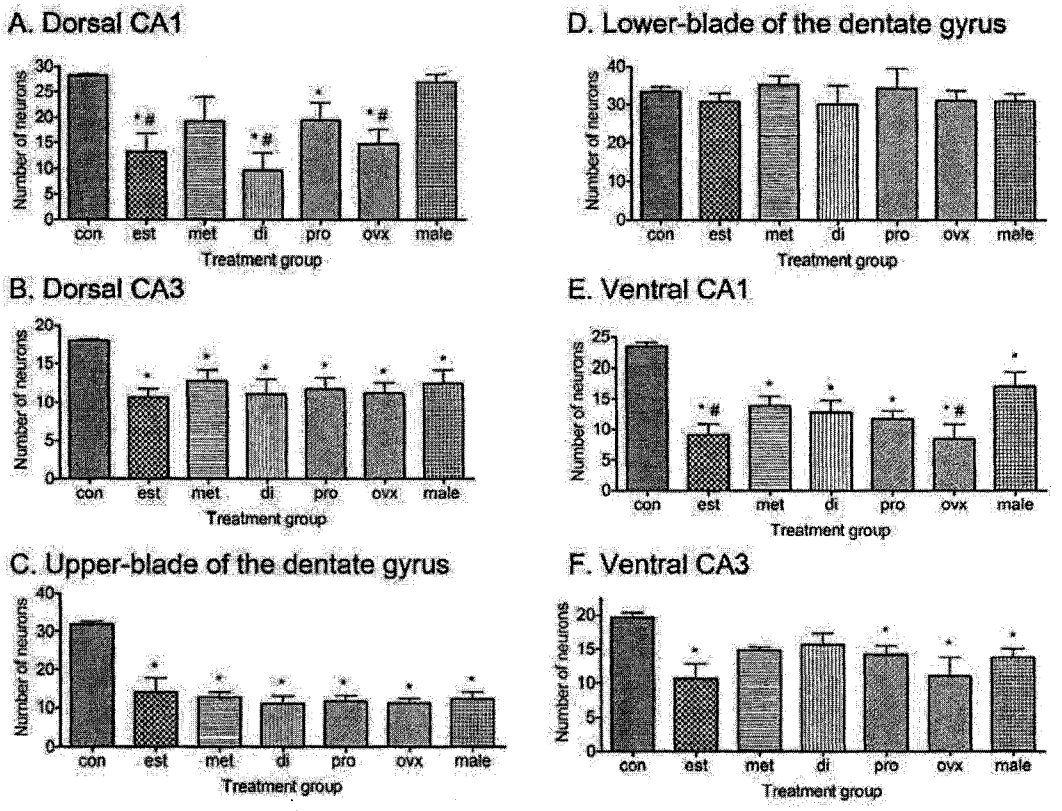

Figure 4.7. Quantification of cresyl violet-stained neurons in the hippocampus 3 wk after pilocarpine-induced status epilepticus. Six different regions in the hippocampus were analyzed: dorsal CA1 (A) CA3 (B), the upper and lower blade of the dentate gyrus (C, D), and the ventral CA1 and CA3 (E, F). There was neuronal loss that was significant from controls and between groups in the indicated regions. One-way ANOVA, significant differences between groups indicated (* $p<0.05$ from controls; # $p<0.05$ from males). Error bars indicate S.E.M. Abbreviations: con, control; est, estrus; met, metestrus; di, diestrus; pro, proestrus; ovx ovariectomized female.

Figure 4.8. Quantification of the number of Fluoro-Jade-positive neurons in the hippocampus 3 wk after pilocarpine-induced status epilepticus.

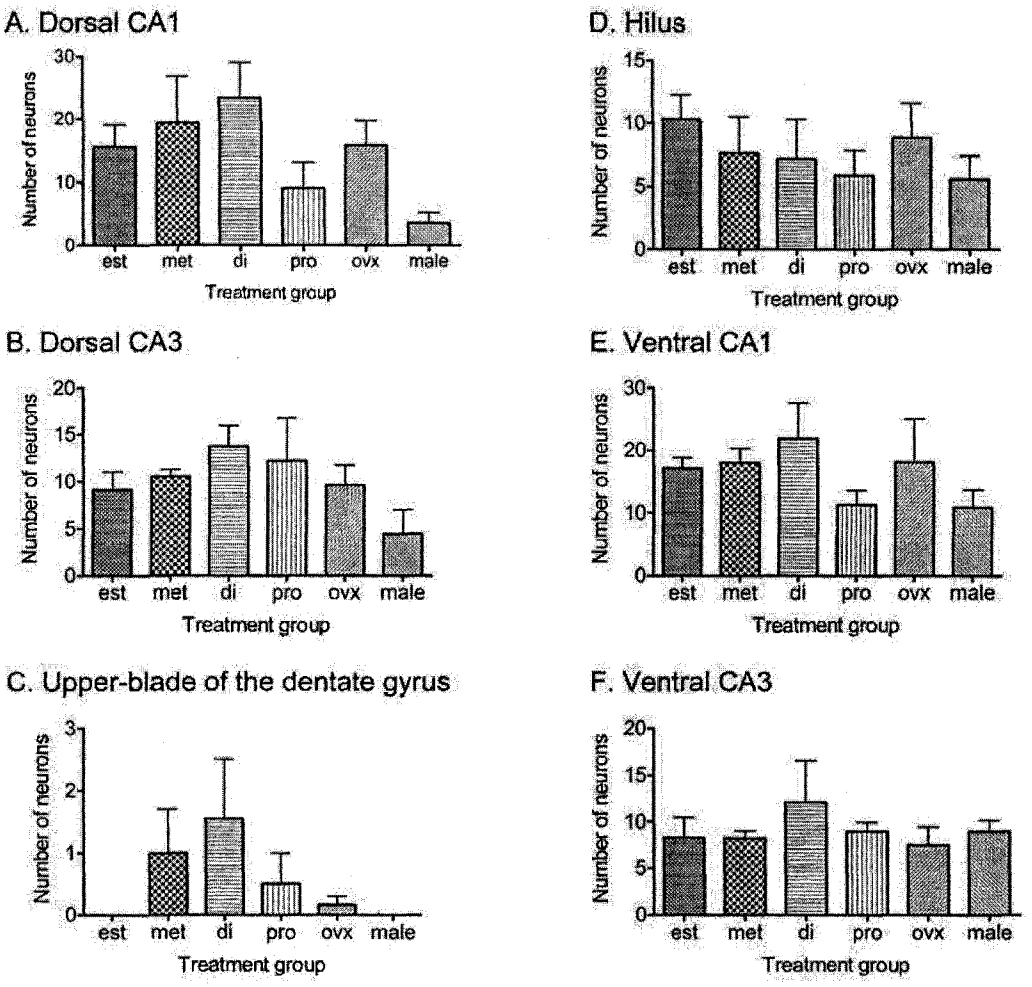

Figure 4.8. Quantification of the number of Fluoro-Jade-positive neurons in the hippocampus 3 wk after pilocarpine-induced status epilepticus. Six different regions in the hippocampus were measured: the dorsal CA1 (A) CA3 (B), the upper and lower blade of the dentate gyrus (C, D), and the ventral CA1 and CA3 (E, F). Control rats ha no positive-stained neurons and were therefore not included on the graph ($p<0.05$ from all groups). At 3 wk after SE, there was no significant differences in the number of Fluoro-Jade-positive neurons between the treatment groups. One-way ANOVA, $p> 0.05$. Data reported as mean ± S.E.M. Abbreviations: est, estrus; met, metestrus; di, diestrus; pro, proestrus; ovx ovariectomized female.

Figure 4.9. Photomicrographs of Fluoro-Jade-positive neurons in the hippocampus 24 h and 3 wk after pilocarpine-induced status epilepticus.

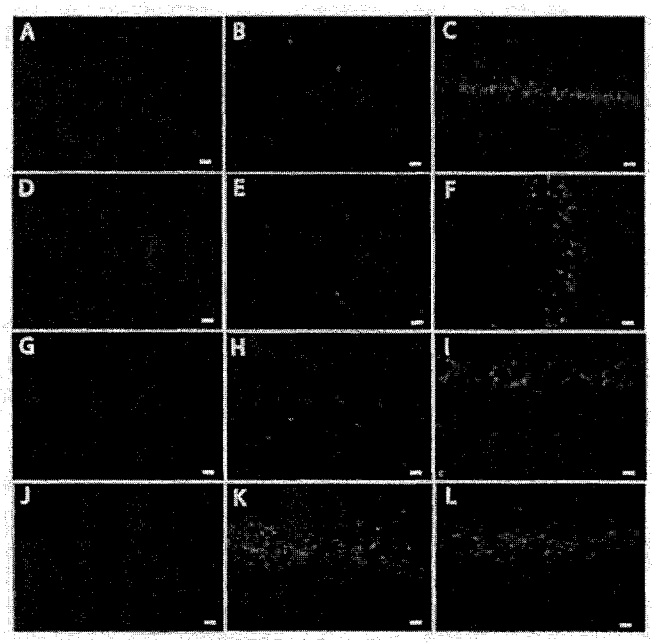

Figure 4.9. Photomicrographs of Fluoro-Jade-positive neurons in the hippocampus 24 h and 3 wk after pilocarpine-induced status epilepticus. There were no Fluoro-Jade-positive neurons in the control rats (A, D, G, J). Fluoro-Jade-positive neurons were present at both 24 hr (B, E, H, K) and 3 wk (C, F, I, L) after pilocarpine-induced status epilepticus. The dorsal CA1 (A-C), dorsal CA3 (D-F), ventral CA3 (G-I), and ventral CA1 (J-L) regions of the hippocampus are shown. Scale Bars: A-L 50 μm.

Figure 4.10. Summary of the number of days spent in each stage of the estrous cycle over a 3 week period.

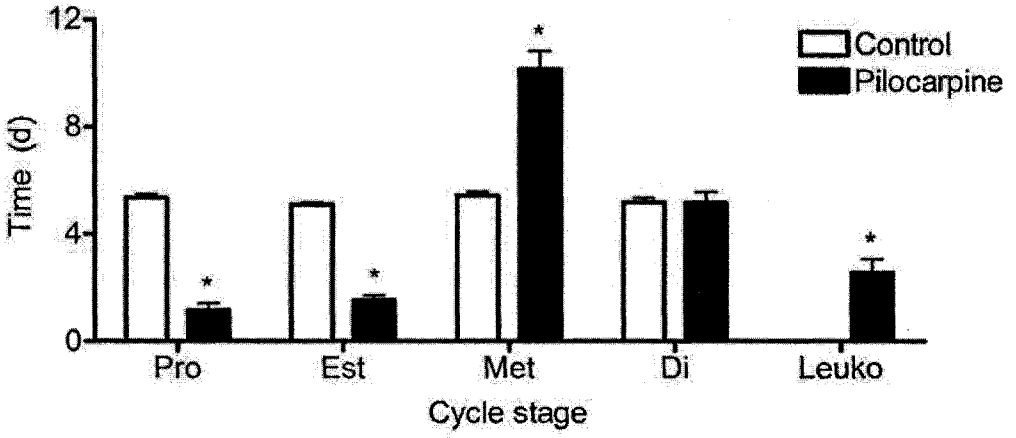

Figure 4.10. Summary of the number of days spent in each stage of the estrous cycle over a 3 week period. The stage of the estrous cycle was determined based on the cytology from vaginal smears. In the 3 wk pilocarpine-treated group (pilocarpine, black bar), the time spent in metestrus was lengthened and the time spent in proestrus and estrus was shortened when compared to the 3 wk saline-treated group (control, open bar). In addition, only the pilocarpine-treated group exhibited cytology consisting of only leukocytes. T-test,* $p<0.05$, t-test. Error bars indicate S.E.M. Abbreviations: est, estrus; met, metestrus; di, diestrus; pro, proestrus; leuko, leukocytes only.

Figure 4.11. Electrographic analyses of recurrent seizure parameters across the estrous cycle after pilocarpine-induced status epilepticus.

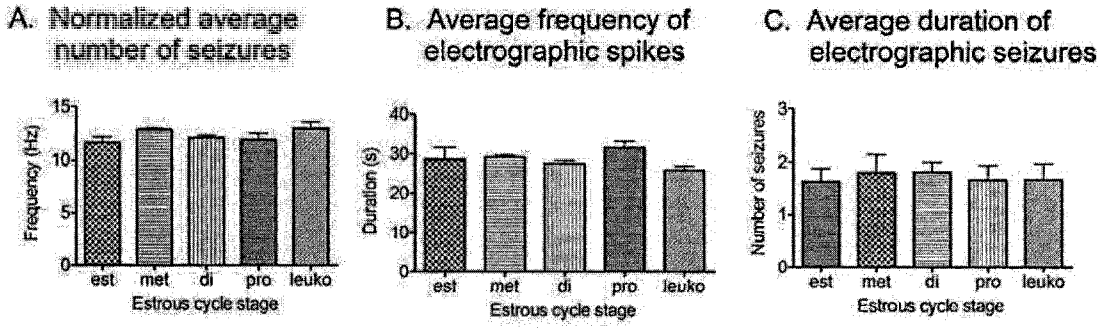

Figure 4.11. Electrographic analyses of recurrent seizure parameters across the estrous cycle after pilocarpine- induced status epilepticus. The estrous cycle was determined as previously described. A) Quantification of the average number of electrographic seizures was tabulated and normalized based on the relative time spent in each cycle stage. B) A summary of the average spike frequency of electrographic seizures on the different days of the estrous cycle. C) A comparison of the average duration of electrographic seizures across the different stages of the cycle. One-way ANOVA, $p>0.05$. Data reported as mean ± S.E.M. Abbreviations: est, estrus; met, metestrus; di, diestrus; pro, proestrus; leuko, leukocytes only.

Chapter 5. Discussion

Status Epilepticus (SE). Pilocarpine was successful in inducing SE in both mice and rats. In the first study, it was important to establish that the GnRH-eGFP mouse strain was susceptible to pilocarpine and hence, a valid model of temporal lobe epilepsy. In the second study, more quantitative measures were used to demonstrate that sex and the estrous cycle had no marked influence on the various quantitative parameters of pilocarpine-induced SE.

Our data do not support previous studies which have reported sex and estrous cycle-dependant differences on SE. However, these studies were based on the behavioral observation of "latency", which is more accurately described as time from injection of the chemoconvulsant to the first behavioral seizure, and often reflects the variability in the administration of the chemoconvulsant (possibly pharmokenetics). This phenomenon is illustrated in a study where different, and sometimes opposite sex differences to induction of SE were obtained after intravenous versus interperitoneal administration of various chemoconvulsants (Pericic and Bujas, 1997). We attempted to address this issue in our methods by using repeated injections of pilocarpine until the rat had

seizures, insuring that enough chemoconvulsant had reached active levels within the brain.

Additionally, discrepancies between previous studies may be attributed to different induction methods of SE. This would indicate that sex differences in susceptibility to SE may actually reflect sex differences in the receptors for the method of induction/chemoconvulsant that is used. In fact, GABA, muscarinic, and NMDA receptors have all been shown to be directly modulated by sex and hormones (Marriott and Korol, 2003; Daniel and Dohanich, 2001; Romeo et al., 2005; Connell et al., 2007; Cyr et al., 2001; Foy et al., 1999; Wilson, 1996; Olsen et al., 1988; Cardoso et al., 2004), and these receptors are the main targets of popular chemoconvulsants (De Deyn et al., 1990; Smolders et al., 1997; Coyle, 1987; Schneider et al., 2006).

Lastly, clinical studies based on large population studies indicate that age and etiology have the greatest influence on SE, and not sex (Towne et al., 1994; Delorenzo et al., 1992; Chin et al., 2004; Vignatelli et al., 2005). Here, we report that males have a higher mortality rate than female rats in proestrus in this study. However, our results indicate that weight was a factor in mortality, and since the weight of male rats was significantly higher than all other treatment groups, we conclude that the increase in mortality is potentially due to variability in dose/administration of the pilocarpine.

In addition, the use of EEG recordings in our study demonstrates that the electrographic parameters we measured *during* SE were not significantly different between groups. A similar electrographic insult would presumably lead

to the development of similar neuronal loss and gliosis, and recurrent seizures, which are hallmark pathologies associated with temporal lobe epilepsy.

Recurrent Seizures. Pilocarpine-induced SE resulted in recurrent spontaneous seizures in both studies. The severity of SE or the extent of neuronal damage did not directly translate into seizure severity in either study. In the second study, the frequency of electrographic spikes during recurrent seizures was negatively correlated with the number of neurons in the upper-blade of the dentate gyrus (animals with higher average frequency had fewer neurons) and positively correlated with the number of Fluoro-Jade-positive neurons in the ventral CA3 (higher frequency results in more degenerating neurons). While certain treatment groups (proestrus at 24 h and males at 3 w) had less neuronal loss, all treatment groups had similar seizure rates after 3 wks. Again, this argues that sex and hormones do not have a significant impact on the acquisition or expression of temporal lobe epilepsy.

Another clinically relevant question to examine was how sex or estrous cycle stage at the induction of SE may influence the progression to recurrent seizures and the associated hippocampal damage. We found that sex and estrous cycle stage did not affect the latency to onset of recurrent seizures, the average number, frequency of electrographic spikes, and duration of recurrent seizure frequency 3 weeks after pilocarpine-induced SE.

Hippocampal Neuronal Damage. While the hippocampus is not the only structure that is damaged in both human and animal models of temporal lobe epilepsy, it is a reliable indicator for both abnormal electrical discharges and

seizures (Margerison and Corsellis, 1966; Mathern et al., 2002; Buckmaster and Dudek 1997; Roch et al., 2002) and is a key hallmark in temporal lobe epilepsy. Both studies have demonstrated progressive neuronal loss which lends further support to the hypothesis that spontaneous seizures and epilepsy leads to progressive neuronal cell loss (Bertram and Cornett, 1993, 1994; French et al., 1993; Mathern et al., 1995; Meldrum, 1997; Hellier et al., 1998; Tasch et al., 1999; Fuerst et al., 2001; Gorter et al., 2001; Roch et al., 2002).

In the first study, pyramidal neuron loss in mice was significant in the CA1 region of hippocampus in the 1 wk (acute) treatment group, with variable neuronal loss in the CA3 region that was non-significant. After 3 mo, the chronic group had progressive neuronal loss that was significant in both the CA1 and CA3.

In the second study, rats had more consistent damage in the dorsal CA3 and ventral CA1 than other areas in the hippocampus 24 hours after pilocarpine-induced SE. After 3 wks, rats showed an increase in neuronal cell loss and gliosis compared to those at 24 hours, with a more pronounced loss in the dorsal CA3, ventral CA1, and upper-blade of the dentate gyrus regions of the hippocampus. This may indicate that these areas are more susceptible to neuronal damage by pilocarpine-induced SE.

Hippocampal damage was more variable 24 h after pilocarpine-induced SE than after 3 wks. Female rats that entered SE during metestrus and diestrus had more hippocampal damage than other groups, and female rats treated during proestrus had significantly less damage than these groups in all (sampled)

areas of the dorsal hippocampus. Since relative levels of estrogen are high the morning of proestrus and low in the morning of both metestrus and diestrus, our data lends further support to the hypothesis that estrogen is neuroprotective in seizure models (Reibel et al., 2000; Veliskova et al., 2000; Hoffman et al., 2003), at least short-term. However, ovx female and male rats (low-to-no circulating estrogen) also showed a trend for less damage, which might indicate that estrogen and the estrogen receptors are not the only factors mediating neuronal protection.

Hippocampal damage was more consistent between groups 3 wks after pilocarpine-induced SE. All of the female groups had similar neuronal cell loss and the differences between the proestrus and ovx treated rats compared to other groups have disappeared. This would suggest that estrogen's neuroprotective effect is only mediated on a short-term basis and does not affect the long-term, progressive neuronal damage that is associated with SE and/or temporal lobe epilepsy.

Interestingly, males had significantly less neuronal damage in both the dorsal and ventral CA1 after three weeks compared to the other treatment groups. These regions (dorsal and ventral CA1), and the lower blade of the dentate gyrus were regions of the hippocampus that did not experience a significant neuronal loss after 3 wks (the count was similar at 3wks vs 24hr). Since male rats had a trend for increased mortality, it is possible that the surviving rats experienced less of an insult, and as a result, had less damage. This may also suggest that males are less susceptible to neuronal damage in

these areas after three weeks, which could be mediated either by testosterone and/or its metabolites, or some other unknown mechanism.

Positive Fluoro-Jade staining was present at both 24 hrs and 3 wks after pilocarpine-induced SE. This indicates that the brain (hippocampus) starts to undergo cell death almost immediately and progressively continues to die up to 3 wks after an insult. The increase in positive neurons at the 3 wk time point would indicate that either that the process of neuronal death caused by the initial injury (SE) had not yet reached a maximum at 24 hrs or that the additional insult of recurrent seizure activity induced additional neuronal loss. While additional time points would be necessary to resolve which mechanism(s) are responsible for the increase in Fluoro-Jade staining, these data again support the hypothesis that epilepsy has temporal progression of neuronal loss (Bertram and Cornett, 1993, 1994; French et al., 1993; Mathern et al., 1995; Hellier et al., 1998; Tasch et al., 1999; Fuerst et al., 2001; Gorter et al., 2001).

Altered Cyclicity. A key question, particularly as it relates to catamenial epilepsy and reproductive problems of women with epilepsy, is whether the estrous cycle is altered after pilocarpine-induced SE. Pilocarpine-induced SE altered ovarian cyclicity in both studies, which supports the hypothesis that repeated seizures/SE disrupts normal reproductive function (Amado et al., 1987; Amado and Cavalheiro, 1998; Bhanot and Wilkinson, 1982; Edwards et al., 1999; Edwards et al., 2000). In the first study, mice stopped cycling normally immediately after pilocarpine-induced SE, and did not resume cycling (no consecutive, 4 day cycle). There was an overall decrease in the time spent in

proestrus and an increase in abnormal cytology (only leukocytes) after 1 wk. The changes in the estrous cycle were not mediated by pilocarpine because mice that received pilocarpine, but did not enter SE, resumed normal cycling. In the second study, female rats also stopped cycling normally immediately after pilocarpine-induced SE. They spent a reduced time in proestrus and estrus and in increased time spent in metestrus after pilocarpine-induced SE. The results from both studies suggest that there are anovulatory estrous cycles, similar to those observed clinically (Herzog et al., 1997) and in other rat models with temporal lobe epilepsy (Bhanot and Wilkinson 1982; Amado and Cavalheiro, 1998).

These abnormal cycles are reflected in the ovarian histology, where mice that experienced pilocarpine-induced SE had an increased number of abnormalities including hemorrhagic follicles and cyst-like formations. It is likely that the chronic acyclicity and presumable alteration in hormones are responsible for the changes in ovarian histology.

Currently, it is unknown how epilepsy and seizures cause reproductive abnormalities. Clinical and animal models data have both provided evidence that seizure activity alters hypothalamic peptide secretion (Trimble, 1978; Pritchard et al., 1983; Dana-Haeri et al., 1983; Bhanot and Wilkinson, 1982; Edwards et al., 1999a,b; Edwards et al., 2000), which may lead to chronic changes in reproductive function. Control of seizure activity either with antiepileptic drugs or surgery relieved reproductive disorders in people with previously uncontrolled seizures (Bauer et al., 2000). It is therefore plausible that seizures arising in

temporal structures such as the hippocampus and amygdala might disrupt normal hypothalamic function via the extensive interconnections between the hypothalamus and limbic system (Fawley et al., 2006).

These experiments also addressed the issue of catamenial epilepsy by analyzing the spontaneous recurrent seizures as they related to the estrous cycle in intact female rats. In this case, the data were grouped together independent of when they were treated during the estrous cycle in order to increase statistical power primarily because we found no obvious differences during our examination of susceptibility to SE as a function of estrous cycle. Furthermore, our data show that when the number of seizures was analyzed across this time period, normalizing for differences in the duration of the different stages of estrous, there was no change in seizure frequency. In addition, we also analyzed whether the frequency of electrographic spikes or duration of spontaneous seizures correlated with estrous cycle stage. Again, we found no significant differences on the different days of the estrus cycle.

One possible caveat is that hormonal changes could be occurring on a faster time scale than our sampling accounts for (hourly or multi-hourly basis within those stages of the estrous cycle) and we cannot be sure that a more detailed analysis of hormonal levels would not reveal a correlation between part of the estrous cycle and seizure frequency.

Another potential caveat is that we only studied recurrent seizures in first three weeks after SE. Given that the latent period was up to a week, and the seizure rate is low, we may not have seen a difference in seizure frequency as a

function of stage of estrous cycle because we had too few seizures. It is conceivable that animals later in the process of epileptogenesis with higher seizure rate might show a relationship to particular parts of the estrous cycle or even hormonal levels.

Thus, although the estrous cycle was altered, there was no clear effect on seizure frequency when considered across estrous cycle. In spite of our concern of the low seizure frequency, the total number of animals in this series was 19, which would partially mitigate the problem of low seizure frequency. Another potential issue is that some (15.8%) animals appeared to show seizures that coincided with particular parts of the cycle, and it is conceivable that since only roughly 30 percent of women have catamenial epilepsy, and that this patient group is not homogenous (i.e., at least three types of catamenial epilepsy) (Herzog et al., 1997), that a larger sample size with a more coordinated analysis of hormone levels could reveal a relationship between hormone state and the frequency of seizures.

GnRH. Changes in the estrous cycles are not mediated by a detectable reduction in GnRH-positive neurons. There were no qualitative change in GnRH-positive fibers between control and pilocarpine treated mice. While this is in contrast to Amado et al., 1993 and Friedman et al., 2002, discrepancies in the data may simply reflect methodological differences, both in the model of epilepsy used and GnRH antibody. However, there is also evidence to suggest that there is functional redundancy in the GnRH network so that only between 12-34% of the population is required for normal reproductive function (Herbison et al.,

2008). This would indicate that a gross reduction in GnRH-positive neurons and fibers would be necessary before it would cumulate as a disruption the estrous cycle. Alternatively, there may be a reduced number of activated GnRH neurons that participate in LH surge activity that is similar to those observed in the age-associated reduction in fertility (Lloyd et al., 1994).

Summary. In summary, the results suggest that the observed changes in reproduction after pilocarpine-induced SE and the resulting epilepsy are not due to a reduction in GnRH-positive neurons, and that they might alternately be due to either altered synaptic input to these neurons, altered regulation of the network, or changes in the HPG axis other than the hypothalamus. The results also suggest that estrous cycle and sex can influence certain aspects of susceptibility, death, and extent of pyramidal neuron loss in the hippocampus. However, once SE is induced, rats experience a comparable amount of electrographic SE and hormones /sex do not seem to be predictors of long-term hippocampal damage or recurrent seizures. This would lend support to the hypothesis that once SE occurs, a cascade of neuropathological alterations including neuronal damage occurs in the brain that leads to recurrent seizures and temporal lobe epilepsy. Intervening with anti-epileptic drug therapies may be crucial not only to retard brain damage and control seizures but also to potentially prevent the reproductive abnormalities associated with seizures and temporal lobe epilepsy.

These studies have established two different rodent models of SE/temporal lobe epilepsy-induced reproductive alterations to explore these

problems. These models may be useful in further studies to examine how to rescue and/or prevent reproductive abnormalities after SE, how anti-epileptic drug therapy may alter the course of these abnormalities and epilepsy, and potentially catamenial epilepsy. The mouse model also provides an excellent resource to examine electrophysiological changes to the GnRH neurons after SE.

Chapter 6: References

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