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Role of the Claustrum in Kindling of Generalized Seizures

by

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B.Sc., Carleton University, 1991
M.Sc., Carleton University, 1993

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Psychology

We accept this dissertation as conforming to the required standard

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ABSTRACT

The precise neuroanatomical pathways underlying seizure genesis and propagation are largely unknown. Current evidence suggests that epileptiform activity may travel along preferred anatomical routes and that some structures may act as "gates" that funnel and spread seizure activity throughout the brain. Burchfiel and Applegate (1989a) have proposed a gating hypothesis to explain kindled seizure propagation. According to this hypothesis, kindling involves three distinct phases that are separated by two crucial gates. The first gate mediates the expression of partial seizures, and the second gate mediates the expression of fully generalized seizures. Little work has been done in determining which anatomical sites contain these gates, in particular the second gate. The following experiments set out to investigate the role that the claustrum may play in mediating the second gate, responsible for the expression of kindled generalized seizures.

Experiments 1 and 2 utilized correlative strategies to address the claustrum's participation in kindling. In Experiment 1, kindling was evoked directly from claustrum and these properties were compared with the nearby structures of the amygdala, insular cortex, and perirhinal cortex. The claustrum generally exhibited much more potent epileptogenic attributes than the other structures, including quick progression to seizure generalization and more vigorous and sustained convulsions. However, the claustrum shared many electrographic and convulsive properties with the insular and perirhinal cortices, including a two phase development of generalized seizures, rapid progression to seizure generalization, and quick onset to limb convulsions.

In Experiment 2, further dissociations were detected between claustrum and amygdaloid kindling by changes in molecular products linked with neural plasticity. Claustrum kindling was associated with generally more intensive expression of claustrum in kindling. Experiments 3 and 4 used more direct approaches to address the role of the claustrum in kindling. In Experiment 3, alternating stimulation between the claustrum and amygdala demonstrated that the claustrum was capable of arresting amygdaloid kindling at the partial seizure stages. This kindling antagonism effect was not observed with stimulation of the superficial insular cortex, perirhinal cortex, or piriform cortex.

In Experiment 4, lesions applied to the claustrum were effective in delaying, but not blocking amygdaloid kindling. These delays were in amygdaloid kindling were produced by small lesions restricted to the ipsilateral anterior claustrum.

Taken together these data suggest that the claustrum may represent the crucial mediator of the second gate responsible for kindled seizure generalization.

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GENERAL INTRODUCTION

For as long as epilepsy has been studied experimentally, clinical and basic researchers have struggled to identify the crucial brain regions responsible for promoting and spreading of seizure activity. Even with the advent of so many new and refined techniques for studying the brain, we appear far from fully understanding the epileptic brain.

The term 'epilepsy' is a broad term that refers to a wide variety of recurrent seizure disorders that are generally classified according to brain region involved as the dominant focus. Common seizure disorders originate from the temporal lobe, which are classified as complex partial seizures (Gastaut, Gastaut, Gonçalves e Silva, & Fernandez-Sanchez, 1975; Penfield, 1954) or more recently as mesial temporal lobe epilepsy (MTLE) (Engel, 1998). It has long been known that patients with MTLE commonly have sclerotic lesions in the temporal lobe structures, including the hippocampus, amygdala, parahippocampal gyrus, temporal pole, and selective temporal cortical areas (Gloor, 1992; Penfield, 1954). Furthermore, most of these temporal lobe structures display abnormal ictal and interictal discharges from intracranial EEG recordings (Baumgartner, Lindinger, Ebner, Aull, Serles, Olbrich, Lurger, Czech, Burgess, & Lüders, 1995; Blume, Borghesi, & Lemieux, 1993; So, Gloor, Quesney, Jones-Gotman, Olivier, & Andermann, 1989; Spencer, Spencer, Williamson, & Mattson, 1990) and direct stimulation of these structures can evoke epileptic symptoms (Feindel & Penfield, 1954; Penfield, 1954).

Despite our knowledge of the neuropathology and the contribution of many of these brain sites to the symptomatology of temporal lobe seizures, the precise neuroanatomical pathways underlying seizure genesis and propagation still evade us. Part of the problem lies in the fact that generalized seizure discharge spreads rapidly and disperses throughout the brain. Many structures eventually become involved in the discharge, making it difficult to determine which propagation pathways are the critical ones. Uncovering the preferred propagation routes and crucial epileptogenic zones would enhance our understanding of the pathophysiology of epilepsy and have profound therapeutic implications. In turn, identifying the anatomical substrates of epilepsy is a necessary prerequisite for understanding the cellular and molecular mechanisms of seizures.

The Kindling Model of Epilepsy

Animal models have significantly contributed to our understanding of the mechanisms of epilepsy. The kindling model of epilepsy has provided some valuable insights into identifying potential brain structures and neural circuits that may play prominent roles in the generation and propagation of seizures. Kindling may be one of the best suited techniques[1] for the investigation of seizure activity spread since it allows for controlled and graded increases in propagation. The strength of the kindling model of epilepsy lies in its ability to *predict* the propagation of seizures throughout the brain.

Kindling refers to the eventual development of persistent seizure activity following repeated exposure to an epileptogenic agent, either an electrical stimulation or pharmacological / chemical agent, to discrete brain areas (Goddard, McIntyre, & Leech, 1969; Racine 1978). In electrical kindling the stimulation is applied, via permanently implanted wire electrodes, in brief trains of electrical pulses to a forebrain site, usually once daily. The stimulation is delivered at an intensity that is strong enough to evoke epileptiform activity in the form of focal discharges. It is this evoked focal discharge that is a necessary and sufficient condition for kindling to occur (Goddard *et al.*, 1969; Racine 1972a,b).

As the kindling stimulation is repeated over successive trials, there is progressive evolution of seizure susceptibility that is manifested in two primary ways. The first is a change in several evoked electrographic measures, including: a reduction in the threshold current required to evoke an afterdischarge (AD); an increase in the focal AD amplitude, frequency, complexity, duration; and a progressive propagation of AD to many regions of

---

[1] Note that kindling can represent *both* a phenomenon and a technique. As a phenomenon kindling can describe various features of progressive seizure states in the brain. As a technique kindling can be used as a method of procedure to carry out various manipulations to the brain.

the brain (Racine, 1972a,b). The second phenomenon is associated with the propagation of AD throughout the brain, which is the appearance of convulsive behaviours. Racine (1972b) developed a five stage progressive behavioural classification scheme to quantify the convulsive profiles of limbic kindling in the rat: 'stage 1' involves automatisms of the facial musculature, including chewing-like jaw movements; 'stage 2' has the same facial automatisms with the addition of head bobbing; 'stage 3' is characterized by the addition of unilateral clonic forelimb movement (usually on the contralateral side from the stimulation); 'stage 4' represents bilateral clonic forelimb movements; and 'stage 5' is characterized bilateral clonus with rearing on the hindlimbs and loss of postural control (due to hindlimb clonus; e.g., McIntyre & Kelly, 1993). Some investigators have added a preceding stage of nonconvulsive symptoms (i.e., 'stage 0') that includes arrest of ongoing behavior followed by vigorous exploratory behavior (e.g., Duchowny & Burchfiel, 1981; Michelson & Buterbaugh, 1985). Stages 1 and 2 appear relatively early in the kindling process and are sometimes referred as partially generalized convulsions, while stages 3 to 5 tend to appear in rapid succession late in the kindling process and are usually referred to as fully generalized convulsions (Racine, Ivy, & Milgram, 1989). Most consider stage 5 to be the end point of the kindling process since most animals reach a steady state of fully generalized seizures. However, with the triggering of many multiple stage 5 seizures animals will display more complex generalized behaviours that tend to be less predictable and reliable (Michael, Holsinger, Ikeda-Douglas, Cammisuli, Ferbinteanu, DeSouza, DeSouza, Fecteau, Racine, & Milgram, 1998; Pinel & Rovner, 1978)

At most limbic sites, kindling is extremely robust and the changes appear to be permanent, transsynaptic, and widespread without any apparent gross tissue damage (Dennison, Teskey, & Cain, 1995; Goddard *et al.*, 1969; Racine, 1978). Early work demonstrated that amygdaloid kindling can persist with relatively little degradation over intervals of 3 to 12 months (Goddard *et al.*, 1969; Wada, Sato, & Corcoran, 1974). Kindling also exhibits the 'transfer effect' whereby the kindling of one site usually facilitates the subsequent kindling of another site in the same brain (Goddard *et al.*, 1969;

Racine, 1972b). Finally, it has been reported that spontaneous recurrent seizures can occur in the absence of an evoked stimulus if kindling stimulation is continued long after the establishment of stage 5 seizures in the rat (Michael *et al.*, 1998; Pinel & Rovner, 1978), in the cat (Wada *et al.*, 1974), and in the primate (Corcoran, Cain, & Wada, 1984). Animals displaying spontaneous seizures also exhibit pathological events such as preictal, ictal, postictal and interictal EEG spiking (Kairiss, Racine, & Smith, 1984), similar to those observed in human epileptics.

Many researchers agree that limbic kindling effectively models partial complex seizures. This is not surprising if one considers that temporal lobe structures are the sites most susceptible to kindling. Support for kindling's validity as a model of complex partial seizures comes from many sources, including: (1) the similarity in EEG patterns of hippocampal and amygdaloid kindled seizures to human complex seizures; (2) the similarities of the behavioural patterns of stage 1 and 2 kindled seizures to those of complex partial seizure origin; (3) the corresponding anticonvulsant pharmacologies between kindled and human complex partial seizures; (4) the presence of secondary foci in kindling as well as in human epilepsies; (5) the occurrence of interictal spike transients in limbic EEG recordings of both kindled and complex partial epileptics; (6) the occurrence of spontaneous seizures in kindled animals; and (7) the contention that kindling-like processes may occur in humans (Racine *et al.*, 1989; Sato, Racine, & McIntyre, 1990). One important distinction between the kindling model and human complex partial seizures is in the process by which the epileptic condition is initially invoked: with kindling the electrographic and behavioural responses are artificially evoked, whereas human epilepsy is a condition brought upon by any number different underlying conditions such as infectious disease, lesion, head injury, metabolic imbalances, genetic predisposition, etc. (Ehrman & Parsons, 1981). Despite the differences in the induction of the seizures, it is important to emphasize that the critical neuronal changes that support the epileptic condition are probably the same in both cases (Racine *et al.*, 1989). In fact, it has been suggested that epilepsy, a progressive disorder, may undergo kindling-like processes whereby the further development of epileptogenic activity may be dependent upon the frequent appearance of preceding epileptiform activity (Hughes, 1985).

A Conceptual Framework of the Kindling Process

As stated in the previous section, one of the most dramatic changes that occur during kindling is the augmented propagation of the epileptiform discharge to other brain sites. As the epileptiform discharge propagates, it progressively recruits a wider range of areas at a very rapid rate. Before one attempts to identify crucial neural circuits or anatomical regions responsible for kindled seizure propagation, one must have an accurate understanding of the fundamental process of kindling (e.g., Burchfiel & Applegate, 1989a; Racine, 1978). In this section, a conceptual framework of the process of kindling will be outlined. In the section to follow, this conceptual framework will be applied to specific anatomical regions that recently have been proposed to be important mediators of seizure propagation.

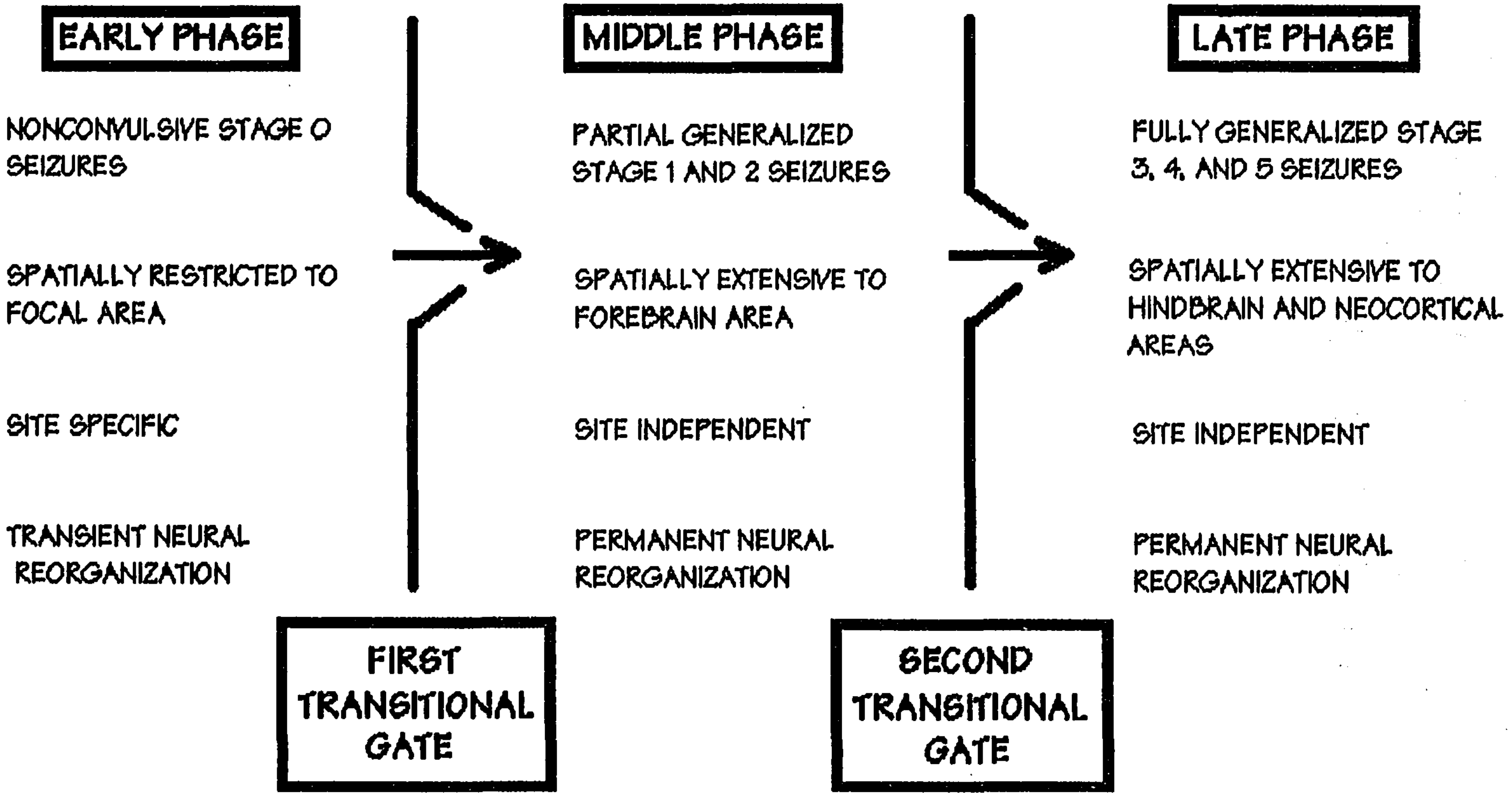
One way to view kindling is as a general, continuous, and single monolithic phenomenon whereby homogeneous and incremental changes of neuronal organization occur that are uniformly distributed over space and time. Burchfiel, Applegate, Samoriski, and Nierenberg (1998) compare this process to that of ripples radiating out from a stone thrown into a pond, such that propagation involves all possible neuronal networks simultaneously and equally. AD propagation to other parts of the brain are passively driven by the primary focal site. With this conceptualization, kindling would essentially involve the same neuronal mechanisms regardless of the structure stimulated. However, as will be revealed in the following sections, there is little evidence to support this notion. Alternatively, kindling can be viewed as involving specific, sequential, and discrete changes in preferential neuronal pathways that are not necessarily spatially or temporally uniform (e.g., Burchfiel & Applegate, 1989a; Burchfiel *et al.*, 1998; Corcoran, 1988a; Gilbert, 1994; Racine, 1978; Racine, Burnham, Gilbert, & Kairiss, 1986; Racine & McIntyre, 1986). With this hypothesis, kindling from a given structure would involve a specific preferential propagation pathway that would tap into a common and independent neuronal circuit(s) responsible for seizure generalization. Expanding on this hypothesis, Burchfiel and Applegate (1989a) have identified three specific sequential phases of seizure propagation that are separated by two distinct "gates". Each phase represents discrete transitions from one state of neural organization to another. In order for kindling to progress from one phase of seizure generalization to the next a gate must open, or a critical threshold must be crossed, to allow the propagated AD to alter the functional organization of a new element of neural circuitry. Outlined below are the three progressive phases of kindling, adapted from Burchfiel and Applegate's (1989a) 'stepwise' kindling hypothesis. Figure 1 schematically summarizes this transitional gating hypothesis of kindling. Note that throughout this dissertation, my use of seizure stages corresponds to the standard scheme developed by Racine (1972b) and not to the somewhat different scheme employed by Burchfiel and Applegate (1989a).

Early Phase - Focal Nonconvulsive Kindling

This 'prekindling' phase is characterized by stage 0, nonconvulsive symptoms that include arrest of ongoing behavior and vigorous exploratory behavior. At this phase the seizure propagation is spatially restricted to the focal area and is driven exclusively by local neurons close to the stimulation site. Any neuronal reorganization at this phase is probably transient and reversible with respect to advancement of kindling. Neuronal changes in this phase are independent of kindled seizure development, such that many of these local changes are not influenced by the kindling process (Burchfiel & Applegate, 1989a; Racine, 1972a).

The local events of the early phase of kindling primarily determine AD characteristics. Specifically, AD threshold and probably AD duration are dissociable from many kindled seizure characteristics. For example, it is well established that electrical stimulation alone can lower AD threshold without influencing the progression of kindling (Racine, 1972a). Similarly, with the transfer effect, after a primary site has been kindled to motor generalization a secondary site can subsequently demonstrate facilitated kindling with no notable change in AD threshold (Burnham, 1976). There is

Figure 1. Model of the transitional gating hypothesis of kindling. Kindling involves two discrete transitions or "gates" that divide the process into three distinct phases of neuronal organization. Adapted from Burchfiel and Applegate (1989a), who hypothesized that piriform cortex is site of the first transitional gate. More recently, Applegate et al. (1998) have suggested that the second transitional gate resides in perirhinal cortex.


EARLY PHASE
NONCONVULSIVE STAGE 0 SEIZURES
SPATIALLY RESTRICTED TO FOCAL AREA
SITE SPECIFIC
TRANSIENT NEURAL REORGANIZATION
MIDDLE PHASE
PARTIAL GENERALIZED STAGE 1 AND 2 SEIZURES
SPATIALLY EXTENSIVE TO FOREBRAIN AREA
SITE INDEPENDENT
PERMANENT NEURAL REORGANIZATION
LATE PHASE
FULLY GENERALIZED STAGE 3, 4, AND 5 SEIZURES
SPATIALLY EXTENSIVE TO HINDBRAIN AND NEOCORTICAL AREAS
SITE INDEPENDENT
PERMANENT NEURAL REORGANIZATION
FIRST TRANSITIONAL GATE
SECOND TRANSITIONAL GATE

evidence that initial AD duration at the focal site may also be independent of kindled seizure generalization. It has been shown that inhibitory neurotransmitters, such as γ-aminobutyric acid (GABA) and noradrenaline (NA) are important for the expression and development of kindled generalized seizures, respectively, but have no effect on local AD durations and/or thresholds (Applegate & Burchfiel, 1988; Corcoran, 1988b; Corcoran & Mason, 1980; Corcoran & Weiss, 1990; Jimenez-Rivera, Voltura, & Weiss, 1987; Karlsson, Klebs, Hafner, Schmutz, & Olpe, 1992; McIntyre, 1980; Shin, Silver, Bonhaus, & McNamara, 1987).

Middle Phase - Partial Generalized Kindling

Entry into the middle phase of kindling is marked by the opening of the first gate, which controls the transition from stage 0 seizures to stage 1 and 2 seizures. Once this transition gate is overcome, focal epileptiform discharge is able to spread extensively to forebrain structures that drive partially generalized motor convulsions, such as facial and head automatisms. Although the precise manner in which forebrain structures drive partial convulsions is not fully understood, some forebrain structures, such as the amygdala, do have direct connections to the masticatory motoneurons of the brainstem (Nakamura & Katakura, 1995; Sasamoto & Ohta, 1982; Takeuchi, Satoda, & Matsushima, 1988) and seizure activity generated in these forebrain regions can drive brainstem motor systems (e.g., Manni, Bortolami, Passatore, Lucchi, & Filippi, 1980; McNamara, 1986). Surmounting this first transition gate represents a breakdown of inhibitory influences, or an increase of excitatory influences, that allows focal AD to propagate to other forebrain structures. Most of the evidence points to NA as the major inhibitory component mediating this forebrain gate (e.g., Bengzon, Kokaia, & Lindvall, 1993; Burchfiel & Applegate, 1989a; Burchfiel, Applegate, & Konkol, 1986; Corcoran & Weiss, 1990; Jimenez-Rivera, Chen, Vigil, Savage, & Weiss, 1989; McIntyre, Kelly, & Dufresne, 1991). However, other inhibitory transmitters such as GABA (e.g., Bradford, 1995; Burnham, 1989; Dalby & Nielsen, 1997; Kokaia, Aebischer, Elmér, Bengzon, Kalen, Kokaia, & Lindvall, 1994; Sato, Morimoto, Okamoto, Nakamura, Otsuki, & Sato, 1990; Ueda & Tsuru, 1995) and excitatory transmitters such as glutamate (e.g., Bradford, 1995; Cain, Desborough, & McKitrick, 1988b; Croucher, Ruffle, & Bradford, 1997; Gilbert, 1988; Löscher, 1998; Mori, Wada, Sato, Saito, & Kumashiro, 1992; Morimoto & Sato, 1992; Ueda & Tsuru, 1995) and acetylcholine (e.g., Baptista, Weiss, Zocchi, Sitcoske, & Post, 1994; Cain, *et al.*, 1988b; Ferencz, Kokaia, Elmér, Keep, Kokaia, & Lindvall, 1998; Wasterlain, Morin, & Jonec, 1982; Westerberg & Corcoran, 1987) have also been implicated in mediating the early stages of kindling. Various neural substrates have been proposed to accommodate the forebrain transition gate. Some of the more prominent candidates will be reviewed in the anatomical section below.

Burchfiel and Applegate (1989a) argue that the middle phase represents the first substantial advancement in kindling. Advancement into the middle phase is the critical transition step that eventually establishes a *permanent* reorganization of neural circuits that support extensive seizure propagation. Homan and Goodman (1988) demonstrated that neural changes underlying partial kindling can persist without decrement over a prolonged period without stimulation. Once this permanent neural reorganization is established, the AD propagation is no longer dependent on the original site of stimulation. In other words, when AD from a given site gains access to the circuitry of the middle phase, that circuity will be altered (reflected by stages 1 and 2 progression) to the point where the subsequent process of kindling proceeds essentially the same, irrespective of the original site of stimulation (i.e., the rate of progression from stage 3 to stage 5 is identical from all sites) (Burchfiel & Applegate, 1989a; Burchfiel *et al.*, 1998). In contrast, the early kindling phase undergoes little or no significant development in kindling since most of the neural reorganization is transient and the evoked ADs are tied to the properties of the site of stimulation.

Support for this discrete advancement in kindling comes from many correlative kindling studies. Burnham (1975) was the first to point out that growth in AD duration in the rat tends to occur in sudden steps, which he attributed to the recruitment of additional circuitry. It is well known that kindling rates vary from different limbic sites. Although it is not often acknowledged, in most kindling studies the variability in kindling rate is not distributed equally between each of the kindling stages; instead, many more stimulation trials are spent in stages 0 to 2 than in the later stages (Burchfiel *et al.*, 1998; Corcoran, 1988a,b; Corcoran, Wada, Wake, & Urstad, 1976b; Duchowny & Burchfiel, 1981; Kirkby, Gilbert, & Corcoran, 1993; Le Gal La Salle, 1981; Löscher, Cramer, & Ebert, 1998; Michelson & Buterbaugh, 1985; Sato & Nakashima, 1975). For example, Le Gal La Salle (1981) compared the number of kindling stimulations required to reach each individual stage of seizure development from different amygdaloid nuclei and reported that kindling rates from different sites were directly proportional to the number of trials spent in stage 1 and 2 seizures, but not stages 3, 4, or 5 seizures. Burchfiel *et al.* (1998) recently surveyed the kindling rates for other limbic sites, including the entorhinal cortex, olfactory bulb, septal nucleus, and piriform cortex, and also confirmed that stages 0 to 2 account for most of the variability in kindling rates. Löscher, Cramer, and Ebert (1998) verified that differences in amygdaloid kindling from seven different rat strains were solely attributable to the amount of time spent in stage 1 seizures. Similarly, in two rat strains selectively bred for susceptibility to amygdaloid kindling, the slower kindling strain spend proportionately more time in stages 0 to 2 than the faster kindling strain; whereas the number of trials spent in stages 3 to 5 were virtually identical in the two strains (McIntyre, Kelly, & Dufresne, 1999). Further evidence from drug studies demonstrates that compounds that either suppress or accelerate kindling tend to exert their effects on the early stages, rather than the later stages of kindling. For example, application of brain-derived neurotrophic factor (BDNF) (Larmet, Reibel, Carnahan, Nawa, Marescaux, & Depaulis, 1995; Reibel, Larmet, Carnahan, Lê, Marescaux, & Depaulis, 1998), various glutamate antagonists (Cain *et al.*, 1988b; Gilbert, 1988; Löscher, 1998), and chemicals that alter NA levels (Corcoran & Weiss, 1990; Michelson & Buterbaugh, 1985; Pelletier & Corcoran, 1993) exert their effects primarily on stages 0, 1, and 2 of kindling.

The kindling antagonism paradigm has also provided strong support for the existence of distinct and discrete transitional phases in kindling. Kindling antagonism refers to a technique in which concurrent stimulation is alternatively delivered to two different forebrain sites, such that one site receives a stimulation on one day and the other site receives a stimulation on the next day (Burchfiel, Serpa, & Duffy, 1982; Kirkby *et al.*, 1993). With this pattern of stimulation, one site can exhibit typical progression to stage 5 seizures whereas the other site may show little or no development of seizures. This suppression of kindling is expressed specifically at either stage 0 or stages 1 / 2 (Burchfiel & Applegate, 1989a). Evidence suggests that the suppression of kindling is not due to the masking of behavioural convulsions, but instead represents an actual *arrest* of the kindling process. For example, it has been demonstrated that once alternating stimulation is terminated and stimulation is delivered to only the suppressed site, the same number of kindling stimulations is required to reach a stage 5 seizure from the suppressed site as would be required from the same site in a naive animal (Burchfiel & Applegate, 1989a; Duchowny & Burchfiel, 1981; Kirkby, Gilbert, Westcott, & Corcoran, 1995). Burchfiel and Applegate (1989a) propose that the arrest in seizure development observed with kindling antagonism reflects an inability of a site to open a transition gate and enter into the next phase of kindling. Suppressed sites that exhibit only stage 0 seizures are blocked from opening the first transition gate and as a result are arrested at the early phase of kindling, while sites that exhibit only stage 1 or 2 seizures are blocked from opening the second transition gate and as a result are arrested at the middle phase of kindling.

These kindling data emphasize a very critical aspect of the middle kindling phase: transition into this phase appears to be principally responsible for differences in the rate of kindling. The velocity of spread of AD from a given focal kindling site is directly proportional to how easily the first forebrain gate is opened to gain access to the middle phase of kindling (Burchfiel & Applegate, 1989a). Slower kindling structures may have a weaker ability to assail the forebrain gate and consequently spend more trials in the early phase of kindling. Faster kindling structures may have a stronger ability to overpower the forebrain gate and thereby spend fewer trials in early phase kindling. Although we lack a clear understanding of the exact properties responsible for propagating AD throughout the brain, the middle kindling phase hypothesis does predict that: (1) evoked ADs must achieve a 'critical mass' to overcome the forebrain gate and; (2) some structures are better endowed to overcome the forebrain gate than others. Achieving this critical mass may depend on numerous factors, such as the intrinsic capabilities of specific local neurons to generate AD and/or the local neuronal organization at the site of stimulation and the distance and complexity of AD propagation between the generation site and the forebrain circuitry (e.g., Gilbert, 1994; Racine *et al.*, 1986; Sato *et al.*, 1990). For instance, neurons at a given site of stimulation may vary in their ability to fire in synchronized high frequency bursts and in their ability to propagate burst activity to recruit other normal neurons into the synchronized bursting. Therefore, both the intrinsic bursting properties of the neurons and their proximity to available routes will determine the site's efficacy in progressing to the forebrain circuitry.

In summary, the middle phase of kindling is characterized by partial convulsive behaviours (stages 1 and 2). This phase of kindling is entered when AD activity generated in the early kindling phase overcomes the forebrain transition gate. It is the transition into the middle phase that accounts for differences in the progression of kindling. Neural organization at this phase is permanent and involves the recruitment of a discrete epileptogenic-susceptible forebrain circuit. The establishment of this kindled neural network appears to be common to all limbic ADs, regardless of where they were originally evoked.

Late Phase - Fully Generalized Kindling

Entry into the late phase of kindling is initiated by the opening of the second gate, which controls the transition from stage 1 and 2 seizures to stage 3, 4, and 5 seizures. Once this second transition gate is traversed, epileptiform discharge is able to spread extensively to motor regions that drive fully generalized motor convulsions; ranging from unilateral clonic forelimb movements to bilateral clonic forelimb and hindlimb movements. These motor regions are probably situated both in hindbrain structures (e.g., Browning, 1987; Burchfiel & Applegate, 1989a; Burnham, 1985; Chiba & Wada, 1997; Hamada, & Wada, 1998; McNamara, Galloway, Rigsbee, Shin, 1984; Wada & Sato, 1974; Wada & Sato, 1975) and neocortical regions (e.g., Corcoran, Urstad, McCaughran, & Wada, 1976a; Fernandez-Mas, Martinez, Gutierrez, & Fernandez -Guardiola, 1992; Kelly & McIntyre, 1996; Racine, 1972b; Wada, Sato, & McCaughran, 1975). Analogous to the first transition gate, surmounting this second gate probably also requires either a breakdown of inhibitory influences and/or an increase of excitatory influences that allows forebrain AD to propagate to the motor regions. Our understanding of the exact mechanisms mediating this hindbrain/neocortical transition gate is not as advanced as for the forebrain transition gate. Recent neural substrates that have been proposed to house the hindbrain/neocortical transition gate will be reviewed in the anatomical section below.

Transition into the late kindling phase, like the previous transition into the middle kindling phase, is a discrete advancement in the kindling process that is independent of the original site of stimulation (Burchfiel & Applegate, 1989a). Prior to this transition, the AD is confined to activation of predominantly forebrain circuitry of the middle kindling phase. As the hindbrain/neocortical gate is opened, another fundamental and permanent change in neural reorganization is established that supports even more extensive seizure propagation to motor regions. The late kindling phase differs from the middle kindling phase in a few important aspects. First, neuronal reorganization of the late kindling phase probably occurs in more than one anatomical location. Since AD propagation extends to both hemispheres and can activate an array of different motor systems, it is likely that multiple sites of neuronal reorganization are established. This notion is substantiated by the observation that during the later stages of kindling EEG recordings from most secondary sites display discharges that are independent of the primary stimulated site (Racine, 1978). Second, progression from the middle to the late kindling phase proceeds essentially at the same rate, irrespective of the original site of stimulation. It appears that once the epileptic-susceptible forebrain circuitry is established, the critical mass required to surmount the hindbrain/neocortical transition gate varies little between different sites. As stated in the previous section, the numbers of kindling stimulations required to progress from stage 3 to 5 are virtually identical for most limbic sites (Burchfiel *et al*., 1998; Le Gal La Salle, 1981; Löscher *et al*., 1998; Sato

& Nakashima, 1975).

Indirect evidence from kindling transfer experiments supports the notion of the establishment of epileptogenic-susceptible circuits outside the forebrain. Various researchers have demonstrated that lesions to the kindling primary amygdala, after the establishment of stage 5 seizures, has no effect on the subsequent transfer effect of kindling to a second site (Cain, 1986; Racine, 1972b; Wada, 1980). These data confirm that kindling in the late phase results in a widespread dispersion of epileptogenic susceptibility that does not reside at the site of stimulation.

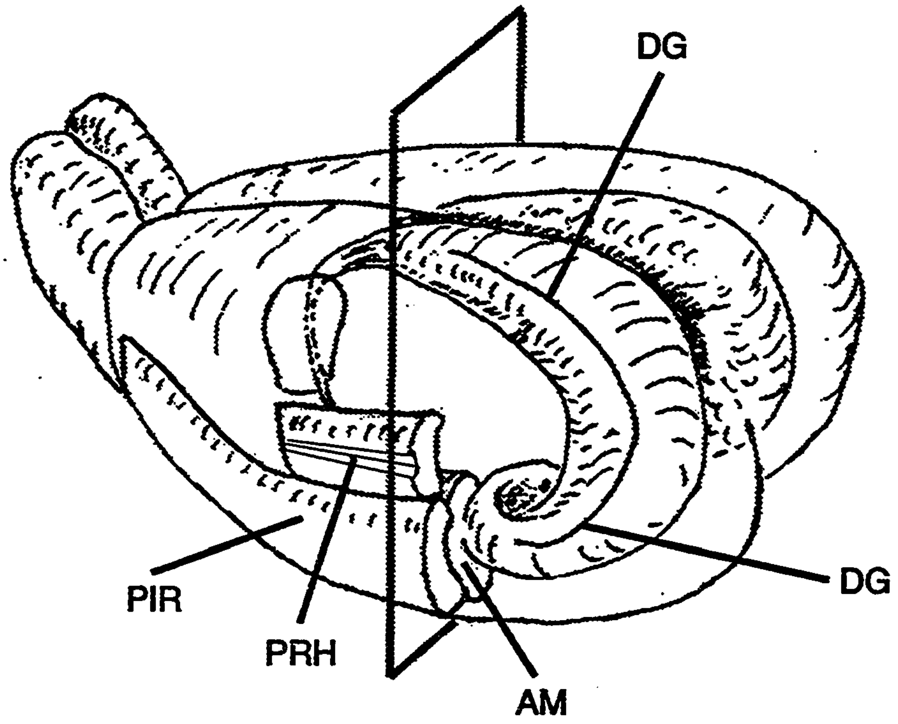
In summary, the late phase of kindling is characterized by fully convulsive behaviours (stages 3, 4, and 5). This phase of kindling is entered when AD activity generated in the middle kindling phase opens the hindbrain/neocortical transition gate. Neural organization at this phase is permanent and involves the recruitment of discrete multiple epileptogenic-susceptible circuits in different motor areas. The establishment of these kindled neural networks appears to be common to all ADs, regardless of where they were originally evoked.

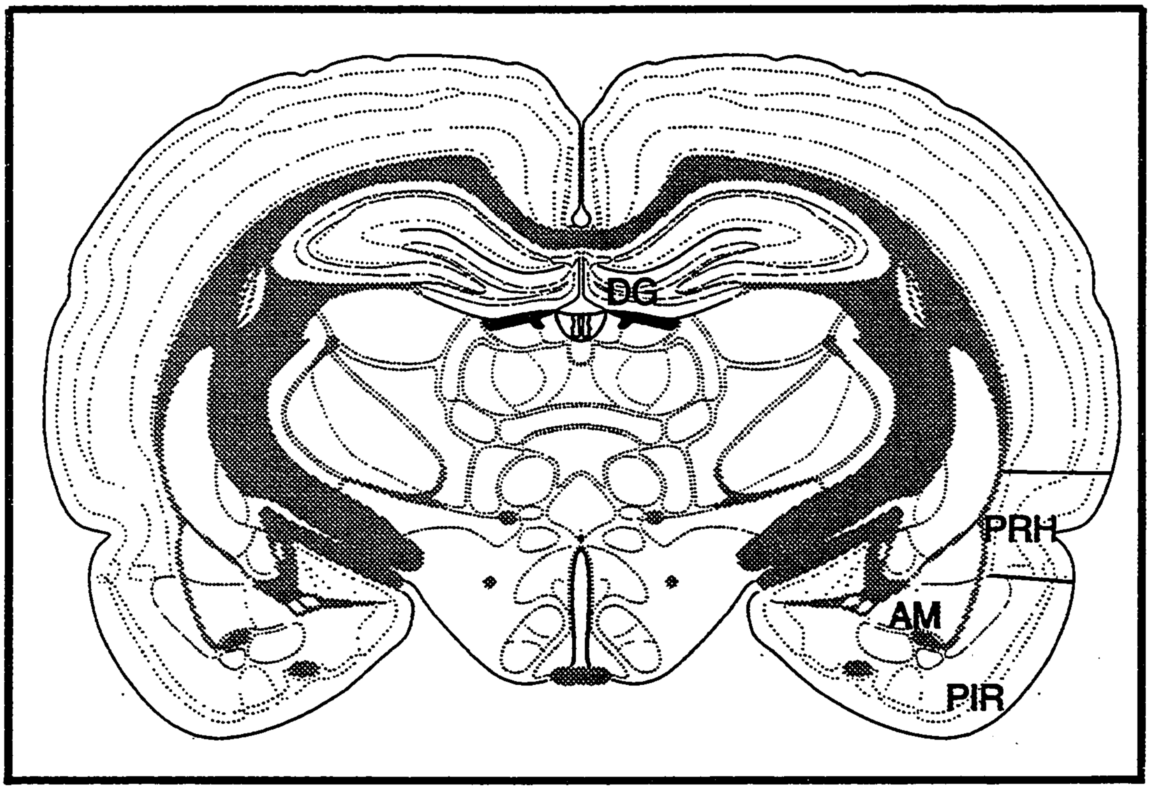
Anatomical Substrates of Kindling

This section will overview some of the anatomical structures that have been implicated as crucial players in kindling. Applying the conceptual framework of the previous section, emphasis will be focused on regions that are thought to encompass a transitional gate that may mediate advancement between the different phases of kindling. Traditionally, there have been two basic approaches used to assess a structure's participation in kindling: the correlative approach measures changes in electrical activity, morphology, neurochemistry, gene transcription and expression, or oxidative metabolism; and the interventive approach utilizes techniques such as electrical stimulation, lesions, or drug infusions. I shall review evidence from both types of study.

Figure 2 depicts both 3-dimensional and coronal plane views of the four anatomical regions discussed in the following section: the dentate gyrus of the

Figure 2. Schematic diagram of the rat forebrain in 3-dimensions and coronal plane views. The four structures depicted are the dentate gyrus of the hippocampus (DG), the amygdaloid complex (AM), the piriform cortex (PIR), and the perirhinal cortex (PRH). Figures adapted from McIntyre and Plant (1989) and Swanson (1992).


DG
DG
PIR
PRH
AM


DG
PRH
AM
PIR

hippocampus, the amygdaloid complex, the piriform cortex, and the perirhinal cortex.

Dentate Gyrus of the Hippocampus

The dentate gyrus (DG) historically has been linked to epileptic susceptibility in both clinical and animal models. Despite the volumes of data implicating the DG in seizure propagation, as will be discussed below, its role in kindling remains elusive.

Anatomy of the DG. The hippocampus is an archicortical structure that can be divided into four distinct areas: dentate gyrus (DG), CA1, CA2, and CA3. Neuronal activity into and through the hippocampus generally flows in one direction, and this major route is referred to as the trisynaptic circuit (Bliss & Lømo, 1973). The circuit begins at the entorhinal cortex, which activates the granule cells of the DG via the perforant path. In turn the DG activates CA3 pyramidal cells via the mossy fibers, and finally CA3 activates CA1 pyramidal cells via the Schaffer collaterals. CA1 pyramidal neurons then feed their axons to the entorhinal cortex via the subiculum, thus completing the trisynaptic circuit (Steward & Scoville, 1976; Witter, 1993). The entorhinal cortex receives inputs from several limbic regions, including the septum, thalamus, hypothalamus, amygdala, claustrum (Fibiger, 1982; Finch, Wong, Derian, Chen, Nowlin-Finch, & Brothers, 1986; Wyss, Swanson & Cowan, 1977), neocortical areas (Insausti, Amaral, & Cowan, 1987; Lopes da Silva, Witter, Boeijinga, & Lothman, 1990; Witter, Room, Groenewegen, & Lohman, 1986), and brainstem (Köhler & Steinbusch, 1982). Due to its location in the trisynaptic circuitry, it has been suggested that the DG is in a ideal position to act as a regulator of normal and epileptic activity passing into the hippocampus via the entorhinal cortex (e.g., Lothman, Stringer, & Bertram, 1992).

Kindling of the DG. It has been proposed that the DG is critically involved in limbic kindling (Dasheiff & McNamara, 1982; Frush, Giacchino, & McNamara, 1986; Savage, Rigsbee, & McNamara, 1985). However, the resistance of the DG to kindling does not entirely support this claim. Compared to most other limbic sites, the DG and the hippocampus require some of the greatest numbers of stimulations to achieve full seizure generalization (e.g., Burnham, 1976; Goddard *et al.*, 1969; Racine, 1978). In general, with hippocampal kindling, AD thresholds are low and kindling rates can vary from 25 to

120 stimulations in the rat depending on the cellular region and the dorsal-ventral location of the stimulation electrode (de Jonge & Racine, 1987; Grace, Corcoran, & Skelton, 1990; Lerner-Natoli, Rondouin, & Baldy-Moulinier, 1984; Racine, Rose, & Burnham, 1977). Several studies have specifically examined kindling from the granule cells of the DG and have concluded that kindling is unstable and resistant to developing fully generalized seizures (de Jonge & Racine, 1987; Grace *et al.*, 1990). The pattern of kindling from the DG is somewhat different from other structures, in that most of the ADs are associated with partial seizures (stage 1 and 2) and progression into stage 5 seizures is abrupt and easily regresses back to partial seizures (Grace *et al.*, 1990).

Lesions of the DG. Lesioning of the granule cells of the DG produces mixed effects on kindling. Early studies demonstrated that colchicine-induced lesions of granule cells or transections of the entorhinal cortex could produce significant delays in kindling progression from different limbic sites, but have no effect on established stage 5 seizures (Dasheiff & McNamara, 1982; Frush, *et al.*, 1986; Savage, *et al.*, 1985; Tsunoda, Mori, Osonoe, Ariga, Saitoh, Kittaka, & Ogata, 1995). McNamara's group concluded from these results that the DG was a critical node in a kindling network that could promote propagation of limbic discharge. Contradictory to this view, many other laboratories demonstrated that DG lesions have no effect on kindling. For example, colchicine lesions have shown to have either no effect (Mitchell & Barnes, 1993; Tsunoda *et al.*, 1995) or can actually *accelerate* kindling (Sutula, Harrison, & Steward, 1986). Aspiration or ibotenic lesions of the entire ventral hippocampus also have no effect on the development of fully generalized convulsions (Racine, Paxinos, Mosher, & Kairiss, 1988b; Tanaka, Kondo, Hori, Tanaka, & Yonemasu, 1991). Similarly, lesions produced by perforant path stimulation will facilitate amygdala kindling (Mazarati & Wasterlain, 1997). These more recent studies suggest that the DG and possibly the entire hippocampus are not essential for limbic kindling. Moreover, the granule cells of the DG may more likely play an antagonistic rather than a facilitating role in the spread of seizure activity (e.g., Barnes & Mitchell, 1990).

Inhibition in the DG. Recall from the conceptual kindling framework section the proposition that before epileptiform activity could be widely propagated, a transition gate had to be opened via collapse of inhibitory mechanism(s). Numerous kindling experiments that have examined the loss of inhibition have focused on the breakdown of GABA inhibitory transmission in the hippocampus (for discussion of GABA-mediated inhibition in the hippocampus, see, for example, McCarren & Alger, 1985; Thompson & Gahwiler, 1989). In many of these experiments, the paired-pulse technique is commonly used to measure changes in both feedback and feedforward[2] inhibition. The paired-pulse procedure involves application of a conditioning pulse that activates a GABA mediated inhibitory response. At various intervals following the conditioning pulse a test pulse is delivered, during a period of evoked recurrent feedback inhibition, which results in the suppression of the response to this second pulse. Many experiments measuring feedback inhibition in the DG following electrical kindling have reported an *increase,* rather than a decrease, in inhibition with the test pulses (Adamec & Stark-Adamec, 1983; Bronzino, Austin-LaFrance, Morgane, & Galler, 1991; Milgram, Michael, Cammisuli, Head, Ferbinteanu, Reid, Murphy, & Racine, 1995; Tuff, Racine, & Adamec, 1983; Voskuyl & Albus, 1987). Furthermore, Maru and Goddard (1987) have demonstrated that kindling also produces potentiation of feedfoward inhibition. Together these results suggest that the DG is actually *resistant* to epileptiform activity. These observations are supported by the recent reports that GABA$_A$ receptors numbers do not change (Lehmann, Ebert, & Löscher, 1996) or even increase (Lopes da Silva, Faas, Kamphuis, Titulaer, Vreugdenhil, & Wadman, 1998; Nusser, Hajos, Somogyi, & Mody, 1998) in the DG following kindling. Overall, most of these findings are inconsistent with the hypothesis that the DG mediates kindling by the breakdown of GABA inhibition.

Bursting in the DG. Correlative evidence from neuronal bursting more strongly implicates the DG in kindling epileptogenesis. Burst discharge is a pattern of high-

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[2] Feedback inhibition limits the spread of excitation among adjacent neuronal populations; while feedfoward inhibition is exerted from upstream distal sites that excite inhibitory neuronal populations that, in turn, limit the spread of excitation among adjacent neuronal populations (see Buzsaki, 1984).

frequency discharge of action potentials that usually ride on a sudden and large membrane depolarization that can last over 100 milliseconds (Ayala, Dichter, Gumnit, Matsumoto, & Spencer, 1973). Stringer, Williamson, and Lothman (1989) were able to evoke bursting responses from the DG that would abruptly become epileptiform, which they referred to as "maximal dentate activation" (MDA). MDA is potentiated by prior kindling (Stringer, Williamson, & Lothman, 1991) which has led to the hypothesis that MDA represents an open 'gate' or switch in the DG that promotes the propagation and amplification of AD throughout the hippocampus (Lothman, *et al.*, 1992; Stringer & Pan, 1998). However, there are several problems with the MDA hypothesis. For example, it is difficult to attribute causation with these correlational data, and the phenomenon is not observed consistently in all rats during kindling (Stringer, 1992). In corroboration of the MDA effect, *in vitro* data demonstrate increased bursting in DG granule cells from kindled animals following various induction techniques (Behr, Lyson, & Mody, 1998; Pan & Stringer, 1996; Patrylo, Schweitzer, & Dudek, 1994; Stanton, Mody, & Heinemann, 1989). Many of these bursting phenomena have been attributed to changes in the intrinsic membrane properties of DG granule cells, leading to enhanced activation of the excitatory *N*-methyl-D-aspartate (NMDA) glutamate receptor (e.g., Köhr, De Koninck, & Mody, 1993; Mody & Heinemann, 1987).

Morphological changes in the DG. Many investigators have searched for morphological changes that could account for kindling, and several have described various morphological changes in the DG following kindling. One of the more provocative is the abnormal sprouting of mossy fibers into the inner molecular layer of the DG. Sutula's group was the first to show that kindling is associated with persistent sprouting of mossy fibers in the DG (Cavazos, Golarai, & Sutula, 1991; Sutula, He, Cavazos, & Scott, 1988). From these experiments it has been proposed that mossy fiber sprouting may play a functional role in the development and/or maintenance of kindling (Sutula *et al.*, 1988). Specifically, the hypothesis posits that sprouted reorganized mossy fibers form a recurrent excitatory feedback circuit, or an epileptic generator, that contributes to the elaboration and propagation of epileptiform discharge (Cronin, Obenaus, Houser, & Dudek, 1992; Sutula, 1990; Tauck & Nadler, 1985). However, problems exist with the mossy fiber sprouting hypothesis: first, as was stated previously, the DG exhibits decreased excitation after kindling; second, it has not been determined whether sprouted mossy fibers actually form functional recurrent excitatory circuits (Represa, Jorquera, Le Galle La Salle, & Ben-Ari, 1993; Ribak & Peterson, 1991; Sloviter, 1991); third, recent studies have documented dissociations between mossy fiber sprouting and kindling (Armitage, Mohapel, Jenkins, Hannesson, & Corcoran, 1998; Corcoran, Armitage, Hannesson, Jenkins, & Mohapel, 1998; Ebert & Löscher, 1995a; Elmér, Kokaia, Kokaia, Lindvall, & McIntyre, 1997; Kokaia, Ernfors, Kokaia, Elmér, Jaenisch, & Lindvall, 1995; Mohapel, Armitage, Hannesson, & Corcoran, 1997; Racine, Adams, Osehobo, Milgram, & Fahnestock, 1998). Fourth, the sprouting that has been reported is typically seen after kindling of generalized seizures (Armitage *et al.*, 1998).

Other persistent morphological changes have been observed in the DG following kindling. Geinisman and colleagues demonstrated relative increases in synapses between the perforant path terminals and dendritic spines of granule cells with kindling (Geinisman, deToledo-Morrell, & Morrell, 1990; Geinisman, Morrell, & deToledo-Morrell, 1988; Geinisman, Morrell, & deToledo-Morrell, 1992). Hovorka, Langmeier, and Mareš (1989) observed a significant redistribution of synaptic vesicles to the vicinity of the synaptic cleft in axospinous synapses in the middle molecular layer of the DG. More recently, some investigators have reported evidence that new neurons are actually generated in the DG following kindling (Bengzon, Kokaia, Elmér, Nanobashvili, Kokaia, Lindvall, 1997; Parent, Janumpalli, McNamara, & Lowenstein, 1998; Scott, Wang, Burnham, De Boni, & Wojtowicz, 1998). Collectively, all of these authors have argued that these morphological changes in the DG, including neurogenesis, could produce the sustained increases in excitatory synaptic drive required to produce kindling. There have been no experiments that have yet tested the functional consequences of these changes in the DG. Furthermore, neurogenesis occurs only after development of stage 5 seizures, not during the earlier phases of kindling.

Molecular changes in the DG. During recent years attention has been focused on the role of growth factors in mediating morphological changes in epileptogenesis. Growth, or neurotrophic, factors may trigger many plastic responses that can lead to critical circuit changes required for kindling (Lindvall, Kokaia, Elmér, Ferencz, Bengzon, & Kokaia, 1998; Reibel *et al.*, 1998). Increases in both mRNA and protein levels in the DG have been reported for nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and for the neurotrophin receptor *trkB* with either one or multiple kindling stimulations (Bengzon, Kokaia, Ernfors, Kokaia, Leanza, Nilsson, Persson, & Lindvall, 1993; Bengzon, Söderström, Kokaia, Kokaia, Ernfors, Persson, Ebendal, & Lindvall, 1992; Elmér, Kokaia, Kokaia, Carnahan, Nawa, & Lindvall, 1998; Ernfors, Bengzon, Kokaia, Persson, & Lindvall, 1991; Merlio, Ernfors, Kokaia, Middlemas, Bengzon, Kokaia, Smith, Siesjö, Hunter, Lindvall, & Persson 1993; Sato, Kashihara, Morimoto, & Hayabara, 1996). BDNF protein levels remain elevated in the DG longer than any other structure (Elmér *et al.*, 1998). Infusion of neurotrophins into the brain produces mixed results, with one group reporting that NGF accelerates hippocampal kindling (Adams, Sazgar, Osehobo, Van der Zee, Diamond, Fahnestock, & Racine, 1997) but another reporting that BDNF retards kindling (Larmet *et al.*, 1995; Reibel *et al.*, 1998).

Other molecular mechanisms have been linked to neuronal plasticity, such as early immediate genes or transcription factors. Specifically, early immediate genes have been proposed to act as crucial intermediates in a cascade linking membrane stimulation to long-term alterations in neuronal activity (Morgan & Curran, 1989). With kindling, the mRNA for *c-fos* or it's post-transcriptional protein Fos[3] have been shown to be rapidly and transiently induced in the DG (Burazin & Gundlach, 1996; Clark, Post, Weiss, Cain, & Nakajima, 1991; Dragunow, Robertson, & Robertson, 1988; Shin, McNamara, Morgan, Curran, & Cohen, 1990). Moreover, the induction of *c-fos* or Fos in the DG has been associated with longer ADs that exceed 30 seconds in duration (Chiasson, Dennison, & Robertson, 1995; Clark *et al.*, 1991; Dragunow *et al.*, 1988; Hosford, Simonato, Cao,

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[3] Note that when referring to the genetic code, one spells out the gene's name in lower-case three-letter words that are italicized (i.e., *fos*), whereas the post-transcriptional protein of a gene is spelled with the first letter in upper-case and non-italicized (i.e., Fos).

Garcia-Cairasco, Silver, Butler, Shin, & McNamara, 1995; Sato, Yamada, Morimoto, Uemura, & Kuroda, 1998). In light of this, there is a debate as to whether changes in *c-fos* truly represent changes in underlying neural plasticity (Labiner, Butler, Cao, Hosford, Shin, & McNamara, 1993; Watanabe, Johnson, Butler, Binder, Spiegelman, Papaioannou, & McNamara, 1996) or whether *c-fos* is merely a consequence of neural activity (Teskey, Atkinson, & Cain, 1991).

Cell loss in the DG. Clinical findings were the first to suggest a link between neuronal cell loss and epileptogenesis, in that hippocampal degeneration is often the hallmark of MTLE (Shin & McNamara, 1994). In fact, many of the morphological changes and neurogenesis mentioned above are thought to be compensatory responses to cell loss. The underlying rationale is that the loss or damage of critical inhibitory neurons shifts the balance towards over-excitation and increased epileptogenic reactivity. Earlier studies failed to find any evidence of neuronal degeneration with kindling (e.g., Goddard & Douglas, 1975; Goddard *et al.*, 1969; Racine, 1972a, 1972b). Using astrocyte activation as a index of possible cellular degeneration, some investigators have identified increases in various astrocyte markers with kindling (Dalby, Rondouin, & Lerner-Natoli, 1995; Hansen, Jorgensen, Bolwig, & Barry, 1990; Steward, Torre, Tomasulo, & Lothman, 1991). However, these changes have been to shown to occur in the absence of any neuronal degeneration in the DG (Khurgel, Switzer, Teskey, Spiller, Racine, & Ivy, 1995). Recently, it has been reported that neurons are lost in the hilus of the DG through both necrotic (excitotoxic cell death) (Cavazos, Das, & Sutula, 1994; Cavazos & Sutula, 1990; Spiller & Racine, 1994) and apoptotic (programmed cell death) mechanisms (Bengzon *et al.*, 1997; Pretel, Applegate, & Piekut, 1997; Zhang, Smith, Li, Weiss, & Post, 1998). The size and density of granule cells have also been reported to be reduced in kindled animals (Hosokawa, Itano, Usuki, Tokuda, Matsui, Janjua, Suwaki, Okada, Negi, Murakami, Konishi, & Hatase, 1995). Nevertheless, more recent studies question whether cell loss is truly occurring with kindling, since it has been demonstrated that decreases in DG cellular density can be accounted for by increases in hilar volume (Bertram & Lothman 1993; Racine *et al.*, 1998; Watanabe *et al.*, 1996), and DG cells that exhibit apoptosis have not been actually identified as neurons. Furthermore, it has been shown that compounds that prevent neuronal degeneration, such as NGF, actually *facilitate* rather than retard kindling (Adams *et al.*, 1997). The issue of neuronal degeneration and kindling remains controversial and unresolved. To date, however, the bulk of the evidence suggests that cell damage in the DG is not the primary mechanism underlying kindling.

To conclude, many of the correlational studies implicate the DG as an important locus for the elaboration and propagation of epileptiform activity to other regions of the brain. In reference to the conceptual kindling framework it could be argued that the DG represents the major component of the first transitional gate responsible for establishing the forebrain epileptogenic circuitry, which mediates the early partial stages of kindling. Changes in neurotrophic factors, early immediate genes, and the occurrence of bursting in the DG support this proposition. However, not all the correlative evidence speaks to the idea that the forebrain gate resides in the DG, since mossy fiber sprouting, neuronal degeneration, and neurogenesis occur only *after* the establishment of fully generalized seizures. Much of the correlative evidence has not been tested for functional consequences, which makes it difficult to make causal interpretations. Data from the intervening kindling studies do not implicate the DG in epileptogenesis. The kindling susceptibility, DG lesion, and paired-pulse inhibition experiments suggest that the DG may actually be resistant to epileptiform propagation. Overall, the DG itself appears not to be critical for limbic kindling and at most may play only a facilitating role in seizure propagation (see, Lothman, 1992).

Amygdaloid Complex

In the earliest attempt to identify the anatomical substrate for kindling, Goddard *et al.* (1969) identified the amygdala (AM) as a critical site due to its rapid kindling progression. Today the AM is the most frequently stimulated and studied structure in kindling research (Cain, 1992). Unlike the DG, there is more cohesive evidence implicating the AM as an important player in seizure propagation.

Anatomy of the AM. The amygdaloid complex represents a heterogenous group of 13 distinct nuclear and cortical structures that include the: accessory basal nucleus, central nucleus, cortical nucleus, basal nucleus, lateral nucleus, medial nucleus, and periamygdaloid cortex (Amaral, Price, Pitkänen, & Carmichael, 1992; Price, Russchen, & Amaral, 1987). Each of these nuclei has a distinct complement of intrinsic and extrinsic connections. Generally, most projections enter the AM via the lateral nucleus, which projects to many other AM nuclei including the basal nucleus. The basal nucleus projects to neocortical and to some subcortical areas, via the amygdalofugal pathway, as well as to the central nucleus. The central nucleus, in turn, projects to the majority of subcortical areas via the stria terminalis (Amaral, *et al.*, 1992; Krettek & Price, 1978; Savander, Go, LeDoux, & Pitkänen, 1995). The AM is interconnected with a wide variety of cortical regions such as the temporal (i.e, perirhinal and entorhinal), frontal, insular, and cingulate association cortices (Amaral, *et al.*, 1992; Krettek & Price, 1974); and subcortical brain regions, including the brainstem, hypothalamus, thalamus, basal forebrain, claustrum, and hippocampus (Amaral, *et al.*, 1992). Since the AM acts as a bidirectional conduit that can relay neural signals between association cortices and subcortical structures, it is in the ideal position to simultaneously influence the excitability of several brain regions at any one time (see Le Gal La Salle, 1982).

Kindling in the AM. Goddard *et al.* (1969) were the first to acknowledge the importance of the AM in kindling when they observed a correlation between the number of kindling stimulations required by other structures and their anatomical distance from the AM. Subsequent work has shown that structures that have prominent connections with the AM kindle as rapidly as or even more quickly than the AM itself (e.g., Cain, 1977; Le Gal La Salle, 1979; McIntyre, Kelly, & Armstrong, 1993; Racine, Mosher, & Kairiss, 1988a). Unlike the DG, kindling from the AM is robust, reliable, and predictable. With AM kindling AD thresholds are low and full seizure generalization can occur after 10 to 15 daily stimulations. Amygdaloid nuclei can differ in their kindling rates, whereby the fastest to the slowest are: central, basolateral/basomedial, lateral, medial, and cortical nucleus (Gilbert, Gilles, & Cain, 1984; Le Gal La Salle, 1981;

Mohapel, Dufresne, Kelly, & McIntyre, 1996).

Lesions of the AM. Lesion studies of the AM do not strongly implicate this structure in kindling. Racine (1972b) showed that lesioning the AM had no effect on subsequent transfer to another site, suggesting that the AM was not important for the establishment of seizures in other sites. Others have shown that AM lesions retard but do not block kindling from the olfactory bulb (Cain, 1977) or bed nucleus of the stria terminalis (Le Gal La Salle, 1979). Smaller incomplete AM lesions have been shown to have either no effect or even accelerate dorsal hippocampal kindling (Araki, Aihara, Watanabe, Yamamoto, & Ueki, 1985; Le Gal La Salle & Feldblum, 1983; McIntyre, Stuckey, & Stokes, 1982). Racine *et al.* (1988) demonstrated that transecting different fibers around the AM could either have no effect, facilitate, or retard kindling from the AM depending on the location of the knife cut.

Inhibition in the AM. Inhibitory neurotransmitters have generally been shown to delay kindling from the AM. NA agonists (see Corcoran & Weiss, 1990; McIntyre, 1981) and GABA complex agonists (see Burnham, 1989) can retard, but not completely block, kindling from the AM. Shinnick-Gallagher and associates have performed extensive research on changes in GABAergic inhibition in AM basolateral neurons following kindling (see Shinnick-Gallagher, Keele, & Neugebauer, 1998). By eliciting synaptic responses with stimulation of the stria terminalis (a feedforward inhibitory pathway), they observed a long-lasting decrease in inhibition in the AM after kindling that was due to: (1) a loss of GABAergic interneurons (Gean, Shinnick-Gallagher, & Anderson, 1989; Callahan, Paris, Cunningham, & Shinnick-Gallagher, 1991); and (2) a diminished sensitivity of $GABA_B$ receptors (Asprodini, Rainnie, & Shinnick-Gallagher, 1992). The authors concluded that these two disinhibition mechanisms act to produce increases in excitatory drive that culminate in epileptic bursting in the amygdaloid neurons.

Bursting in the AM. Similar to the DG, prominent cellular bursting has been reported in the AM with kindling. Basolateral amygdaloid neurons in kindled rats tend to exhibit longer and higher frequency bursts as compared to controls (Gean, *et al.*,1989; Racine, Newberry, & Burnham, 1975; Racine & Zaide, 1978; Shoji, Tanaka, Yamamoto, Maeda, & Higashi, 1998; Tsuru, 1985). Shinnick-Gallagher and colleagues have shown that kindling induced epileptic bursting in basolateral AM neurons are not mediated by NMDA receptors, as they are with DG granule cells, but are instead mediated by the non-NMDA glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (Rainnie, Asprodini, & Shinnick-Gallagher, 1992). More current studies also have implicated presynaptic metabotropic glutamate receptors in AM bursting (Shinnick-Gallagher *et al.*, 1998).

Morphological and molecular changes in AM. Once one looks beyond the hippocampus, there is less direct evidence of morphological changes with limbic kindling. Only one study, examining the ultrastructure characteristics of axondendritic and axospinous synapses, has reported a decrease in the density of synapses formed with dendrites in the medial AM with kindling (Nishizuka, Okada, Arai, & Iizuka, 1991). Growth factors that lead to morphological changes have been identified in the AM with kindling. After several stage 5 seizures BDNF mRNA expression, but not protein expression, are increased in the AM (Bengzon, *et al.*, 1993; Elmér *et al.*, 1998). Note that the expression of these neurotrophins occurs in the AM only after generalized seizures have been evoked, whereas these changes can appear in the DG after only one single non-convulsive stimulation. Intraventricular administration of NGF retards kindling from the AM (Funabashi, Sasaki, & Kimura, 1988; Van der Zee, Rashid, Le, Moore, Stanisz, Diamond, Racine, & Fahnestock, 1995), while BDNF has no effect (Reibel *et al.*, 1998). Transgenic knockout mice that have mutations in the gene that produces BDNF or neurotrophin-3 (NT-3) exhibit delayed AM kindling (Elmér, Kokaia, Ernfors, Ferencz, Kokaia, & Lindvall, 1996; Kokaia, *et al.*, 1995; Lindvall *et al.*, 1998). The delays in kindling for these mutant mice are due to the requirement of more stimulation trials in the partial seizure stages (i.e., middle kindling phase). These results suggest that neurotrophic factors in the AM may play an important role in establishing kindling.

Similar to the DG, kindling leads to activation of early immediate genes in the AM (Chiasson *et al.*, 1995; Dragunow *et al.*, 1988). Furthermore, the advancement of kindled seizures from partial to generalized states corresponds to a progressive increase in the distribution of *c-fos* expression, starting in the AM (partial) and moving bilaterally to the hippocampus (generalized) (Chiasson *et al.*, 1995; Clark *et al.*, 1991; Dragunow *et al.*, 1988; Hosford *et al.*, 1995). In more recent experiments, the expression of *c-fos* was reduced with antisense oligodeoxynucleotides, resulting in an acceleration in AM kindling (Chiasson, Hong, & Robertson,1998; Rocha & Kaufman, 1998). From these results it could be argued that *c-fos* expression in the AM may reflect an attempt by the brain to reverse the events that led to kindling.

Cell loss in the AM. Various studies have identified correlates of degenerative changes in the AM with kindling. As was discussed above, GABAergic interneurons are lost in the basolateral nucleus of the AM (Callahan *et al.*, 1991; Lehmann, Ebert, & Löscher, 1998) and dendritic densities are reduced in medial nucleus of the AM after kindling (Nishizuka *et al.*, 1991). Decreases in amygdaloid GABA somatostatin-immunoreactive neurons also occur with kindling (Tuunanen, Halonen, & Pitkänen, 1997). Some researchers have looked for changes in reactive astrocytes as indicator of neuronal death/degeneration, since astrocytes are known to remove degenerated synapses from neurons (Khurgel & Ivy, 1996; Steward *et al.*, 1991). Using immunocytochemical labelling of glia-fibrillary-acidic-protein (GFAP), an index of activation of astrocytes, many reports have detected increased levels in the AM after kindling (Hansen *et al.*, 1990; Khurgel, Racine, & Ivy, 1992; Khurgel, *et al.*, 1995; Racine *et al.*, 1989). However, it appears that these astrocytes represent an adaptive response to seizures and are not necessarily crucial for the establishment of kindling (Khurge & Ivy, 1996). Overall, in comparison to the DG, there is less direct substantial evidence of neuronal death occurring in the AM with limbic kindling.

To conclude, much of the evidence indicates that the AM may play an important role in kindling. Both the intervenient and correlative data point to the AM as a potential candidate for accommodating the first forebrain transition gate of the kindling framework. For instance, the kindling rates from the AM are some of the quickest in the brain, the AM has a propensity to develop bursting following kindling, and plastic changes occur readily in the AM with kindling. In spite of this, not all the evidence is overwhelming. The lesion, morphological, and neuronal degeneration data weakly implicate the AM as a crucial epileptogenic site. Many of the correlative studies have shown these changes occur in the AM *after* the establishment of kindled generalized seizures. There seems to be little question that the AM is closely associated with the forebrain transition gate, but it may not actually contain the forebrain gate. As it will be discussed in the next section, some researchers attribute the AM's epileptogenic properties to its intimate association with the neighbouring piriform cortex (see Löscher & Ebert, 1996).

Piriform Cortex

In recent years there has been growing interest in the role of the piriform cortex (PIR) in the development and maintenance of limbic kindling. The evidence listed below makes the PIR one of the best contenders for establishing the epileptogenic forebrain neural circuitry of early phases of kindling.

Anatomy of the PIR. The PIR is the largest area of the mammalian olfactory cortex and extends over a vast distance on the lateral and ventral surface of the rat forebrain. The PIR is a phylogenetically old structure comprising only three neuronal layers (neocortical areas are six layered). However, just below layer III sits the endopiriform nucleus, which is heavily interconnected with the PIR and is sometimes described as 'layer IV' of the PIR (Haberly, 1990). Instead of topical projections usually found with neocortical areas, the PIR has very diffuse inputs. Projections generally move in two directions: from anterior to posterior, and from the surface superficial layers to the deeper structural layers (Haberly, 1990). The PIR receives its major input from the olfactory bulb and various other subcortical structures, including the basal forebrain, thalamus, hypothalamus, and brainstem (Haberly, 1990). Output from the PIR arises from axons from layer III pyramidal cells that project to the insular cortex, entorhinal cortex, subiculum, amygdala, hypothalamus, and mediodorsal thalamus (Carlsen, De Olmos, & Heimer, 1982; Datiche & Cattarelli, 1996; Haberly, 1990; Krettek & Price, 1978; Kuroda, Murakami, Kishi, & Price, 1992). Its unique intrinsic association fiber system and abundant connections to the limbic system make the PIR ideal for the spread of seizure activity to other forebrain sites.

Kindling in the PIR. The PIR is very sensitive to kindling, both in its low thresholds for AD and its rapid rate of seizure evolution (Cain, Corcoran, Desborough, & McKitrick, 1988a; Mohapel *et al.*, 1996; Racine, 1975). Full seizure generalization can be established within 8 to 12 stimulations, depending on the region of the PIR. Löscher and colleagues recently mapped the kindling susceptibilities of the PIR on both laminar and anterior/posterior dimensions and discovered that most stimulation sites in layer III were very sensitive, particularly the rostral portion of the posterior PIR, to electrical stimulation (Hönack, Wahnschaffe, & Löscher, 1991; Löscher, Ebert, Wahnschaffe, & Rundfeld, 1995). Interestingly, the differences in sensitivity were primarily due to changes in pre- and post-kindling afterdischarge thresholds (ADT) and not to the number of stimulations required to evoke a stage 5 seizure (i.e., kindling rate) (Löscher *et al.*, 1995).

There is evidence that the PIR can act not only as a site from which kindling can be evoked, but also may be involved in the generation and propagation of epileptic discharge induced from other limbic sites that receive kindling stimulation. For example, stimulating various limbic structures can readily trigger large and independently driven AD spikes (Burchfiel & Applegate, 1991; Burchfiel *et al.*, 1998; Ebert, Rundfeldt, & Löscher, 1995), dramatically increase spontaneous baseline firing (Teskey & Racine, 1993), and increase interictal spiking (IIS)[4] (Kairiss *et al.*, 1984; Racine *et al.*, 1988a), suggesting that epileptogenesis depends on these limbic sites accessing the neuronal network of the PIR. Note that many of these changes occur in the *ipsilateral* PIR, suggesting that many limbic sites in one hemisphere may be functionally coupled via the PIR of the same hemisphere (Löscher & Ebert, 1996). This is supported by the observation that the initial few stimulations applied to most limbic sites triggers AD in the ipsilateral PIR long before they trigger AD in their corresponding contralateral limbic

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[4] IIS are considered the hallmark of many epilepsies, whereby a steady increase in IIS is thought to eventually culminate in seizures (Wieser, Bjeljac, Khan, Müller, Siegel, & Yonekawa, 1994).

sites (Ebert *et al.*, 1995b). Furthermore, Burchfiel and associates examined kindling transfer between the PIR and various ipsilateral limbic sites and found a high degree of transfer between many limbic sites to the PIR, but not from the PIR to these limbic sites. Based on these results, it was argued that most limbic sites converge upon the PIR during kindling, which explains the rapid transfer to the PIR. However, PIR kindling does not converge upon these limbic sites, which would explain the lack of significant transfer from the PIR (Burchfiel *et al.*, 1998).

Lesions of the PIR. In support of the kindling data, results obtained in all models of convulsive status epilepticus (prolonged seizure bouts that result in brain damage) suggest that the PIR is especially vulnerable to pathological damage, due to its propensity to exhibit hyperexcitable epileptic discharge (Milgram, Green, Liberman, Riexinger, & Petit, 1985; McIntyre & Plant, 1989). McIntyre and Kelly (1990) have used the status epilepticus treatment as a means to selectively lesion the PIR and discovered that rats were unable to develop secondarily generalized seizures upon subsequent kindling of the hippocampus or olfactory bulb. Furthermore, electrolytic and transection lesions of both the anterior and posterior portions of the PIR retard kindling from the septum or AM (Racine *et al.*, 1988b). However, caution must be exercised in interpretation of this PIR lesion data since the lesions in these studies were not exclusively restricted to the PIR. More selective lesions of only the anterior portion of the PIR produce no effect on AM kindling (Cain *et al.*, 1988a; Ludvig & Moshé, 1988), and selective lesions of only the posterior PIR also produce no effect on AM kindling, but do lower ADT (Wahnschaffe, Ebert, & Löscher, 1993).

Inhibition in the PIR. Several experiments have examined the effects of kindling on changes in PIR inhibition. Racine, Moore, and Evans (1991) looked at changes in paired-pulse facilitation in layer II of the PIR following kindling in the olfactory bulb, and reported an increase in inhibition. In contrast, Haberly and Sutula (1992) measured extracellular/intracellular potentials and membrane current source densities in the PIR and reported a decrease in inhibition, due to the reduction in the long-lasting inhibitory postsynaptic potential (IPSP) component in pyramidal neurons, following kindling from the lateral olfactory tract. In a nonkindled rat this long-lasting IPSP was shown to widely propagate throughout the basal forebrain and the authors speculate that this process may play a critical role in limiting seizure activity by containing positive feedback in the PIR (Haberly & Sutula, 1992). Further, it has been proposed that a compromise in this particular IPSP mechanism may represent the 'opening' of the first transitional forebrain gate (Löscher & Ebert, 1996).

Bursting in the PIR. The PIR is exceptionally susceptible to the development of epileptiform bursting following kindling. As mentioned previously, IIS appear first in the PIR and AM regardless of the location of the applied kindling stimulation (Kairiss *et al.*, 1984; Racine *et al.*, 1988a). *In vitro* work has demonstrated that layer II neurons of the PIR can display greater spontaneous bursting and an evoked burst response to stimulation of the AM, after AM kindling (McIntyre & Wong, 1985, 1986). Hoffman and Haberly (1991) observed long latency epileptiform bursting potentials from olfactory bulb kindled rats. These bursting responses were shown to originate from the anterior endopiriform cortex , layer III of the PIR , and the adjaecent claustrum (Hoffman & Haberly, 1991, 1996). Many of these bursting responses in the PIR with kindling are thought to be initiated by the GluR2 subunit of the AMPA receptor and facilitated by the NMDA receptor (Hoffman & Haberly, 1996; Prince, Conn, Blackstone, Huganir, & Levey, 1995). Additionally, epileptiform bursting has been linked to dynamic alterations in high-affinity glutamate uptake transporters. With AM kindling some glutamate transporter proteins are down-regulated after development of partial seizures, while others only are up-regulated after development of fully generalized convulsive seizures from the PIR/AM region (Miller, Levey, Rothstein, Tzingounis, & Conn, 1997).

Pirreda and Gale (1985) described a site localized within a very small region of the anterior PIR/endopiriform cortex (deep prepiriform cortex) at which single injections of a chemoconvulsant can initiate generalized seizures. Gale (1988) named this susceptible region the "area tempestas" and hypothesized that it played a pivotal role is kindling seizure generation and propagation. Several studies have failed to substantiate this claim because: kindling in the area tempestas is no more sensitive than from any other PIR sites or from the AM (Cain *et al.*, 1988a; Hönack et al., 1991; Löscher *et al.*, 1995; Morimoto, Dragunow, & Goddard, 1986; Zhao & Moshé, 1987); infusion of chemiconvulsants into the area tempestas fails to facilitate AM kindling (Morimoto *et al.*, 1986; Stevens, Phillips, & de Beaurepaire, 1988); and bilateral or unilateral lesions of this anterior PIR site have no effect on kindling or established seizures triggered from the AM (Cain *et al.*, 1988a; Ludvig & Moshé, 1988). Together, these studies suggest that the area tempestas does not critically participate in either the generation or the propagation of kindled forebrain seizures. Interestingly, other structures adjacent to the deep prepiriform cortex such the claustrum have been implicated in generating epileptiform bursting (Hoffman & Haberly, 1991), and it is conceivable that the area tempestas's epileptic susceptibility may instead be due to effects of the treatments on the nearby claustrum.

Molecular and metabolic changes in PIR. Although there has been no direct evidence of morphological alterations in the PIR with kindling, changes do occur in neurotrophins that are suggestive of plastic restructuring. BDNF, NGF, and *trkB* receptor mRNA expression levels increase in the PIR following the establishment of generalized kindled seizures (Benzgon *et al.*, 1992, 1993; Ernfors *et al.*, 1991). Recall that a single brief stimulation induces BDNF mRNA only in the DG granule cells, whereas a single stimulation causes a *decrease* of BDNF mRNA in the PIR. Unlike the AM, the PIR exhibits larger and more rapid increases of NGF and BDNF mRNA and proteins with 40 rapid recurring hippocampal seizures (Elmér *et al.*, 1998).

Autoradiographic studies of cerebral metabolism during the early stages of kindling have demonstrated a strong association between sites that exhibit high metabolic activity and sites that readily exhibit IIS (Ackermann, Chugani, Handforth, Moshé, Caldecott-Hazard, & Engel, 1986). The 2-deoxyglucose method for calculating the local cerebral metabolic rate for glucose is used to determine seizure severity and duration in various anatomical locations following kindling (Engel, Wolfson, & Brown, 1978). During AM partial seizure kindling the ipsilateral PIR consistently exhibits increased glucose utilization (Ackermann *et al.*, 1986; Engel *et al.*, 1978).

These 2-deoxyglucose studies parallel the patterns of expression seen with *c-fos*.

As with the AM, during the initial stimulation trials *c-fos* expression occurs exclusively in the AM and PIR and eventually spreads bilaterally to other limbic structures as kindling progresses to generalized seizures (Chiasson *et al.*, 1995; Clark *et al.*, 1991; Dragunow *et al.*, 1988; Ebert & Löscher, 1995b), regardless of which forebrain site is stimulated (Burchfiel *et al.*, 1998). Burchfiel and colleagues (1998) have further shown that the transition point of the spread of Fos expression from the ipsilateral PIR to the contralateral structures occurs at the time that the first stage 1 / 2 kindled seizure is evoked. Ebert and Löscher (1995b) found more pronounced *c-fos* expression in the posterior rather than the anterior portions of the PIR with kindling, which corresponds to the posterior PIR's greater sensitivity to kindling stimulation (Löscher *et al.*, 1995).

<u>Cell loss in the PIR.</u> There is some evidence that points to degenerative processes in the PIR. After partial and full kindling there is an elevation in GFAP in the ipsilateral PIR and AM (Hansen *et al.*, 1990; Khurgel *et al.*, 1992; Racine *et al.*, 1989). The largest increases of GFAP occur in the PIR, but return to control levels within two months (Hansen, Jorgensen, Bolwig, & Barry, 1991). More recently, Lehmann, Ebert, and Löscher (1998) reported that kindling of the basolateral AM led to a pronounced decrease in the number of GABA immunoreactive neurons in the ipsi- and contralateral AM, while a significant reduction in GABA cells were observed only in the ipsilateral PIR at the transition zone between anterior and posterior portions (area of the PIR adjacent to the AM).

To conclude, the evidence reviewed above strongly implicates the PIR in the genesis of kindling. Much of the correlative evidence reveals the PIR to be a highly excitable region that is very susceptible to kindling stimulation and readily exhibits IIS and spontaneous bursting. When provoked, the PIR will exhibit epileptiform bursting. Furthermore, the results of *in vivo* and *in vitro* studies suggest that kindling from other sites enhances the excitability of the PIR, implying that these other sites may all converge onto the PIR. Together, these changes in excitability may allow the PIR to function as an amplifier that generates and propagates seizures throughout in the forebrain. Because of this evidence some researchers have proclaimed the PIR to be the site of the first transition gate responsible for facilitating, intensifying, and spreading of seizures from other foci in the forebrain (Burchfiel & Applegate, 1989b; Löscher & Ebert, 1996). Two important aspects of the data support this notion: first, many of the correlative changes occurred prior to the establishment of fully kindled seizures; second, many of the changes occurred unilaterally.

Perirhinal Cortex

The perirhinal cortex (PRH) is the one of the more recent structures implicated in kindling. Thus we have the least amount of information about this region in comparison to the other structures reviewed so far. Notwithstanding its paucity, the evidence acquired to date suggests that the PRH participates in establishing the later stages of generalization during kindling.

Anatomy of the PRH. There is lack of consensus in the literature regarding the boundaries of the PRH in rats. Essentially the rat PRH surrounds the posterior portion of the rhinal sulcus and is bounded by the entorhinal cortex, temporal association areas, and the postrhinal/parahippocampal cortex (Burwell, Witter, & Amaral, 1995). Apart from the PRH being a six layered neocortical region, little more is known about the cytoarchitectonic features and how they contribute to the flow of information. The connections of the PRH can be defined by three major features (Suzuki, 1996). First, the PRH has robust interconnections with the hippocampus via the entorhinal cortex, such that nearly 40% of the direct input to the entorhinal arises from the PRH. Second, the PRH receives prominent input from diverse unimodal and polymodal association cortices, including the frontal, parietal, insular, and PIR cortices (Burwell *et al.*, 1995) and projects back to many of these cortical areas in the rat (McIntyre, Kelly, & Staines, 1996). Third, the PRH has pronounced interconnections with the AM (Burwell *et al.*, 1995; Stefanacci, Suzuki, & Amaral, 1996), and various other subcortical regions including the nucleus accumbens, fundis striati, thalamus, and the central grey (McIntyre *et al.*, 1996). These neuroanatomical data suggest that the PRH is a zone of convergence from both cortical and subcortical structures. From a functional perspective the PRH is ideally positioned to carry epileptiform activity from subcortical to cortical motor regions.

Kindling in the PRH. McIntyre, Kelly, and Armstrong (1993) were the first to kindle the PRH and found that this structure could develop full seizure generalization within 4 stimulations, significantly faster than the PIR or AM (Mohapel *et al.*, 1996). Additionally, it was found that the onset of the kindled clonic convulsion from the PRH was almost instantaneous with the onset of the electrical stimulation (McIntyre *et al.*, 1993). These authors have argued that the brief latencies to clonus reflect the PRH's ability to both synchronize discharge and to propagate it with sufficient intensity to regions that can support motor convulsions (Kelly & McIntyre, 1996; McIntyre *et al.*, 1993). More recently, T. Sato *et al.* (1998) examined PRH kindling and confirmed the rapid latencies to clonus and convulsion profiles of McIntyre's data, but failed to replicate the rapid kindling rate (i.e., Sato observed that 12 stimulations were required to kindle the first generalized seizure). Similar to the PIR, the PRH may also promote epileptic discharge induced from other limbic sites that receive kindling stimulation. Buchanan & Bilkey (1997) demonstrated partial positive transfer between the PRH and AM, in that partial seizures were evoked sooner in AM kindling by prior kindling of fully generalized seizures from the ipsilateral PRH.

Lesions of the PRH. In attempt to assess the role of the PRH in kindled motor seizures, Kelly & McIntyre (1996) selectively lesioned the PIR and PRH by inducing status epilepticus via 60 minutes of continuous electrical stimulation to a kindled AM focus. They performed a forebrain commissurotomy to prevent epileptiform discharge from moving easily between hemispheres, and found that lesion damage incurred only to the PIR did not interfere with the ability of a kindled focus from the dorsal hippocampus to activate motor substrates in the contralateral hemisphere. Damage to both PIR and PRH, however, prevented the hippocampal focus from accessing motor substrates from the contralateral hemisphere (Kelly & McIntyre, 1996). The authors concluded that an intact PRH was required for epileptiform activity to propagate from the hippocampus to the contralateral motor substrate necessary for seizure generalization. Support for this proposition comes from a recent study in which the PRH in one hemisphere was inactivated by infusion of KCl, resulting in regression of contralateral kindled AM seizures from generalized to partial convulsions (Ferland, Nierenberg, & Applegate, 1998).

Bursting in the PRH. Several drug and electrophysiological studies have suggested the PRH is an important site for the amplification and distribution of limbic seizure discharges. Focal application of GABA agonists into the PRH have been shown to block chemically induced seizures from the area tempestas (Halonen, Tortorella, Zrebeet, & Gale, 1994). McIntyre and Plant (1993) discovered that in slices taken after AM kindling, the PRH displayed greater spontaneous and evoked bursting responses when the NMDA glutamate receptors where augmented with a magnesium free perfusion. Matsumoto and colleagues recorded field potentials from slices taken after AM kindling and found that the PRH exhibited greater spontaneous bursting and strong synchronizing activity following tetanic stimulation (Matsumoto, Yamada, Morimoto, Bilkey, & Kuroda, 1996). Kelly and McIntyre (1996) examined the intrinsic responses of different neurons in the PRH and discovered that neurons in deep layer 5 had very low spike thresholds and a propensity to intrinsic bursting.

Molecular changes in PRH. Some recent research has examined the pattern of Fos protein expression following PRH kindling to identify the relevant regions involved in the propagation of seizures from the PRH. Most of the Fos labelling with full PRH kindling occurred in the ipsilateral hemisphere of stimulation, where the AM, PIR, PRH, entorhinal cortex, insular cortex, parietal cortex, and frontal cortex exhibited positive labelling (Ferland *et al.*, 1998; Sato *et al.*, 1998). More interestingly, Fos labelling in the contralateral site was more discrete and was predominantly confined to the PRH, insular cortex, and frontal motor cortex (Ferland *et al.*, 1998; Sato *et al.*, 1998). Again these data strongly suggest that the PRH is important for recruiting contralateral structures that are responsible for seizure generalization. Applegate and colleagues further demonstrated that the activation of these same contralateral sites was not necessarily dependent on selfsustained AD in the PRH (i.e., on kindling stimulation), since they were able to show similar Fos patterns via application of a strong electrical current to the PRH that would elicit clonic motor convulsions without eliciting self-sustaining AD (Ferland *et al.*, 1998).

There have been no investigations that have looked at morphological, structural, or neurodegenerative changes in the PRH with kindling. There is some suggestion that plastic changes do occur since full kindling increases BDNF levels in the PRH (Bengzon *et al.*, 1993; Sato *et al.*, 1996).

To conclude, the evidence accumulated thus far implicates the PRH in kindling. Applegate, Burchfiel, and associates argue that the PRH represents the second hindbrain/neocortical transition gate that is responsible for progression into the later generalized stages of kindling (Applegate, Burchfiel, Ferland, & Nierenberg, 1998). There is strong support for this proposition. First, there is rapid onset to motor convulsions with PRH kindling that suggests an anatomically close association with motor substrates. These PRH motor circuits can be tapped into without even generating self-sustaining AD (Ferland *et al.*, 1998). Furthermore, the lesion and Fos data suggest that the PRH probably has access to multiple *bilateral* motor substrates, since it appears to propagate epileptiform activity between both hemispheres. Second, lesioning of the PRH did not completely abolish all convulsions but was only capable of degrading them to the partial seizure stages, which implies that a unique circuit responsible for seizure generalization (i.e., the hindbrain/neocortical transition gate) was blocked, whereas the circuit responsible for the establishment of partial seizures (i.e., the forebrain transition gate) was not. Kelly and McIntyre (1996) found a complete disappearance of all full and partial motor convulsions only when *both* the PRH and PIR were damaged.

The Claustrum and Kindling

For many years the claustrum (CLA) has received little attention from investigators, and it still remains poorly understood despite its prominent presence in the mammalian brain. Functionally, the CLA is thought to participate in the integration of sensory, motivational, emotional, and mnemonic information via its reciprocal connections with the cortex and subcortical limbic structures (e.g., Markowitsch, Irle, Bang-Olsen, & Flindt-Egebak, 1984). There have been very few studies that have looked at the involvement of the CLA in epilepsy or animal seizure models.

Anatomy

The CLA is a telencephalic structure that is located deep in the cerebral hemispheres, just underneath the cortex adjacent to the rhinal sulcus. The rat's CLA has an irregular elliptical shape with its major axis oriented from ventromedial to dorsolateral. In the coronal plane the CLA extends anteriorly from the tip of the striatum and extends posteriorly, as the size of the nucleus gradually decreases, to the most posterior portion of the thalamus (Minciacchi, Molinari, Bentivoglio, & Macchi, 1985). The rodent CLA can be segmented into two parts, according to the most prominently visible cell groups: the ventromedial (anterior) section that adjoins the prepiriform cortex, and the dorsolateral (posterior) section that underlies the insular/PRH cortex (Kowiański, Dziewiątkowski, Kowiańska, & Moryś, 1999; Minciacchi *et al.*, 1985; Witter, Room, Groenewegen, & Lohman, 1988). The anterior CLA has a ventral border that merges with the dorsal endopiriform nucleus, and contains polymorphous, fusiform, and small multipolar cells. The posterior CLA is bounded medially by the external capsule and laterally by the insular/ PRH cortex where it merges with these deep cortical layers, and also contains similar but slightly larger (pyramidal rather than fusiform) and more densely packed cells (Kowiański *et al.*, 1999; Minciacchi *et al.*, 1985; Sadowski, Moryś, Jakubowska-Sadowska, & Narkiewicz, 1997).

Very few detailed studies are available on the anatomical organization of the rat CLA and most of our knowledge has come from cat and monkey studies. In these species the CLA projects to virtually all regions of the ipsilateral cerebral cortex and receives many reciprocal cortical inputs back. In rodents, as in other species, there appears to be somewhat of a topographical organization of the claustrocortical system with neocortical, limbic cortical, and paralimbic cortical regions (e.g., Markowitsch *et al.*, 1984; Norita, 1977; Sloniewski, Usunoff, & Pilgrim, 1986b; Witter *et al.*, 1988). The anterior CLA has reciprocal connections with the frontal cortex (including motor areas), entorhinal cortex, and possibly the anterior olfactory nucleus and prepiriform cortex; while the posterior CLA is interconnected with the visual cortex, orbitofrontal cortex, insular cortex, PRH, anterior limbic, and cingulate cortex in the rat and cat (Clasca, Avendano, Roman-Guindo, Llamas, & Reinoso-Suarez, 1992; Markowitsch *et al.*, 1984; Minciacchi *et al.*, 1985; Witter *et al.*, 1988). The CLA also has bilateral topographic reciprocal connections with many of the same neocortical and limbic cortical regions across the corpus callosum in the rat and cat (Minciacchi *et al.*, 1985; Norita, 1977; Witter *et al.*, 1988). Although not as prevalent, or well studied, the CLA also has many subcortical interconnections. In the rat, the thalamus, hypothalamus, and brain stem (monoaminergic nuclei) project to the CLA (Sloniewski, Usunoff, & Pilgrim, 1986a), and in the cat and primate reciprocal connections occur with the AM, thalamus, basal ganglia, and substantia nigra (Andersen, 1968; Arikuni & Kubota, 1985; Flindt-Egebak & Olsen, 1978; Krettek & Price, 1978; Sloniewski *et al.*, 1986a). To summarize, the intricate organization and anatomy of the CLA suggests that this nucleus can readily interact with many different cortical activities. Thus it may act as a powerful interhemispheric relay mechanism between subcortical and cortical structures.

Involvement in kindling

The unique connectivity of the CLA makes it an attractive candidate as a critical substrate for epileptogenesis from limbic sites. Indeed, strong electrical stimulation of the CLA can induce contralateral head and body turning with tonic extension of the contralateral forelimb in cats (Gabor & Peele, 1964), which is identical to the behavioural pattern of a stage 4 seizure in an AM kindled cat (Wada & Sato, 1974). Wada and associates are the only group to investigate the role of the CLA in kindling (Wada, 1998). Their approach was to examine the effects of unilateral CLA lesions on AM kindling in both cats and primates. In cats they looked at the effects of unilateral CLA lesions either prior to or following completion of ipsilateral AM kindling and found that they could destabilize but not completely prevent or eliminate motor seizure development (Kudo & Wada, 1990; Wada & Kudo, 1997). This inability to entirely eliminate kindled motor convulsions in the felines could be attributed to the fact that epileptiform activity was capable of migrating to the non-lesioned hemisphere and activating its intact motor substrates (Wada & Kudo, 1997). However, with primates, the same CLA lesion applied following ipsilateral AM or cingulate cortex kindling was able to effectively *eliminate* generalized motor convulsions and reduce them to non-convulsive partial seizures (Wada & Tsuchimochi, 1992, 1997). Additionally, unilateral CLA lesions were able to degrade bisymmetrical generalized seizures to partial onset secondarily generalized seizures in cats and primates treated with D,L-allylglycine, which allows seizures to be triggered photically (Kudo & Wada, 1995; Wada & Tsuchimochi, 1992, 1997).

Objectives

Very little work has been done to investigate the second transitional gate responsible for establishing the late kindling phase. Recall that only one structure has been proposed to house this gate, the PRH. Compared to the PIR, our understanding of the PRH's role in kindled seizure generalization is sparse at best.

The objective of my research was to determine whether the PRH alone can account for the second transitional gate responsible for generalized seizures, or whether another structure can better represent this transitional gate. Wada's data strongly implicate the CLA as an alternative candidate to the PRH. Its anatomical features suggest that the CLA could act as an interhemispheric relay between many of the crucial limbic structures implicated in kindling, such as the AM, PIR, and PRH, and the cortical and brainstem regions that contain the motor convulsive substrates. The research reported in my dissertation focuses primarily on the epileptic properties of the dorsolateral CLA, because this region has close associations with the deep laminar layers of the PRH. Moreover, based on McIntyre's reports, the deep layers of the PRH exhibit the greatest susceptibility to kindling stimulation (Felstead, Kelly, & McIntyre, 1995) and exhibit strong intrinsic bursting properties that have been linked to the amplification and distribution of seizure discharge (Kelly & McIntyre, 1996). I attempted to determine the degree to which the kindling properties of the deep layers of PRH can be attributed to the dorsolateral CLA. The ultimate objective was to refine our understanding of the locus for the seizure generalization transitional gate by examining the epileptogenic properties of the CLA in relation to the PRH.

The following experiments were designed to address the unique contribution of the rat CLA to kindling. These experiments characterized the electrographic and behavioural profiles of kindling when stimulation is directly applied to the CLA; examine changes in various molecular markers linked to neural plasticity with CLA kindling; determine how CLA kindling interacts with alternating kindling from other limbic sites (i.e., kindling antagonism); and determine the effect of CLA and deep cortical lesions on limbic kindling.

GENERAL METHODS

Procedures common to all experiments are described in this section; deviations from these methods are described in the appropriate sections.

Subjects

Male Long-Evans rats (Charles River, Quebec), weighing between 250-350 g at the time of surgery, were used in these experiments. Food and water were freely available and testing occurred during the light portion of the 12 hr light/dark cycle.

Surgery

Surgical procedures were conducted according to the guidelines of the Canadian Council on Animal Care. Following at least 7 days of colony room habituation and daily handling, rats were anaesthetized with sodium pentobarbital (65 mg/kg, i.p.) and placed in a stereotaxic frame. All rats had bipolar stimulating/recording electrodes implanted bilaterally into various limbic regions. The electrodes were constructed from two strands of 127μm diameter Teflon-coated nichrome wire, attached to female amphenol pins. The tips of the wire electrodes were scraped with a razor blade to remove the Teflon coating and were positioned side-by-side. A stainless steel jeweller's screw, which was secured to the right anterior pole of the skull, served as the ground/reference electrode. Four additional jeweller's screws were anchored to the skull to which the electrodes were affixed with dental acrylic. In these experiments electrodes were aimed at any of the five limbic regions: the amygdala (AM), claustrum (CLA), insular cortex (INS); piriform cortex (PIR), or perirhinal cortex (PRH). The stereotaxic coordinates were: AM, 0.4 mm posterior from bregma, 4.5 mm lateral to midline, and 8.4 mm ventral from dura; CLA, 0.2 mm anterior from bregma, 4.0 mm lateral to midline with the electrode angled laterally at 14^0, and 7.0 mm ventral from dura; INS, 0.2 mm anterior from bregma, 4.0 mm lateral to midline with the electrode angled laterally at 13^0, and 7.0 mm ventral from dura; PIR, 0.2 mm posterior from bregma, 4.0 mm lateral to midline with the electrode angled at 10^0, and 9.0 mm ventral from dura; PRH, 0.4 mm posterior from bregma, 4.0 mm lateral to midline with the electrode angled laterally at 13^0, and 7.0 mm ventral from dura. The incisor bar for all placements was set at +5.0 mm.

Kindling

Following a one week postsurgical recovery period, the thresholds for afterdischarge (ADT) were determined. Rats were connected to the recording/stimulating lead and placed in a clear plexiglass testing chamber (33 X 37 X 50 cm). Before ADT determination, the baseline EEG was recorded for approximately 30 sec. Electrical stimulation (Grass S88 stimulator) consisted of a 1-sec. train of constant current balanced biphasic square-wave pulses (1-msec. duration, 60/sec.) delivered to one of the electrode sites at an initial current of 20 μA (base-to-peak), and increased to higher intensities (40, 60, 80, 100, 150, 200 μA; and by 100 μA increments to 10 000 μA) at 1-min intervals until an AD was evoked. ADT was arbitrarily defined as the minimum stimulation intensity required to trigger an AD that outlasted the stimulation by at least 5 sec. Twenty-four hrs following the first ADT determination, the same procedure was repeated for the contralateral electrode site.

Twenty-four hrs later, once-daily kindling sessions began, with the same stimulation parameters used to determine the ADT. The kindling stimulation was applied at one current intensity increment below the ADT of the previous day. If that stimulation was insufficient to trigger AD, then, after one min., it was increased by one intensity until an AD was triggered. This method insured that the kindling site was stimulated with an intensity near its daily ADT. The AD duration and the behavioral seizure stages (Racine, 1972b) were recorded after each stimulation trial. Briefly, stage 1 involves chewing movements, stage 2 includes the addition of head bobbing, stage 3 includes the addition of unilateral clonic forelimb movement, stage 4 involves bilateral clonic forelimb movements, and stage 5 includes the addition of rearing on the hindlimbs and loss of postural control (Racine, 1972b). Daily stimulation typically continued until three generalized stage 5 seizures were evoked.

Histology and Data Analysis

At the completion of kindling, rats were deeply anaesthetized and perfused intracardially with saline. Brains were removed and stored in 10% formalin for at least one week before sectioning. Frozen coronal sections 40 μm thick were taken from the region of the electrode tracks and/or lesions and stained with cresyl violet.

Data were analysed (SPSS, version 6.1.2) with one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test, with an alpha level of 0.05. Planned comparisons between groups were made with independent sample *t*-tests. Differences were considered statistically significant at $\alpha = 0.05$. Values are given as MEAN ± S.E.M.

EXPERIMENT 1

Kindling in the Claustrum

No information is available about the kindling properties of the CLA. Therefore, this experiment sets out to characterize the electrographic and behavioural characteristics of CLA kindling. Electrodes were placed in the dorsolateral CLA region, which is bordered medially by the external capsule and laterally by the insular cortex (INS) or PRH. Many of the deep laminar layers of the INS and PRH merge with the dorsolateral CLA (Minciacchi *et al.*, 1986); therefore one of the main objectives of this experiment is to determine whether CLA kindling is distinct from these adjacent cortical structures.

The following experiments compared the kindling profile of the dorsolateral CLA with that of INS, deep layers of the PRH, and AM. The first experiment examined the kindling and EEG profiles, including the kindling rates, convulsion latencies and durations, and AD spike durations, frequencies, and amplitudes. This experiment also examined the transfer of kindling from the CLA, INS, or PRH, to the AM. In a second experiment rats were suspended in a harness so that all four limbs could be clearly observed during convulsions. Suspending the rats allowed for more detailed documentation of the clonic and/or tonic components in both forelimb and hindlimb convulsions (see Chen, Noffel, Cottrell, Hwang, & Burnham, 1996).

Experiment 1a: Electrographic Profiles

Methods

Groups

All rats carried 2 electrodes, one in each hemisphere. One electrode was implanted into the AM and the other was implanted into either the contralateral dorsolateral CLA, INS or deep laminar PRH. Seizures were kindled from one site in all rats, and in some rats seizures were kindled from both sites to examine transfer, until a minium of 3 "robust" consecutive stage 5 seizures were evoked. At the completion of kindling rats were perfused and electrode placements were assessed, resulting in 34 rats being placed into one of four kindled groups: AM (n=8), CLA (n=10), INS (n=8), and PRH (n=8).

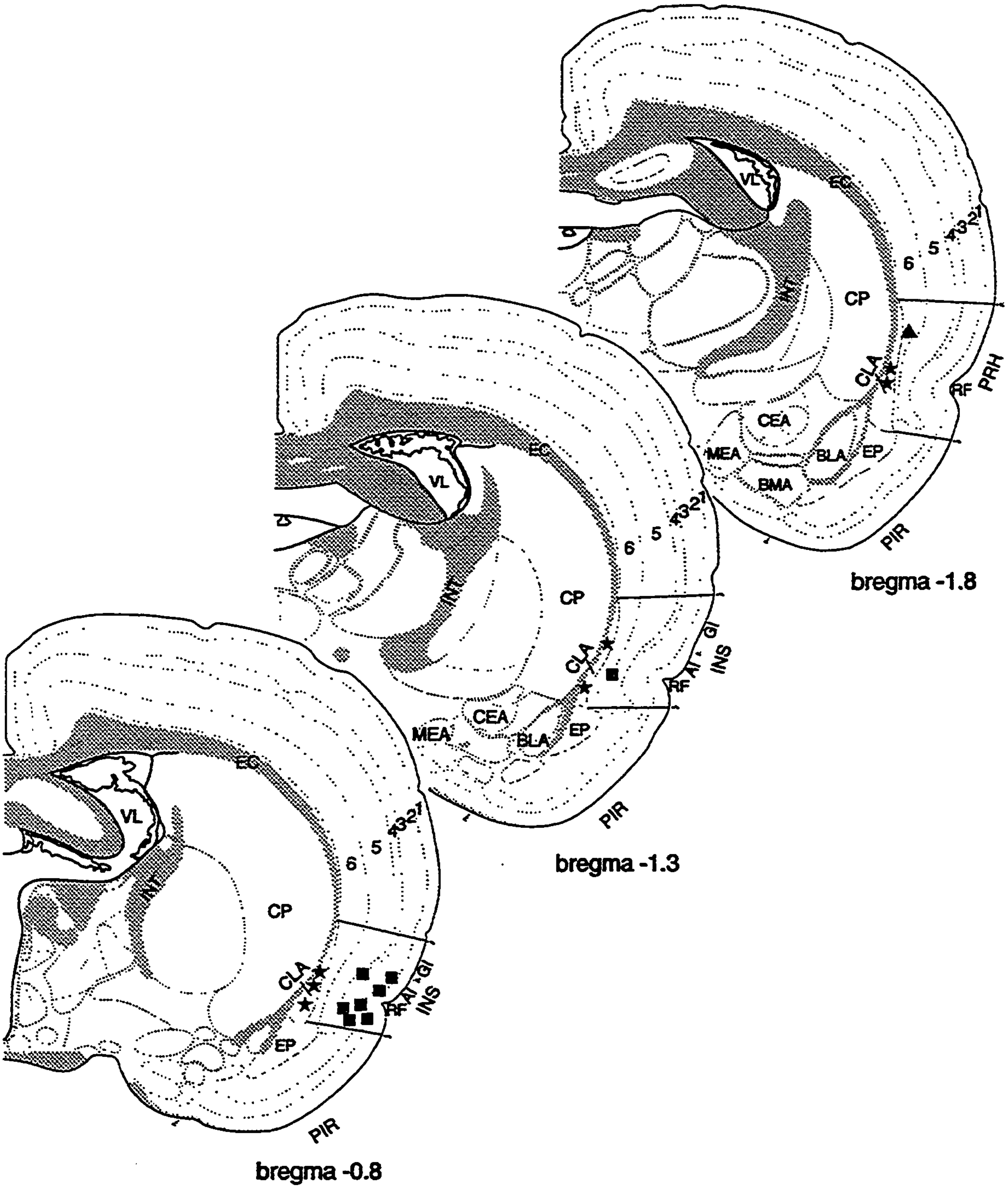
Results

Schematic locations of all the electrode tips are presented in Figure 3. Anatomical distinctions and boundaries were derived from the rat brain atlases of both Paxinos and Watson (1986) and Swanson (1992). All of the AM placements were located in or near the basal nuclei. The INS placements were located primarily in the posterior agranular regions (approximately between bregma -0.3 to -1.3 mm) and broadly distributed across the full range of laminar layers. All PRH placements were restricted to the deep laminar layers of the anterior portion of the cortex (approximately between bregma -1.8 to -2.7 mm). CLA placements were located in the dorsolateral aspect, adjacent to the deep cortical layers of the INS and PRH (approximately between bregma -0.4 to -1.8 mm). Although the dorsolateral CLA was not easily distinguishable from deep cortical layers, it could be identified by its more intensive staining, since the CLA region has a higher cellular density and lacks the spatial orientation of the cells in the cortical laminar layers in the rat (see Kowiański *et al.*, 1999).

Afterdischarge Thresholds

Assessment of the ADTs (see Table 1) suggested that the AM group had the lowest mean initial threshold values. However, due to the large variances in initial ADT in the cortical regions and in the CLA, ADTs only from the INS were statistically different from the AM ($F(3,30) = 3.95$; $p < 0.02$). Over the course of kindling ADTs for all groups dropped significantly ($t(30) = 2.48$, $p < 0.02$); however, the INS group's final ADT decreased the least, since it remained significantly higher than the AM's final ADT ($F(3,30) = 2.88$; $p < 0.05$).

Figure 3. Schematic diagram of coronal sections of the rat brain, adapted from Swanson (1992), illustrating the electrode tip locations for each rat in the 4 kindled groups. Note that a few of the electrode tips overlap. Solid circles (●) indicate electrode placements in the amygdala (AM); solid stars (★) indicate placements in the claustrum (CLA); solid squares (■) indicate placements in the insular cortex (INS); and solid triangles (▲) indicate placements in the perirhinal cortex (PRH). Abbreviations: AI, agranular insular cortex; BLA, basolateral nucleus of the amygdala; BMA, basomedial nucleus of the amygdala; CEA, central nucleus of the amygdala; CP, caudate-putamen; EP endopiriform nucleus; EC, external capsule; GI, granule insular cortex; INT, internal capsule; MEA, medial nucleus of the amygdala; PIR, piriform cortex; RF, rhinal fissure; VL, lateral ventricle.


VL
EC
4 3 2 1
6
5
INT
CP
CLA
RF
PRH
CEA
MEA
BLA
EP
BMA
PIR
bregma -1.8
VL
EC
6
5
4 3 2 1
INT
CP
CLA
AI
GI
INS
RF
CEA
MEA
BLA
EP
PIR
bregma -1.3
EC
VL
4 3 2 1
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INT
CP
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PIR
bregma -0.8

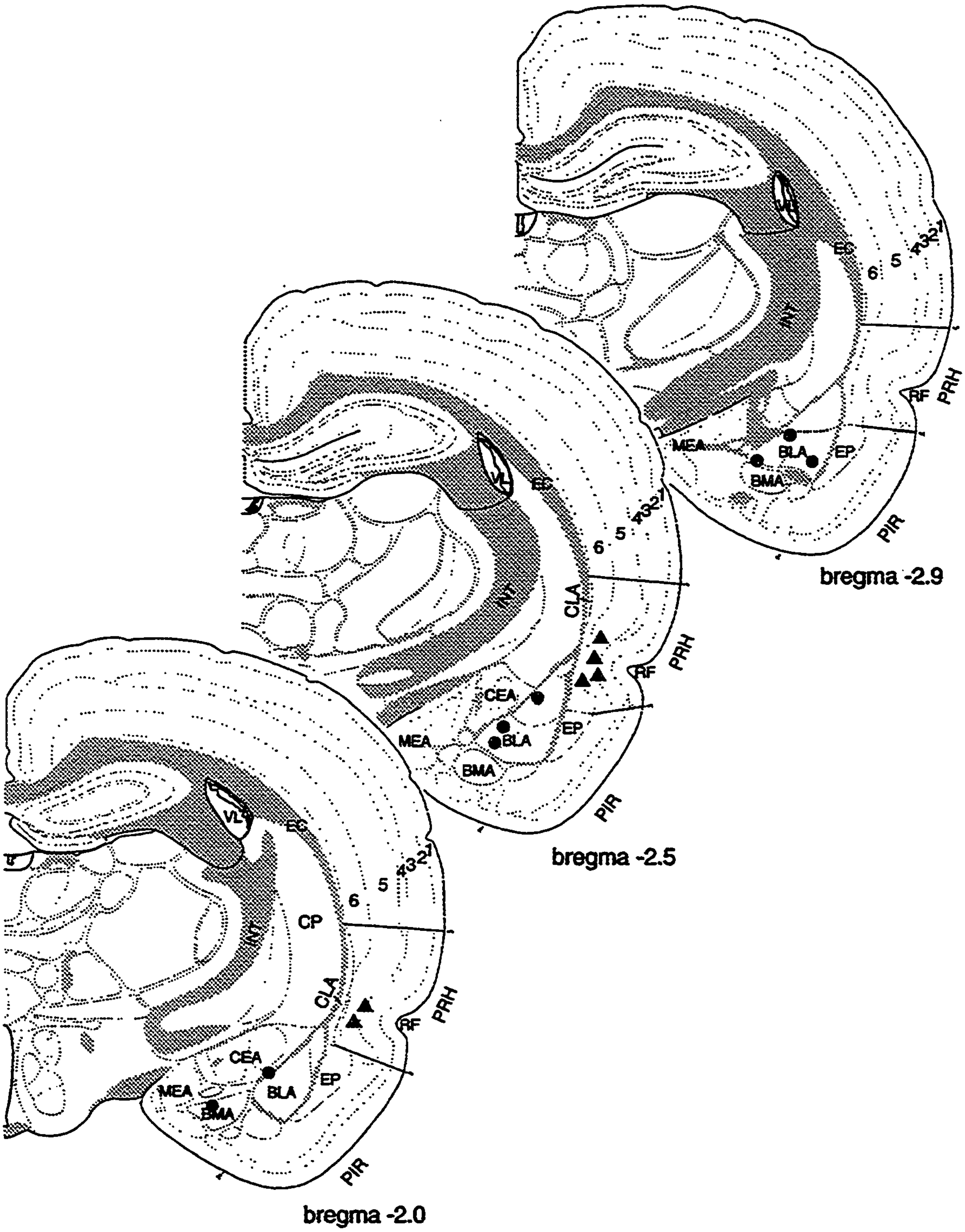

VL
EC
6
5
4
3
2
1
INT
RF
PRH
MEA
BLA
EP
BMA
PIR
bregma -2.9
VL
EC
6
5
4
3
2
1
INT
CLA
CEA
RF
PRH
MEA
BLA
EP
BMA
PIR
bregma -2.5
VL
EC
6
5
4
3
2
1
CP
INT
CLA
RF
PRH
CEA
MEA
BLA
EP
BMA
PIR
bregma -2.0

Table 1.

Mean (± S.E.M.) initial and final afterdischarge thresholds (ADTs) and several different measures of kindling rates, including: the number of daily stimulations to the first bilateral clonus; and the number of daily stimulations and total cumulative after-discharge durations (ADD) to a stage 5 seizure with afterdischarge (AD) that exceeds 30 sec., and to a stage 5 seizure with a 2 fold increase in ADD from the preceding stage 5 seizure. Kindled groups compared are the amygdala (AM), claustrum (CLA), insular cortex (INS), and perirhinal cortex (PRH).

Group	*n*	Intial ADT (μA)	Final ADT (μA)	1st Bilateral clonus (# of trials)	1st Stage 5 - AD > 30s (# of trials)	1st Stage 5 -2x ADD (# of trials)	Cumulative ADD to stage 5 -AD > 30s	Cumulative ADD to stage 5 - 2x ADD
AM	8	86 ± 24[INS]	100 ± 24[INS]	12.0 ± 0.8*	12.3 ± 0.9	12.0 ± 0.8	400.5 ± 56.6*	393.3 ± 57.9[INS]
CLA	10	1715 ± 1005	170 ± 38	2.8 ± 0.4	13.7 ± 1.1	15.3 ± 1.6	215.5 ± 17.4	267.9 ± 33.9
INS	8	5381 ± 1472	1453 ± 727	3.9 ± 0.9	12.3 ± 1.4	12.8 ± 1.3	190.6 ± 21.4	216.9 ± 24.9
PRH	8	3050 ± 1308	368 ± 142	4.3 ± 0.9	11.5 ± 1.7	13.5 ± 2.0	195.5 ± 28.2	269.3 ± 47.6

* Significantly different from all other groups, $p < 0.0002$.

[INS] Significantly different from the INS group, $p < 0.05$.

Kindling Rates

Kindling rates and convulsion profiles revealed an atypical pattern of development of seizure generalization from the CLA, INS, and PRH, as compared from the AM. These 3 structures progressed through two distinct phases of stage 5 generalized seizure development.

The initial (early) stage 5 phase of the CLA, INS, and PRH was characterized by very rapid progression to the generalized seizure stages 4 and 5 with little or no partial seizure expression. As shown in Table 1, the CLA, INS, and PRH all required fewer mean stimulation trials to reach the first bilateral forelimb clonus (i.e., stage 4, 5) compared with the AM ($F(3,30) = 32.68$, $p < 0.0001$). Furthermore, there was a trend for the CLA to kindle more rapidly than the other cortical groups. However, post hoc analysis demonstrated no significant differences between the CLA, INS, or PRH (see table 1). These nonsignificant results were confirmed with planned comparison tests between these 3 structures (CLA vs. INS: $t(9.3) = -1.11$, $p = 0.29$; CLA vs. PRH: $t(9.3) = -1.52$, $p = 0.16$; INS vs. PRH: $t(14) = -0.30$, $p = 0.77$). Subsequent analysis revealed low statistical power 0.23 ($\eta^2 = 0.09$) for these tests and, using the power charts of Feldt and Mahmoud (1958), it was determined that a sample size of approximately 25 would be required to increase the power to a modest level of 0.71. This issue will be returned to in Experiment 1b.

With repeated daily stimulation the short generalized stage 5 seizures from the CLA, INS, and PRH would abruptly transform into more elaborate and prolonged convulsions. Table 1 captures the transition to this late stage 5 phase by presenting two different measurement criteria: one lists the first stimulation trial to exhibit a stage 5 seizure that exceeds 30 sec. in AD; the other lists the first stage 5 seizure with a two-fold increase in AD duration from the immediately preceding stage 5 seizure. With both of these measurement criteria, the kindling rates for the CLA, INS, and PRH were similar to the that of the AM ($F(3,30) = 0.57$, $p = 0.64$; $F(3,30) = 0.93$, $p = 0.44$, respectively). Another conventional measurement for kindling rate, the total cumulative AD durations to the first stage 5 seizure, was also used to assess differences in susceptibility to kindling. Table 1 demonstrates that the AM group required significantly more mean cumulative AD than all the other groups to reach the first late phase criterion of stage 5 seizures with AD durations exceeding 30 sec. ($F(3,30) = 8.86$, $p < 0.001$), and that the AM group required significantly more mean cumulative AD only than the INS group to reach the second criterion of a stage 5 seizure with a two-fold increase in AD durations ($F(3,30) = 3.00$, $p < 0.05$). All further references to the late stage 5 phase of kindling used a combination of these two criteria to categorize the seizures.

Convulsive Profiles

Further distinctions were detected between the early and late phases of stage 5 seizure development from the CLA, INS, and PRH with respect to latency of onset for forelimb clonus, forelimb clonus durations, and AD durations. Figure 4 plots the differences between all groups in these 3 parameters over the first 8 stage 5 seizures trials. Over the 8 convulsion trials the CLA, INS, and PRH groups demonstrated very short latencies from stimulation onset to initiation of forelimb clonus, which were significant more rapid than the AM group ($F(3,30) = 23.56$, $p < 0.0001$; means of the first 3 stage 5 trials) (see Table 2). Additionally, these 3 groups had significantly shorter forelimb clonus durations and AD durations when compared to the AM ($F(3,30) = 19.87$, $p > 0.0001$; $F(3,30) = 30.76$, $p < 0.0001$; respectively) (see Table 2). Once the CLA, INS, and PRH stage 5 seizures progressed into the late stage 5 phase, the mean latency to forelimb clonus remained unchanged and was still significantly shorter than the AM ($F(3,29) = 22.95$, $p < 0.0001$; means of the first 3 late stage 5 trials), while the forelimb clonus and AD durations increased to match the durations of the AM ($F(3,29) = 1.62$, $p = 0.21$; $F(3,29) = 2.72$, $p = 0.06$; respectively) (with the exception that the CLA had a significantly shorter AD duration).

Electroencephalographic Profiles

Figure 5 illustrates the changes in AD spiking frequencies, amplitudes, and durations, for all 4 groups over the first 15 stimulation trials (note that frequency and amplitude measures were obtained by sampling from 5 sec. of the most prominent spike

Figure 4. Mean (± S.E.M.) convulsive profile parameters over the first 8 generalized bilateral clonic (stage 4 / 5) seizures for all 4 groups. A: latency to forelimb clonus; B: duration of forelimb clonus; C: afterdischarge (AD) duration during the stimulation trial. Kindled groups compared are the amygdala (AM), claustrum (CLA), insular cortex (INS), and perirhinal cortex (PRH). Single asterisk (*) denotes all groups significantly different from the AM group ($p < 0.03$) and double asterisks (**) denotes CLA and INS significantly different from AM and PRH groups ($p < 0.03$).

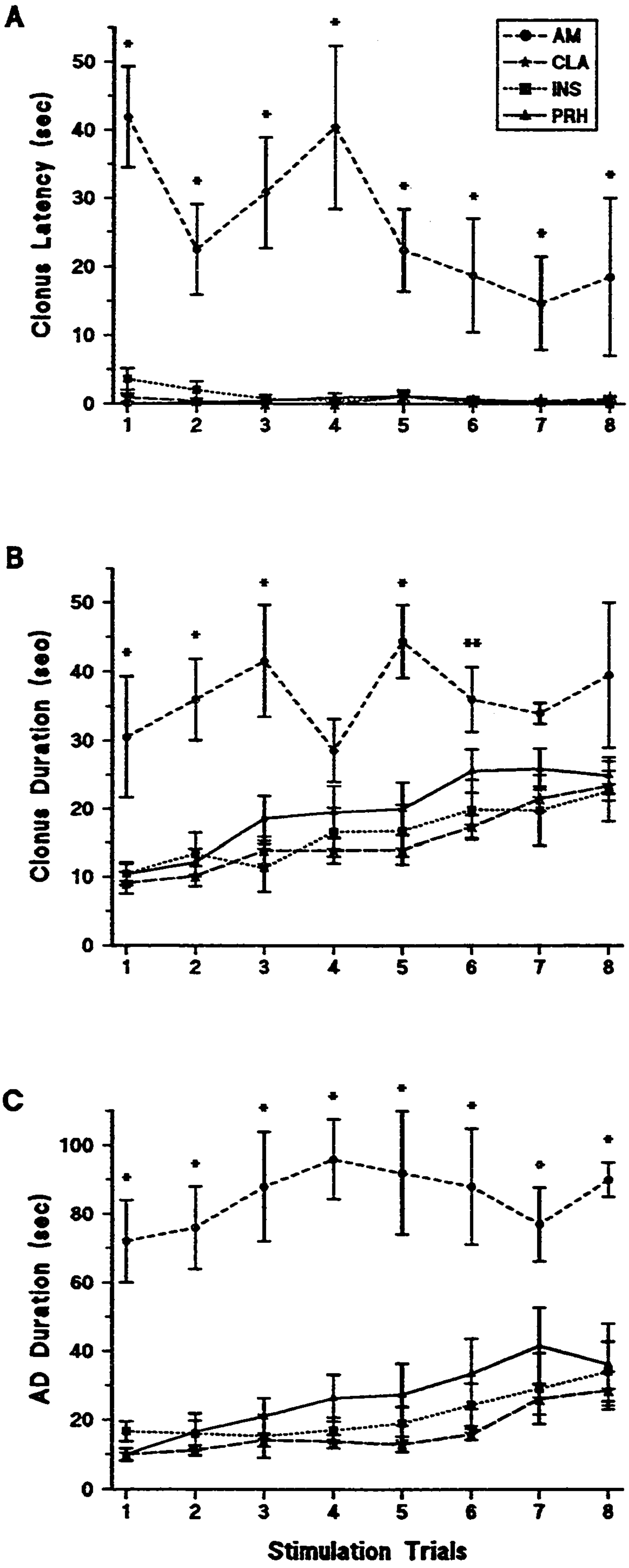

A
AM
CLA
INS
PRH
Clonus Latency (sec)
0
10
20
30
40
50
*
B
Clonus Duration (sec)
0
10
20
30
40
50
*
**
C
AD Duration (sec)
0
20
40
60
80
100
*
1
2
3
4
5
6
7
8
Stimulation Trials

Table 2.

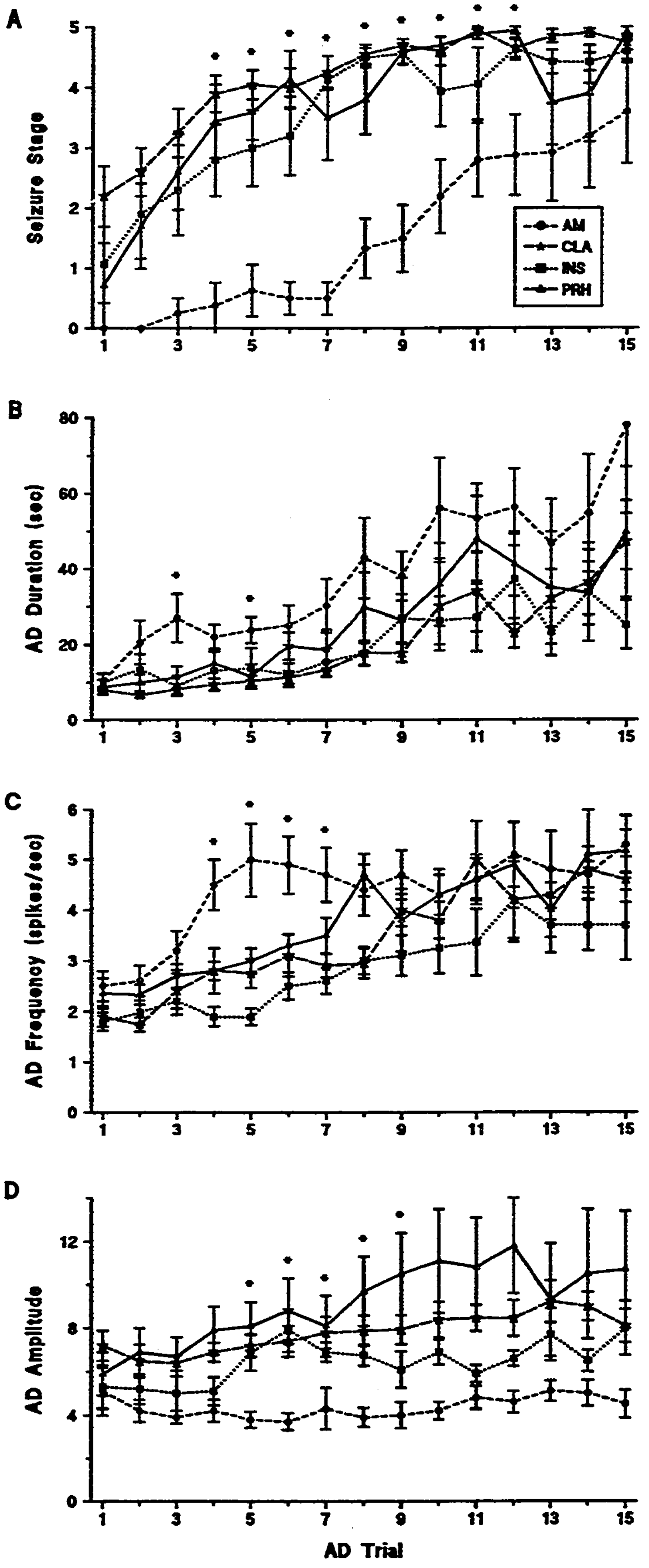
Mean (± S.E.M.) convulsive profile parameters (sec.) over the first 3 stage 5 seizures (early phase) and over the first 3 stage 5 seizures that have afterdischarge (AD) durations that exceed 30 sec. (late phase). Measurements include the latency to forelimb clonus, duration of forelimb clonus, and AD duration during the stimulation trials. Kindled groups compared are the amygdala (AM), claustrum (CLA), insular cortex (INS), and perirhinal cortex (PRH).

Group	*n*	Early phase stage 5 seizures			Late phase stage 5 seizures		
		Latency to clonus	Duration of clonus	AD duration	Latency to clonus	Duration of clonus	AD duration
AM	8	31.7 ± 6.6*	36.0 ± 4.7*	79.6 ± 11.3*	31.7 ± 6.6*	36.0 ± 4.7	79.6 ± 11.3^{CLA}
CLA	10	0.4 ± 0.2	10.7 ± 1.0	11.0 ± 1.1	0.7 ± 0.4	32.3 ± 1.8	56.7 ± 3.5
INS	8	2.1 ± 1.0	11.7 ± 2.1	15.9 ± 3.6	0.8 ± 0.4	31.1 ± 1.6	55.6 ± 5.9
PRH	8	0.2 ± 0.2	13.7 ± 1.8	15.8 ± 3.5	0.8 ± 0.3	26.6 ± 2.7	57.6 ± 5.6

* Significantly different from all other groups, $p < 0.0001$.

^{CLA} Significantly different from the CLA group, $p < 0.05$.

Figure 5. Mean (± S.E.M.) seizure stage and EEG parameters over the first 15 stimulation trials for all 4 groups. A: seizure stages; B: afterdischarge (AD) durations (sec.); C: AD frequencies (spikes/sec.); D: AD amplitude (proportion above baseline). Kindled groups compared are the amygdala (AM), claustrum (CLA), insular cortex (INS), and perirhinal cortex (PRH). Asterisk (*) denotes all groups significantly different from the AM group ($p < 0.05$).


A
Seizure Stage
0
1
2
3
4
5
AM
CLA
INS
PRH
B
AD Duration (sec)
0
20
40
60
80
C
AD Frequency (spikes/sec)
0
1
2
3
4
5
6
D
AD Amplitude
0
4
8
12
1
3
5
7
9
11
13
15
AD Trial

wave form pattern). Initially, with the first few stimulations all groups demonstrated similar EEG patterns, wherein the AD spikes were generally of the simple biphasic configuration with the same mean frequencies, amplitudes and durations (see Figures 5 & 6). On the fourth stimulation trial, at the point that most of the CLA, INS, and PRH rats displayed stage 5 seizures (early phase), AD spikes developed notched or double spike configurations, and the mean frequencies and amplitudes for these 3 groups correspondingly increased to values significantly greater than the AM (see Figures 5 & 6). The significant mean differences in AD spike frequency and amplitude of these 3 groups from the AM group continued for the next 5 subsequent stimulation trials ($F(3,30) = 8.61, p < 0.001$; $F(3,30) = 6.53, p < 0.002$; respectively). On about the 12th stimulation trial, the mean at which the AM group displayed stage 5 seizures, EEG measures no longer differed among the groups (see Figure 5).

Table 3 compares AD spike frequencies, amplitudes, and durations between the first mean 3 early and late phases of stage 5 seizures. Compared to the early stage 5 phase of the CLA, INS and PRH, AM stage 5 seizures exhibited more complex wave forms (see Figure 6). Also during this phase the AM displayed higher mean AD spiking frequencies than the other 3 groups, and in turn, the PRH displayed a higher mean AD spiking frequency over the CLA and INS ($F(3,30) = 22.65, p < 0.0001$). No significant mean differences were detected in AD spike amplitudes among the 4 groups during the early stage 5 seizures ($F(3,30) = 1.85, p = 0.16$). Once the CLA, INS, and PRH stage 5 seizures progressed into the late phase all groups displayed similar AD spike frequencies ($F(3,29) = 0.95, p = 0.43$) and AD spike amplitudes ($F(3,29) = 1.36, p = 0.28$) (see Table 3); however, the AM demonstrated less complex wave configurations than the other groups (see Figure 6).

Transfer Kindling

A subset of rats from the CLA, INS, and PRH groups received kindling stimulation in the contralateral AM to test for transfer. All groups had developed at least 3 late stage 5 phase convulsions before stimulation to the secondary site AM was applied, 1 day following the last primary site stimulation. There were no significant differences in

Figure 6. Changes in wave form, frequency and amplitude of afterdischarge (AD) spikes in the amygdala (AM), claustrum (CLA), insular cortex (INS), and perirhinal cortex (PRH) as a result of electrical kindling stimulations to each of these sites. Comparisons are made between representative AD spikes from: the initial afterdischarge threshold (ADT) stimulation trial; the first stage 5 seizure (early phase) stimulation trial; and the first stage 5 seizure that had an AD duration exceeding 30 sec. (late phase) stimulation trial. During the initial stimulation trial all groups had very similar AD spiking patterns. With the first stage 5 seizure trial the AM demonstrated appreciably more complex wave forms and increased frequency and amplitudes than all other groups. Only until the CLA, INS, and PRH progress into the late stage 5 seizure phase do the AD spiking patterns match or even exceed the complexity of the AM.

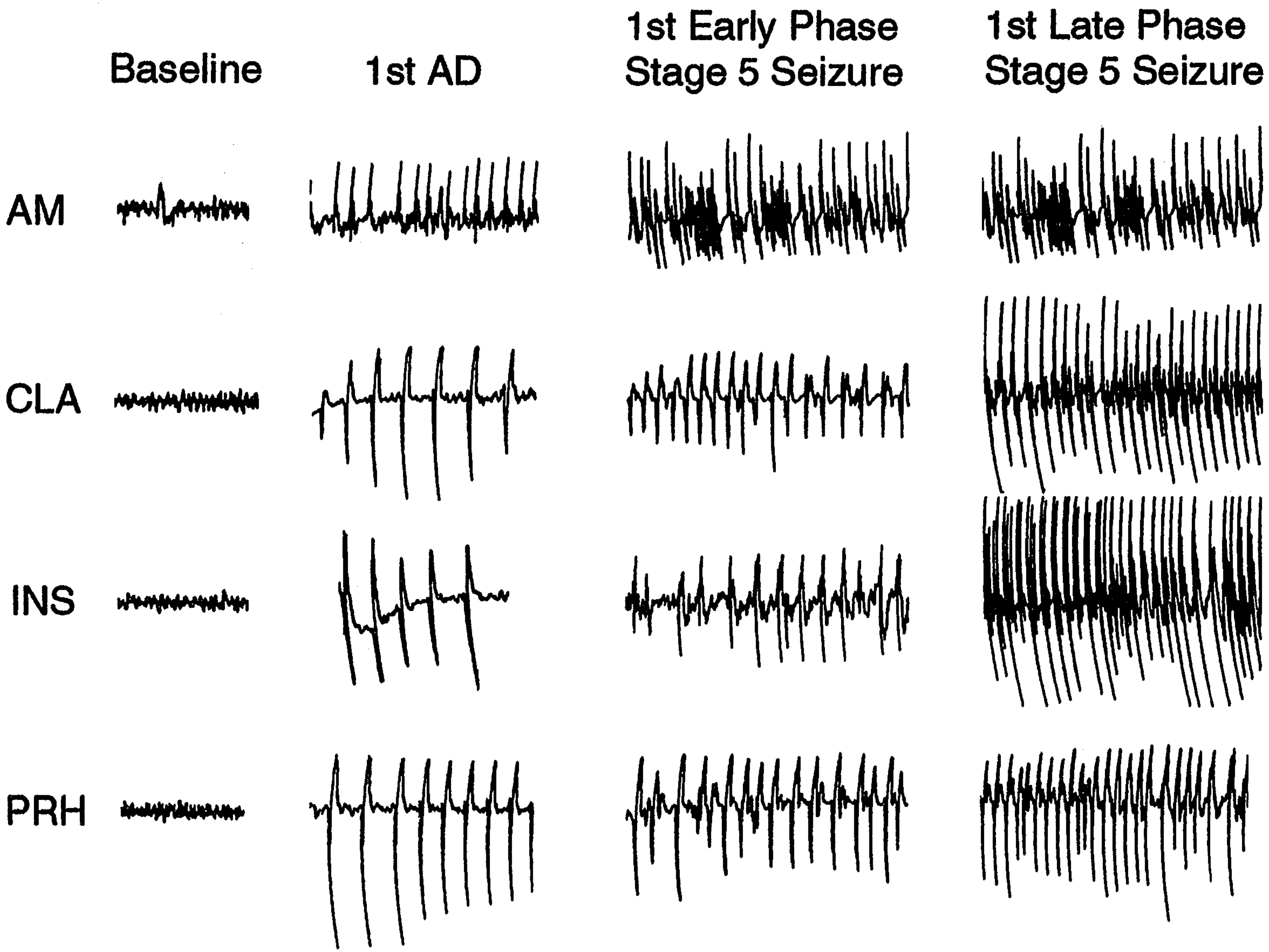

Baseline
1st AD
1st Early Phase Stage 5 Seizure
1st Late Phase Stage 5 Seizure
AM
CLA
INS
PRH

Table 3.

Mean (± S.E.M.) electroencephalographic parameters over the first 3 stage 5 seizures (early phase) and over the first 3 stage 5 seizures that have afterdischarge (AD) durations that exceed 30 sec. (late phase). Measurements include the AD spike frequency (spikes/sec.), AD spike amplitude (proportion over baseline bursting) and AD duration (sec.) during the stimulation trials. Kindled groups compared are the amygdala (AM), claustrum (CLA), insular cortex (INS), and perirhinal cortex (PRH).

Group	*n*	Early phase stage 5 seizures			Late phase stage 5 seizures		
		AD frequency	AD amplitude	AD duration	AD frequency	AD amplitude	AD duration
AM	8	5.0 ± 0.3*	5.9 ± 0.5	79.6 ± 11.3*	5.0 ± 0.3	5.9 ± 0.5	79.6 ± 11.3$^{\text{CLA}}$
CLA	10	2.5 ± 0.2	7.3 ± 0.5	11.0 ± 1.1	4.7 ± 0.4	8.5 ± 0.7	56.7 ± 3.5
INS	8	2.5 ± 0.2	6.3 ± 0.5	15.9 ± 3.6	4.2 ± 0.5	8.7 ± 2.1	55.6 ± 5.9
PRH	8	3.3 ± 0.2^	7.9 ± 1.1	15.8 ± 3.5	4.8 ± 0.4	9.9 ± 2.1	57.6 ± 5.6

* Significantly different from all other groups, $p < 0.0001$.

^ Significantly different from CLA and INS groups, $p < 0.05$.

$^{\text{CLA}}$ Significantly different from the CLA group, $p < 0.05$.

the mean number of daily stimulation trials required to evoke the first stage 5 seizure in the contralateral AM between the CLA (11.2 ± 0.8), INS (8.8 ± 1.2), or PRH (9.5 ± 1.0) ($F(2,12) = 1.56$, $p = 0.25$). Furthermore, there was no significant savings in AM kindling stimulations following prior kindling from any of these structures ($F(3,19) = 2.48$, $p = 0.09$).

In summary, kindling from the CLA, INS, and PRH exhibited very similar susceptibilities to epileptogenesis, convulsive and EEG characteristics, and transfer effects. More specifically, these 3 sites also shared an unusual two phase progression through stage 5 seizures: (1) an early phase that was characterized by rapid onset, short convulsive and AD durations, and low AD spike frequencies and amplitudes; and (2) a late phase that was virtually identical to AM stage 5 seizures with respect to the longer onset, longer convulsion durations, increased complexity in AD spiking patterns, and possibly longer AD durations, with the important exception of shorter latencies to forelimb clonus.

It is clear from the first experiment that stage 5 seizures are composed of two distinct elements with CLA, INS, and PRH kindling. Furthermore, the seizures triggered during final stage 5 phase kindling from these sites are not completely identical to AM stage 5 seizures. In the second section of this experiment I set out to better assess subtle differences between dorsolateral CLA and deep laminar PRH kindling and to refine our understanding of the 2 phase development of stage 5 seizures in these structures by characterizing convulsions using a more sensitive procedure.

Experiment 1b: Behavioural Profiles

Methods

Groups

As in experiment 1a all rats carried 2 electrodes, one in the AM and the other in either the contralateral dorsolateral CLA or deep laminar PRH. Seizures were kindled from only one site until a maximum of either 3 consecutive early stage 5 phase (early) or

3 consecutive late stage 5 phase (late) seizures were evoked. At the completion of kindling rats were perfused and electrode placements were assessed, resulting in 25 rats being placed into one of 5 kindled groups: AM (n=5), early CLA (n=5), late CLA (n=5), early PRH (n=5), and late PRH (n=5).

Suspension and Kindling

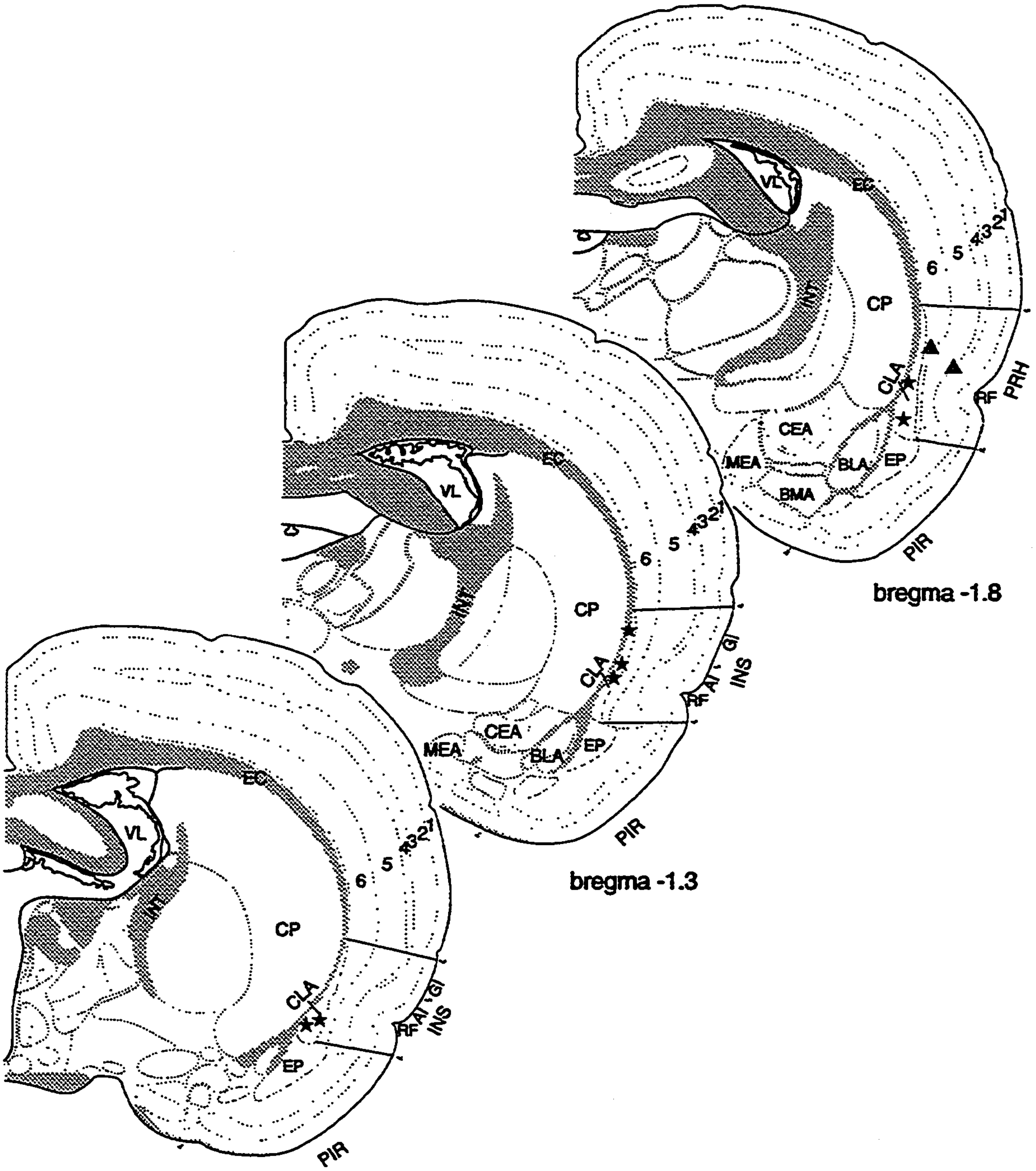
Four days following surgery for implantation of electrodes, rats were handled and habituated in a suspended harness for 10 consecutive days. The harness was constructed from fish netting with four holes for each of the limbs and a large Velcro strip to fasten the harness around the midsection of the rat. The harness permitted unimpeded movement of all limbs. Upon first exposure in the suspended harness rats displayed moderate levels of distress (vocalization and struggling to escape). However, these distress responses disappeared over the 10 day habituation period.

On the 11th day after surgery ADTs were assessed (according to the procedures outline in the general methods section) in freely moving rats. The day after the last ADT assessment, kindling trials proceeded with the rats suspended in the harness for each trial. During each stimulation trial the suspended rats were videotaped, such that a clear record of the convulsions could be obtained. Videotaping was initiated about 5 sec. before the onset of the stimulation and was continued about 1 minute after the offset of the convulsion. Videotapes of the seizures were examined for forelimb and hindlimb convulsive behaviours.

Results

Schematic locations of all the electrode tips are presented in Figure 7. Four of the AM placements were located in the basolateral nucleus, and one was located in the medial nucleus. Consistent with Experiment 1a, all PRH placements were restricted to only the deep laminar layers of the anterior portion of the cortex (approximately between bregma -1.8 to -2.8 mm), and CLA placements were located in the dorsolateral aspect,

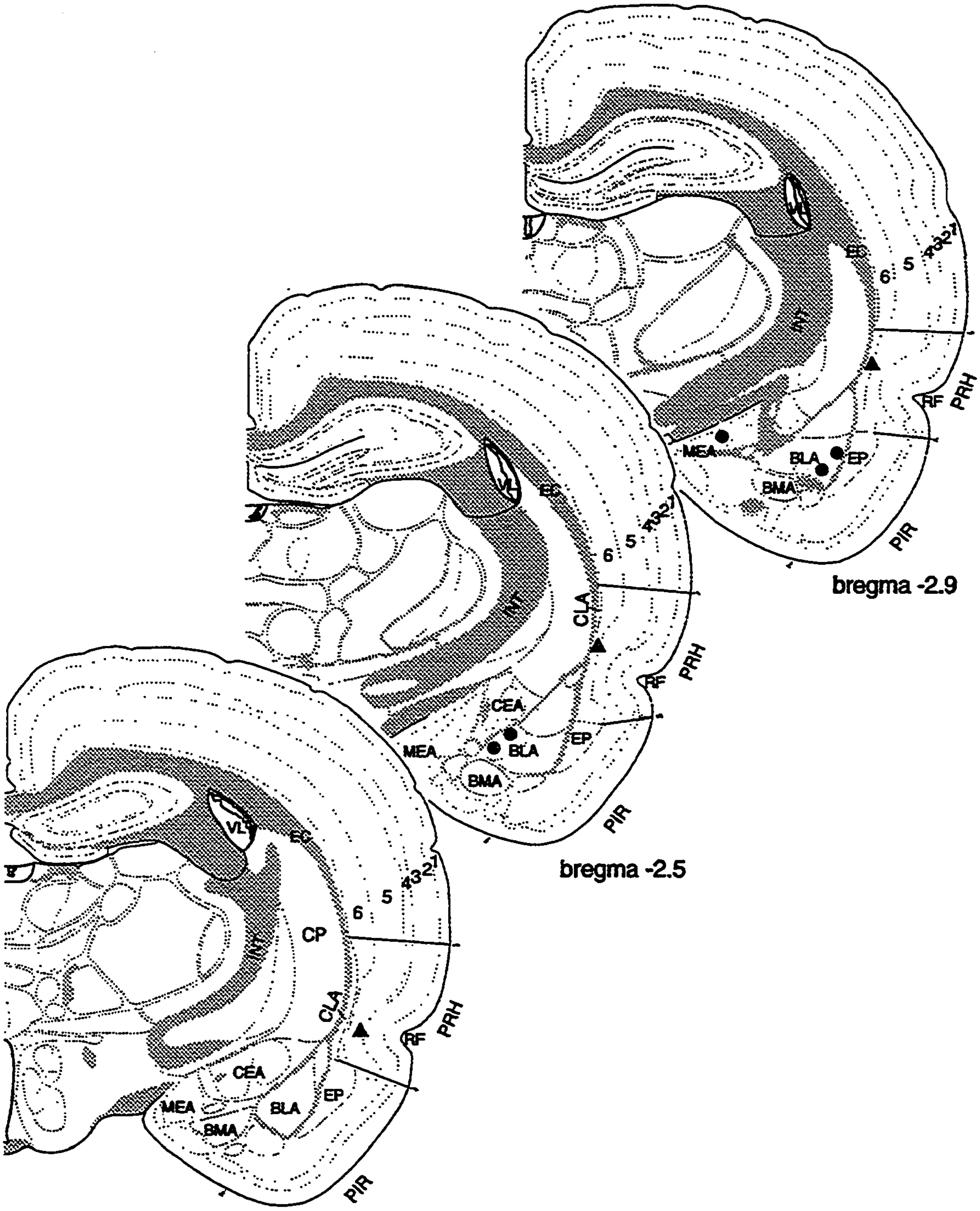
Figure 7. Schematic diagram of coronal sections of the rat brain, adapted from Swanson (1992), illustrating the electrode tip locations for each rat in the 3 kindled groups. Note that a few of the electrode tips overlap. Solid circles (●) indicate electrode placements in the amygdala (AM); solid stars (★) indicate placements in the claustrum (CLA); and solid triangles (▲) indicate placements in the perirhinal cortex (PRH). Abbreviations: AI, agranular insular cortex; BLA, basolateral nucleus of the amygdala; BMA, basomedial nucleus of the amygdala; CEA, central nucleus of the amygdala; CP, caudate-putamen; EP endopiriform nucleus; EC, external capsule; GI, granule insular cortex; INT, internal capsule; MEA, medial nucleus of the amygdala; PIR, piriform cortex; RF, rhinal fissure; VL, lateral ventricle.



bregma -1.8

bregma -1.3

bregma -0.8



bregma -2.9

bregma -2.5

bregma -2.0

adjacent to the deep cortical layers of the INS and PRH (approximately between bregma -0.4 to -1.8 mm).

Kindling Profiles

ADTs from this experiment were similar to those reported for Experiment 1a. Initial AM ADTs were lower compared to the CLA and PRH groups; however, due to large variability no significant differences were detected (F(4,20) = 0.99, p = 0.44). There were no significant differences among all groups in final ADTs (F(4,20) = 1.18, p = 0.35).

Kindling in suspended rats was identical to kindling in the nonsuspended rats of Experiment 1a. Both the CLA and the PRH exhibited rates of kindling faster than the AM, which was reflected by the significantly fewer mean daily stimulations required to the first stage 5 seizure (F(2,22) = 52.16, p < 0.0001) (see Table 4). Once again, there appeared to be a trend for the CLA to develop stage 5 seizures more rapidly than the PRH (2.4 ± 0.3 versus 3.4 ± 0.4 stimulations, respectively). However, due to insufficient statistical power no significant differences were detected (*t*(18) = -1.83, p = 0.83). In attempt to boost the power of this test, the samples of the CLA and PRH groups from Experiments 1a and 1b were combined. With the increased sample size it was discovered that the CLA required a mean of 2.5 ± 0.2 stimulation trials to the first stage 5 seizure, which was significantly fewer than the 3.9 ± 0.5 stimulation trials required by the PRH (*t*(22.2) = -2.55, p < 0.02). The kindling rates to the late stage 5 phase in the CLA and PRH groups were similar to the kindling rates to the first stage 5 seizure in the AM group (F(2, 12) = 0.54, p = 0.59).

Convulsive Profiles

Suspending rats during kindling allowed for more detailed assessment of both forelimb and hindlimb convulsive behaviours; the profiles of behaviors displayed are summarized in Table 5. The AM group displayed significantly longer mean latencies to both forelimb and hindlimb clonus from the onset of stimulation than all other groups (F(4,20) = 6.40, p < 0.002; F(4,20) = 3.94, p < 0.02; respectively) (see Table 4). It is important to note that suspended AM kindled rats had shorter latencies to forelimb clonus

Table 4.

Mean (± S.E.M.) number of daily stimulations to the first bilateral clonus seizure and to the first stage 5 seizure with an after-discharge (AD) that exceeds 30s; and mean latencies, forelimb and hindlimb clonus durations, and AD durations over the first 3 stage 5 seizures. Kindled groups compared are the amygdala (AM), early stage 5 phase claustrum (early CLA), late stage 5 phase claustrum (late CLA), early stage 5 phase perirhinal cortex (early PRH), late stage 5 phase perirhinal cortex (late PRH).

Group	*n*	1st Bilateral clonus (# of trials)	1st Stage 5 - AD > 30s (# of trials)	Latency to forelimb clonus (sec.)	Latency to hindlimb clonus (sec.)	Duration of forelimb clonus (sec.)	Duration of hindlimb clonus (sec.)	AD duration (sec.)
AM	5	11.4 ± 1.3*	11.4 ± 1.3	5.7 ± 2.0*	11.9 ± 4.6*	42.8 ± 6.6	37.3 ± 4.0	49.3 ± 5.2
early CLA	5	2.2 ± 0.6		0.2 ± 0.1	1.5 ± 0.5	16.0 ± 1.0^	14.3 ± 1.2^	15.0 ± 1.2^
late CLA	5	2.6 ± 0.4	13.6 ± 1.7	0	1.7 ± 0.6	35.9 ± 4.3	34.8 ± 3.5	65.8 ± 11.4
early PRH	5	4.0 ± 0.7		0.7 ± 0.5	3.3 ± 0.7	15.7 ± 3.0^	16.1 ± 3.0^	14.9 ± 2.9^
late PRH	5	2.8 ± 0.4	13.4 ± 1.9	1.0 ± 0.4	4.1 ± 1.0	32.4 ± 3.1	30.2 ± 3.2	53.3 ± 11.1

* Significantly different from all other groups, $p < 0.02$

^ Significantly different from AM, late CLA & PRH, $p < 0.001$

(5.7 sec.) as compared to nonsuspended rats (31.7 sec.) in Experiment 1a, suggesting that suspension may be a more sensitive procedure for detecting clonus latencies. Neither forelimb or hindlimb latencies to clonus differed between the CLA and PRH, and both consistently remained short regardless of early or late stage 5 phases. Overall, it was found that mean forelimb latencies to clonus for all groups were significantly shorter than mean hindlimb latencies to clonus ($F(1,48) = 5.22$, $p < 0.03$). Mean forelimb clonus, hindlimb clonus and AD durations were significantly shorter for the early stage 5 phase CLA and PRH groups ($F(4,20) = 9.10$, $p < 0.001$; $F(4,20) = 11.65$, $p < 0.0001$; $F(4,20) = 9.47$, $p < 0.001$; respectively), and were not different between late stage 5 phase CLA and PRH, and AM groups (see Table 4).

During kindling, suspended rats exhibited normal expression of stages 1 to 2 partial seizures. However, the limb convulsions of stages 3 to 5 generalized seizures in suspended rats were different from those in nonsuspended rats. The forelimb and hindlimb convulsive activity varied across 2 major dimensions: (1) clonus versus tonus; and (2) normal posturing versus extension versus flexion. Limb clonus could further be characterized by the dimensions of fast versus slow (forelimbs only) and symmetry versus asymmetry. Figure 8 provides the percentage expression of each of these limb convulsion dimensions for each kindled group.

Forelimbs. With respect to mean percentage time expressing forelimb clonus, both early and late CLA groups demonstrated significantly more time in clonus than the AM group ($F(4,20) = 4.80$, $p < 0.01$) (see figure 8), and the combined early/late CLA groups spent more time in forelimb clonus (98.8 ± 0.7%) than the combined early/late PRH groups (90.4 ±2.7%; $F(2,22) = 10.53$, $p < 0.001$). From the total forelimb clonus, the mean percentage time in fast clonus (defined as 5 to 10 clonic movements per sec.) was found to be significantly longer in the late CLA group compared to all other groups ($p < 0.05$) (see Figure 8). There were no significant differences among groups in the mean percentage time spent in forelimb asymmetrical clonus ($F(4,20) = 0.52$, $p = 0.72$) (see Figure 8). Unlike forelimb clonus, there were no significant differences among groups in the mean percentage time spent in forelimb tonus ($F(4,20) = 0.61$, $p = 0.66$)

Table 5.

Sequence of stage 5 forelimb and hindlimb convulsive behaviours in kindled suspended rats. Kindled groups include the amygdala (AM), early stage 5 seizure phase claustrum (early CLA), late stage 5 seizure phase claustrum (late CLA), early stage 5 seizure phase perirhinal cortex (early PRH), late stage 5 seizure phase perirhinal cortex (late PRH). Values in brackets represent the percentage of rats that exhibited the behaviour.

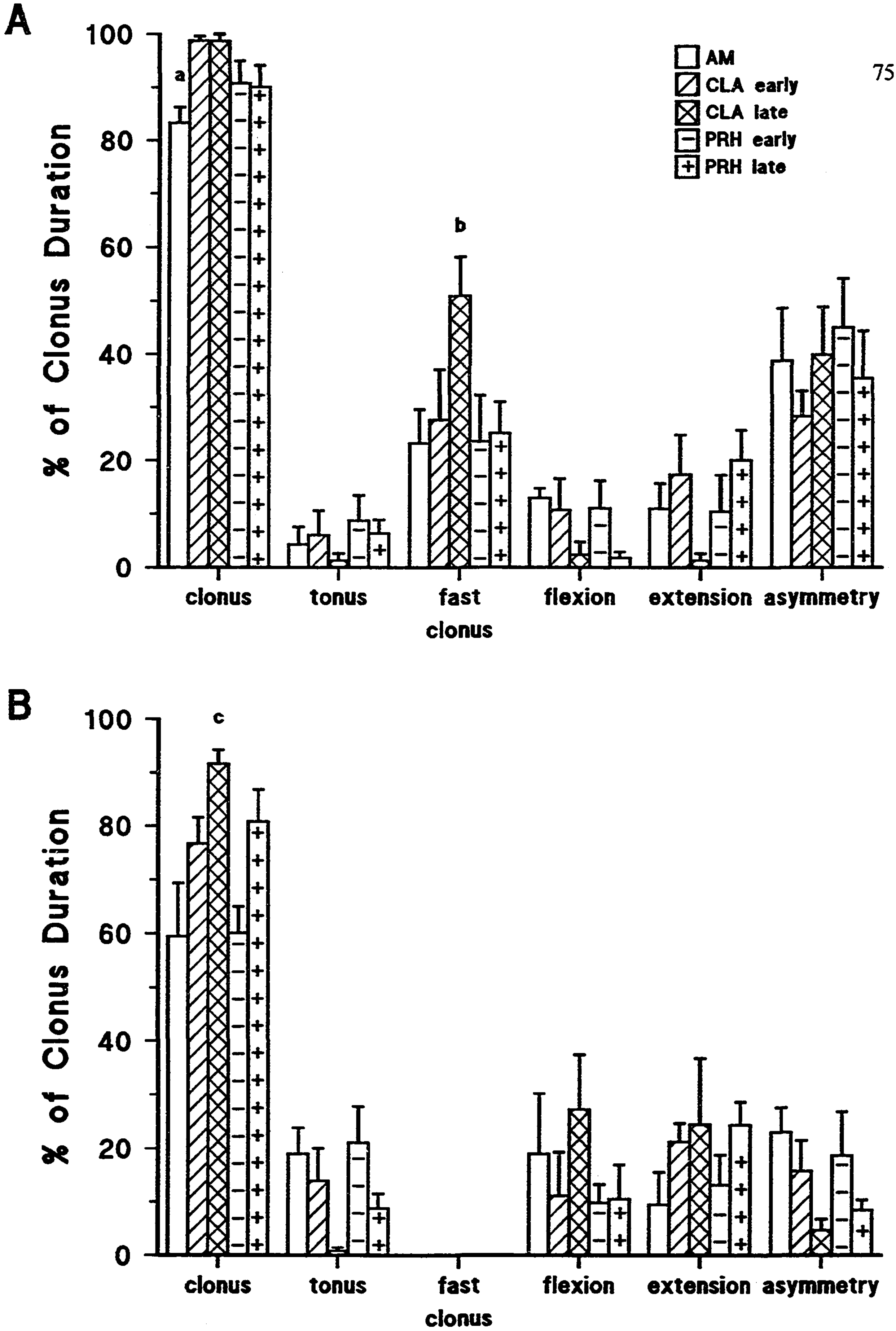
Group	**Forelimbs**	**Hindlimbs**
AM	Slow symmetrical clonus (100%)	Slow symmetrical clonus (60%)
	Fast symmetrical clonic flexion (100%)	Tonic extension (67%)
	Asymmetrical clonic extension (75%)	Slow symmetrical clonus (80%)
	Slow asymmetrical clonus (85%)	
CLA early	Slow symmetrical clonus (93%)	Slow symmetrical clonus (100%)
	Fast symmetrical clonus (75%)	Slow symmetrical clonic flexion (62%)
	Slow symmetrical clonus (55%)	
CLA late	Fast symmetrical clonus (100%)	Slow symmetrical clonus (100%)
	Fast asymmetrical clonus (80%)	Slow symmetrical clonic flexion (92%)
	Slow symmetrical clonus (80%)	Slow symmetrical clonus (88%)
PRH early	Slow symmetrical clonus (100%)	Slow symmetrical clonus (100%)
	Fast symmetrical clonus (63%)	Slow symmetrical clonic flexion (66%)
	Slow clonic extension (50%)	Slow symmetrical clonus (80%)
PRH late	Slow symmetrical clonus (100%)	Slow symmetrical clonus (80%)
	Fast symmetrical clonus (80%)	Slow symmetrical clonic flexion (92%)
	Slow symmetrical clonic extension (64%)	Slow symmetrical clonus (85%)
	Slow symmetrical clonus (70%)	

(see Figure 8). With respect to limb flexion there were no significant differences among the individual groups (F(4,20) = 1.97, p = 0.14) (see Figure 8); however, the combined late CLA/PRH groups exhibited significantly less mean percentage of time in flexion (2.1 ± 1.2%) as compared to the AM (13.0 ± 1.8%) and combined early CLA/PRH groups (10.9± 3.7%; F(2,22) = 4.32, p < 0.03). No significant differences were detected in the mean percentage time of forelimb extensions (F(4,20) = 1.68, p = 0.19) (see Figure 8).

Hindlimbs. With respect to hindlimb clonus both the late CLA (91.6 ± 2.6%) (see Figure 8) and combined early/late CLA groups (86.2 ± 3.6%) exhibited a higher mean percentage time than the AM group (59.5 ± 9.8%; F(4,20) = 5.12, p < 0.006; F(2,22) = 4.50, p < 0.03). Furthermore, the combined late CLA/PRH groups demonstrated longer mean hindlimb clonus times (86.2 ± 3.6%) than either the AM or combined early CLA/PRH groups (68.4 ± 4.3%; F(2,22) = 6.65 p < 0.006). There were no significant differences among groups in the mean percentage time spent in asymmetrical hindlimb clonus (F(4,20) = 0.81, p = 0.53) (see Figure 8). The late CLA group displayed lower mean percentage time spent in hindlimb tonus than the AM group, but this value only approached statistical significance (F(4,20) = 2.89, p = 0.05) (see Figure 8). However, the combined late CLA/PRH groups displayed significantly less hindlimb tonus (4.8 ± 1.9%) than either the AM (18.9 ± 5.0%) or combined early CLA/PRH groups (17.5 ± 4.5%; F(2,22) = 4.43, p < 0.02). No significant differences were detected in the mean percentage time of hindlimb flexions (F(4,20) = 0.95, p = 0.46) (see Figure 8). No significant differences were found across all groups in mean percentage time of hindlimb extensions (F(4,20) = 2.14; p = 0.11) (see Figure 8). However, the combined late CLA/PRH groups displayed significantly less mean extension time (6.6 ± 1.4%) than either the AM (23.0 ± 4.6%) or early CLA/PRH groups (17.1 ± 4.8%; F(2,22) = 4.38, p < 0.03).

Table 5 lists the predominant sequences of forelimb and hindlimb convulsions observed in all 5 groups of suspended rats. With each sequential step the percentage of rats that displayed the convulsive elements is listed in Table 5. It is clear that major differences exist between groups. In the AM 4 distinct forelimb convulsive steps were

Figure 8. Mean (± S.E.M.) percentage expression of various forelimb and hindlimb convulsive behaviours. A: forelimb behaviours; B: hindlimb behaviours. Percent values are based on the proportion of the total cumulative expression time of the behaviour over the total clonus duration time. Kindled groups compared are the amygdala (AM), early stage 5 seizure phase claustrum (early CLA), late stage 5 seizure phase claustrum (late CLA), early stage 5 seizure phase perirhinal cortex (early PRH), late stage 5 seizure phase perirhinal cortex (late PRH). (a) Significantly different from early CLA and late CLA ($p < 0.01$). (b) Significantly different from all groups ($p < 0.05$). (c) Significantly different from AM and early PRH ($p < 0.03$).


A
% of Clonus Duration
100
80
60
40
20
0
□ AM
▨ CLA early
☒ CLA late
⊟ PRH early
⊞ PRH late
a
b
clonus
tonus
fast clonus
flexion
extension
asymmetry
B
% of Clonus Duration
100
80
60
40
20
0
c
clonus
tonus
fast clonus
flexion
extension
asymmetry

observed, beginning with a slow (2-3 clonic movements per sec.) symmetrical clonus that was equivalent to Racine's stage 3 and 4 seizure. The AM forelimb convulsions then became faster (5-10 clonic movements per sec.) with flexion, followed by an asymmetrical clonus with the limbs extended, and ending with slow asymmetrical clonus. About 90% of the AM kindled rats showed this convulsive sequence. Note that forelimb tonus was observed, but appeared inconsistently. In the hindlimbs, AM kindled rats demonstrated 3 distinct steps, beginning with slow (1-5 clonic movements per sec.) symmetrical clonus, followed by extension tonus (full extension to the back of the body with toes pointed), and ending in slow symmetrical clonus again. About 69% of the rats exhibited this convulsive sequence. Rats kindled to the early stage 5 phase in the CLA demonstrated only 3 distinct forelimb convulsive steps: slow, to fast, and back to slow symmetrical clonus (about 74% of rats showed this sequence). In the hindlimbs the early CLA group exhibited only 2 steps, slow symmetrical clonus to slow symmetrical clonus with limb flexion clonus (about 81% of rats showed this sequence). Note that both forelimb and hindlimb tonus was observed in the early CLA groups but appeared inconsistently. Once the CLA progressed into the late stage 5 phase, more dramatic changes occurred in forelimb clonus. The initial slow clonus component was lost and a new step emerged of fast asymmetrical clonus (about 87% of rats showed this sequence). In the hindlimbs the late CLA group exhibited the same 2 steps of the early group, with the addition of a slow symmetrical clonus step (about 93% of rats showed this sequence). Limb tonus was rarely seen with these late CLA rats. The early stage 5 phase PRH group demonstrated a forelimb sequence similar to that of the early CLA group, with the exception of a limb extension on the last slow clonus step (about 71% of rats showed this sequence). The sequence of hindlimb clonus in the early PRH group was very similar to the 3 steps observed with CLA kindling (about 82% of PRH rats showed this sequence). Again, as with the early CLA, inconsistent limb tonus was observed. The only change observed with the progression to late stage 5 phase PRH kindling was the addition of a step of slow symmetrical forelimb clonus (about 79% showed the forelimb sequence, and about 86% of the rats showed the hindlimb sequence). Limb tonus was rarely seen in late PRH kindled rats.

In summary, suspending rats during kindling leads to a more accurate assessment of generalized seizures, without any apparent influence on the seizures themselves. With these sensitive measurements it was shown that many convulsive differences exist between different structures and between different phases of generalized seizure development. In particular it was shown that the CLA exhibited a greater ratio of time in fore-and hindlimb clonus, and forelimb clonus was generally faster than in any other structure examined. These behavioural findings, combined with the with the findings that the CLA shows short latencies to limb clonus and the quickest susceptibility to developing generalized seizures, suggest that CLA kindling is distinct from either AM kindling or PRH cortical kindling. Further distinctions were shown between the early and late stage 5 phases of CLA and PRH. Compared to Experiment 1a, many more dissimilarities were observed between the late stage 5 phases of the CLA/PRH and stage 5 seizures of the AM, including: a lower ratio of forelimb flexions, a higher ratio of hindlimb clonus, and lower ratios of hindlimb tonus and limb extensions.

Discussion

The primary objective of this experiment was to ascertain whether kindling from the dorsolateral CLA is distinct from kindling from other structures. In Experiment 1a CLA kindling exhibited many differences from AM kindling, but not many from INS kindling or deep laminar PRH kindling. Like the INS and PRH, the CLA demonstrated a two phase development of stage 5 seizures, rapid progression to seizure generalization, extremely brief latencies to forelimb clonus, similar patterns and development of evoked AD spike characteristics, and the lack of positive transfer to contralateral AM kindling. Therefore, from these data there was no indication that kindling from the three structures differed from each other. However, in Experiment 1b the CLA did stand out from the other structures with respect to selective stage 5 convulsive properties. Suspended CLA kindled rats demonstrated a greater degree of forelimb and hindlimb clonus and more vigourous forelimb clonus than either AM or PRH kindled rats. The discrepancies between Experiments 1a and 1b in discriminating between the CLA and PRH may be related to differences in *focal* versus *distal* measurements of kindling. With measures that involved local properties at the focus, such as AD spike duration, wave patterns, frequency, and amplitude (and possibly kindling rate), the CLA, INS, and PRH were indistinguishable. However, on measures that involved distal motor structures far removed from the focus, such as the convulsive patterns of the forelimbs and hindlimbs, the CLA, PRH, and AM exhibited some clear distinctions from each other.

Further differences in kindling were identified between the CLA and the PRH. By combining CLA and PRH groups from both experimental sections, it was found that kindling from the CLA was *faster* than from the PRH. Note that a larger sample size was needed to detect differences in kindling rates between these two structures. This is probably due to the fact that many rats had required as few as one stimulation to the first stage 5 seizure. Since one stimulation trial was the lowest value that could be obtained, kindling at this ceiling level could have contributed to the low statistical power of the tests.

It can be argued from the data that the CLA may be more intimately associated with the motor substrates responsible for limbic generalized seizures than the AM or even the PRH. Three major results support this notion. First, the CLA has the fastest kindling rates, which suggests it can access the motor substrates more quickly than any other cortical limbic structure. Second, the CLA exhibits more intense and vigorous driving of the limbs during convulsions, which suggests that it is intrinsically better endowed to drive the motor substrates. Third, stimulation of the deep cortical layers of the PRH does not result in the particular kindling characteristics of the CLA, which suggests that the characteristics of CLA kindling cannot be attributed to PRH activation. It is important to mention that these data do not rule out the participation of the INS in these CLA effects. However, a critical role for the INS in CLA kindling seems unlikely, since some of the CLA electrode tips were adjacent only to the PRH, at some distance from the INS.

Even though subtle differences were detected between the CLA, INS and PRH, many more commonalities in kindling profiles were found. The present experiment is the first to show that the characteristics of PRH kindling are not unique to this structure. The properties of PRH kindling that have been taken as evidence for the critical involvement of the PRH in seizure generalization, namely rapid kindling rates and brief latencies to clonus duration (Kelly & McIntyre, 1996; McIntyre *et al.*, 1993), also apply to the CLA and INS. There are no data in the literature on kindling from the INS, but a few researchers have alluded to its potential importance for limbic seizure generalization (Applegate *et al.*, 1998; Burchfiel *et al.*, 1998). For example, bilateral lesions of the INS, orbital cortex, and anterior portions of the PRH have been shown to retard AM kindling (Corcoran *et al.*, 1976a), while infusions of NMDA antagonists into the INS (designated as PRH by the authors) blocked the expression of AM kindled seizures (Holmes, Bilkey, & Laverty, 1992). Indirect evidence has shown strong Fos labelling in the INS with contralateral PRH kindling (Ferland *et al.*, 1998). Further investigations are needed to determine the degree to which INS and PRH kindling are functionally related.

Another critical finding from these experiments was the identification of two discrete phases of stage 5 seizure development with CLA, INS, and PRH kindling. The early phase was characterized by rapid onset to bilateral generalized convulsions, short latencies to forelimb and hindlimb clonus, short convulsive and AD durations, simple sequential patterns of clonus, and low AD spike frequencies and amplitudes. The late phase shared some, but not all, characteristics with AM stage 5 seizures, such as the number of stimulations required to evoke generalized seizures and the AD spike characteristics. In contrast, differences with the AM existed in many of the distal properties that are responsible for motor seizure expression, including the shorter latencies to forelimb and hindlimb clonus, lower incidence of limb flexions and extensions, higher occurrence of hindlimb clonus, and different sequential patterns of clonic behaviours.

Certain aspects of the two phases of stage 5 development from the CLA, INS, and PRH resemble anterior neocortical kindling. Like the early stage 5 phase, neocortical kindling displays high ADTs, short AD durations, forced motor responses during stimulation, immediate onset of clonus with stimulation, and short clonus durations (Burnham, 1975; McIntyre, 1979; Racine, 1975). With repeated stimulations neocortical kindling also develops a distinct later phase of generalized seizures. These late phases share various properties as well, including sudden increases in AD and clonus durations, and clonus patterns that parallel AM kindling (Altman & Corcoran, 1983; Burnham, 1975; McIntyre, 1979; Seidel & Corcoran, 1986). However, some critical differences exist between neocortical and CLA/INS/PRH kindling. For example, neocortical kindling exhibits strong forelimb tonic components during the early stimulation periods (Racine, 1975) that are not readily observed with CLA, INS, or PRH kindling. Furthermore, the onset to the later stage 5 phase seizures is two to four times slower in the neocortex (Seidel & Corcoran, 1986). Therefore it appears that PRH, INS, and even CLA kindling demonstrate properties of both anterior neocortical and AM kindling.

Of the few papers published on PRH kindling, none have encountered or directly addressed the two phase development in stage 5 seizures that I reported in this experiment (see: Buchanan & Bilkey, 1997; Ferland *et al.*, 1998, McIntyre *et al.*, 1993; Mohapel *et al.*, 1996; Sato *et al.*, 1998). For example, McIntyre and associates observed only one type of stage 5 seizure with PRH kindling. Their PRH stage 5 seizures exhibited the same rapid kindling rates as my early stage 5 phase seizures; however, their convulsive durations, AD durations, and ADTs more closely resembled my late stage 5 phase seizures (McIntyre *et al.*, 1993; Mohapel *et al.*, 1996). The inconsistencies between our results may be attributable to the stimulation parameters used in McIntyre's laboratory: a 2 sec. train of 60 Hz sine-wave (McIntyre *et al.*, 1993). Sine-wave kindling stimulation arguably produces a wider field of neuronal activation than the traditional 1-sec. train of biphasic square-wave pulses (1-msec duration, 60 pulses per sec.) stimulation (see Ranck, 1975; Yoemans, 1990) used in my kindling experiments. Therefore, my square-wave kindling stimulation may have been discrete enough to evoke only the early stage 5 phase seizures, while the broader activation field of the sine-wave stimulation may have masked the early phase expression or blended together the two phases into one. Interestingly, McIntyre and colleagues (Felstead *et al.*, 1995) reported that the deep laminar layers of the PRH kindle the fastest, requiring 2 or 3 stimulations to the first stage 5, compared to the superficial layers of the PRH. McIntyre's deep PRH kindling rates correspond closely to my CLA kindling rates. It is conceivable that McIntyre's sine-wave stimulation was actually activating adjacent CLA neurons, which would account for the quicker kindling rates in the deep PRH. Recall that my study found the deep PRH to kindle at a slower rate of 4 to 6 stimulations, which happens to match the rates reported for the more superfical layers of the PRH (Felstead *et al.*, 1995).

Other studies of PRH kindling have utilized the more traditional biphasic square-wave stimulation technique used in my experiment. Buchanan & Bilkey (1997) examined the transfer of kindling from the PRH to the AM and, in agreement with my results, found no evidence of positive transfer. Furthermore, they reported two kinds of kindling patterns with PRH stimulation: one type where convulsions and AD durations were brief, which they classified as "focal cortical" seizures; and another type were AD durations would sudden increase, which they classified as "cortico-generalised." These two classifications appear very similar to the descriptions of the two stage 5 phases of my results. However, these classifications occurred in separate rats and were not sequential, as were my two phases. Buchanan & Bilkey's (1997) results may have been complicated by the fact that they reported having trouble eliciting AD over 5 sec. in length. Many of their placements were located in the superficial layers of the PRH, and based on my own unpublished observations, very high intensities of square-wave stimulation (about 10 000 μA) are required to evoke an AD. If AD durations do not exceed 5 sec., many more stimulation trials are required to evoke the first generalized convulsion. Sato and collegues (1998) observed similar patterns of PRH kindling, where rats would initially exhibit "moving arrest or exploratory behavior" for 10 stimulation trials and then suddenly develop stage 5 convulsions at about 12 stimulations. All these studies suggest that variations in stimulation parameters can have a dramatic effect on the profiles of kindling from the PRH.

On a final note, kindling in suspended rats offered a superior method for assessing subtle differences in limb convulsive activity. Only one other research paper has looked at kindling in suspended rats. Chen *et al.* (1996) examined convulsive patterns from well established stage 5 seizures in AM kindled rats and reported findings similar to mine. There is one major discrepancy between our findings, related to the extent of tonus observed. Chen *et al.* (1996) listed two additional patterns of tonus in both the forelimb and hindlimbs that were not observed in my study. Some tonus was observed with my stage 5 AM seizures, but their occurrence was inconsistent and very brief. However, Chen *et al.* (1996) did note that only about 66% of their rats expressed the full tonus profiles. Furthermore, their rats had experienced significantly more generalized convulsions than my rats, which may account for the differences in our results. It is difficult to make direct comparisons between our studies, since Chen *et al.* (1996) did not provide data on the durations of each of the convulsive patterns.

EXPERIMENT 2

Molecular Correlates of Claustrum Kindling

Experiment 1 revealed that dorsolateral CLA kindling, as well as deep layer cortical kindling, exhibits two distinct phases of generalized stage 5 seizure development. The present experiment attempted to elucidate some of the potential mechanisms and anatomical substrates that may underlie this two phase phenomenon of dorsolateral CLA kindling, and to compare these changes with AM kindling.

Evidence implicates the transcriptional regulation of genes as one of the pivotal mechanisms of neuronal plasticity. One such transcription factor, *c-fos*, is rapidly and transiently increased in the brain in response to various brain stimuli, including seizures (Morgan & Curran, 1989). However, with kindling, there is controversy to whether *c-fos* is a true marker of plasticity or just simply an indicator of neuronal activity (see Teskey *et al.*, 1991). Recently new members of the early immediate gene family have been identified, the FosB and ΔFosB proteins, that have been more conclusively linked to seizure induced neuronal plasticity (Hiroi, Marek, Brown, Ye, Saudou, Vaidya, Duman, Greenberg, & Nestler, 1998; Mandelzys, Gruda, Bravo, & Morgan, 1997). Unlike Fos, FosB and ΔFosB proteins are sustained for much longer periods of time, which may enable them to mediate many long-term adaptations in the brain (Pennypacker, Hong, McMillian, 1995). In this experiment immunohistochemical techniques were used to assess the anatomical locations of Fos and FosB/ΔFosB protein induction following early and late stage 5 phase CLA kindling. These results were compared to AM kindling.

Growth factors, or neurotrophins, are proteins that regulate growth, proliferation, development, differentiation, maintenance, and regeneration of neurons (Thoenen, 1995). One such brain neurotrophin, BDNF (brain derived neurotrophic factor), has been linked to neuronal plasticity involved in kindling (see Lindvall *et al.*, 1998). The second part of this experiment utilized *in situ* hybridization techniques to examine the selective anatomical changes in BDNF mRNA following kindling in the two phases of the CLA and in the AM.

Methods

Groups

All rats carried 2 electrodes, one into the AM and the other into the contralateral dorsolateral CLA. Rats that received stimulation were kindled from only one site until a maximum of either 3 consecutive early stage 5 phase (early) or 3 consecutive late stage 5 phase (late) seizures were evoked. Two hrs after completion of the third stage 5 seizure rats were perfused, according to the procedure listed below. Two hrs has been demonstrated to be an optimal time point for detecting maximal changes in mRNA BDNF expression (see Bengzon, *et al.*, 1993) and in Fos protein expression (see Dragunow *et al.*, 1988) with kindling. Electrode placements were assessed before rats were placed in one of the following kindled groups: AM (n=3), early CLA (n=3), late CLA (n=3), and non-stimulated implanted control (n=3).

Tissue Preparation

Rats were anesthetized with chloral hydrate and perfused intracardially with 0.1M phosphate buffered saline (PBS, pH 7.4) at 4^{0}C followed by freshly prepared 4% paraformaldehyde in 0.1M PBS at 4^{0}C. Rat brains were then immediately removed, postfixed for 2 hours in the paraformaldehyde solution, cryoprotected in 30% sucrose at 4^{0}C for 3 days, flash frozen in a dry ice slurry at -50^{0}C, and stored at -70^{0}C until sectioned. Each brain was processed for both immunohistochemical and hybridization procedures.

Immunohistochemistry

Brains were sectioned coronally on a cryostat at 40 to 50 μm thickness and stored in well plates containing 1% Na azide PBS until staining. Two antibodies were used in this experiment. The Fos polyclonal antibody (Zeneca, Cambridge Research Biochemicals) was raised in sheep and directed against a synthetic peptide corresponding to amino acid 3-16 mapping at the amino terminus of human p62. The FosB/ΔFosB polyclonal antibody (Santa Cruz Biotechnology, Inc.) was raised in rabbit and directed against a peptide corresponding to amino acids 75-150 mapping at the amino terminus of Fos B of human origin.

Free floating sections were prepared using a conventional avidin-biotin-immunoperoxidase technique. In this procedure sections were pretreated for 30 min. in 0.2% H_2O_2 and incubated in 5% normal goat serum for 60 min. Sections were then incubated overnight at room temperature with the primary antiserum at dilutions of 1:1000. The primary antibodies were localized using Vectastain Elite reagents (Vector laboratories, Nurlingame, CA). The reaction product was developed using a modified nickel-enhanced diaminobenzidine (DAB) method (sections incubated in 30 ml of 2.5% ammonium nickel sulphate, 0.05% DAB, and 2 μl of 30% H_2O_2 for 5 min. at room temperature).

In Situ *Hybridization*

Brains were sectioned coronally at 12 μm on a cryostat and affixed to poly-l-lysine coated slides and stored at -70°C until use. Sections of similar anatomical planes from each different kindling group were placed on the same slide. The positioning of each group section on the slide was systematically rotated, and each anatomical area of interest was represented in triplicate. BDNF cRNA probes (from rat cDNA, Regeneron Pharmaceuticals) were labelled with 33S-CTP and applied to slides at 2.0 x 10^6 cpm. Slides were incubated at 50°C for 24 hours, washed in saline-sodium-citrate (SSC) buffer, RNAse treated, dried, and exposed to film.

Data Quantification

Sections stained for FosB/ΔFosB positive neurons were mounted on a Zeiss microscope, which was equipped with a 10x objective lens and a stabilizing light source. Digital images of the sections were captured by a camera mounted on the microscope, incorporated into image analysis system software (Northern Eclipse, Empix Imaging, Inc.), and converted to gray scale. Two areas of interest were quantified, the PIR and the DG. The PIR measurements were sampled within bregma -1.3 to -3.6 mm (Paxinos & Watson, 1986). Positively stained cell counts were made in a fixed defined area that encompassed primarily layer 2. Measurements were made from both hemispheres and on

2 separate sections that were at least 50 μm apart. The DG measurements were sampled within bregma -2.6 to -4.2 mm (Paxinos & Watson, 1986). Due to the tightly packed nature of the neurons in the DG, it was difficult to conduct individual cell counts. Therefore, optical density measurements of the granule cell layer were made by assigning numbers to each pixel according to its gray scale value (0 = black and 255 = white). Each density measurement value was adjusted for background staining.

BDNF mRNA values were quantified by determining the spectrophotometric density of the gray scale values from the exposed film. Gray scales were calibrated by 35S brain paste standards. DG mRNA densities were measured from approximately the same bregma range as specified for the FosB/ΔFosB quantification, while CLA and PIR densities were sampled from bregma +2.2 to +0.2 mm. Each section, from both hemispheres, was represented in the analysis in triplicate.

Results

Kindling Profiles

All AM placements were located in the basolateral nucleus. CLA placements were located in the dorsolateral aspect, adjacent to the deep cortical layers of the INS and PRH (approximately between bregma -0.4 to -1.8 mm) (placements not shown).

The early CLA group required a mean of 3.0 ± 1.2 stimulation trials to the first stage 4 / 5 seizure, which was significantly lower than mean of 11.0 ± 0.6 stimulations for the AM group ($F(1,4) = 38.40$, $p < 0.004$). However, contrary to the results of Experiment 1, the AM group had a significantly quicker kindling rate compared to the number of stimulations to the first late stage 5 phase seizure of the late CLA group (16.3 ± 1.2; ($F(1,4) = 16.00$, $p < 0.02$). The early CLA group had a mean AD duration of 15.6 ± 2.0 sec. for the last 3 stage 5 seizures, which was significantly shorter than the AD duration for the AM group of 50.3 ± 9.4 sec. ($t(4) = 3.64$, $p < 0.03$) and for the late CLA group of 66.1 ± 17.8 sec ($t(4) = -2.83$, $p < 0.05$). The AM and late CLA groups did not significantly differ in their stage 5 AD durations ($t(4) = -0.79$, $p = 0.48$).

Fos and FosB/ΔFosB Profiles

Compared to the controls, all kindled groups demonstrated robust immuno-positive Fos staining in the ipsilateral PIR and moderate staining in the frontal, parietal, INS, PRH, entorhinal cortices, and the AM. Contralaterally, Fos staining was detected primarily in the PAH, AM, temporal and parietal cortices. Sparse to no Fos staining was detected throughout all regions of the hippocampus in both hemispheres.

Table 6 summarizes the qualitative distribution of immuno-positive FosB/ΔFosB cells for each of the kindled and control groups. With kindling, the most abundant staining occurred in the cortical areas, including the frontal, parietal, INS, PRH, PIR, and entorhinal cortices. Staining appeared to be generally stronger on the hemisphere ipsilateral to the kindling focus. Cortical positive staining was restricted to the superficial layer 2 and 3, with the exception of late phase CLA kindled rats who showed additional staining of layers 5 and 6 in the parietal, INS and PRH cortices. Note that even the implanted control rats exhibited low to moderate levels of staining in some of the cortical areas. Many subcortical structures exhibited no or very little positive staining with kindling, with the exception of the basolateral and cortical nuclei of the AM, the CLA, and the hippocampus. Subcortical staining was most prominently observed in AM and late CLA groups.

Differences in mean positive FosB/ΔFosB cell numbers existed between the different kindling treatments. Quantitative analysis of the mean cell numbers in the PIR demonstrated that all kindled groups had greater amounts of stained cells than controls, both ipsilateral ($F(3,8) = 12.80$, $p > 0.003$) and contralateral ($p < 0.05$) to the site of kindling stimulation (see Figures 9 and 10). Moreover, the late CLA kindled rats exhibited significantly greater numbers of stained neurons than all other kindled groups ipsilateral to the site of stimulation ($p < 0.05$) (see Figure 10). In the DG a different profile of FosB/ΔFosB staining emerged (see Figure 11). Only the late CLA group showed significantly higher mean densities of granule cells, in both hemispheres, compared to the other 2 kindled and control groups ($F(3,8) = 10.84$, $p < 0.004$) (See

Table 6.

Anatomical distribution of immuno-positive FosB/ΔFosB neurons ipsilateral to the site of stimulation. Groups include non-stimulated implanted controls, amygdala (AM) kindled, early stage 5 seizure phase claustrum (early CLA) kindled, and late stage 5 seizure phase claustrum (late CLA) kindled. Symbols indicate the density of labelled neurons, varying from no positive cells (-) to extensive positive cells (+++).

Structure	**Control**	**AM**	**early CLA**	**late CLA**
Amygdala				
basolateral n.	-	+	++	++
central n.	-	-	-	-
cortical n.	+	++	++	+++
medial n.	-	+	-	+
Hippocampus				
CA1	-	+	-	+
CA3	-	+	-	++
dentate gyrus	+	+	+	+++
Cortex				
cingulate	-	+	++	+
retrosplenial	+	+	+	+
frontal	+	++	++	+++
parietal	++	+++	++	+++
insular	+	+++	++	+++
perirhinal	-	+++	+++	+++
piriform	+	+++	+++	+++
endopiriform n.	-	+	+	++
entorhinal	+	++	++	++
Claustrum	-	+	+	++

Figure 9. Representative photomicrographs of immuno-positive FosB/ΔFosB staining in the piriform cortex, ipsilateral to the site of stimulation. A: non-stimulated implanted control, B: amygdala (AM) kindled, C: early stage 5 seizure phase claustrum (early CLA) kindled, and D: late stage 5 seizure phase claustrum (late CLA) kindled. All rats were killed 2 hours after their last stage 5 seizure. Arrow heads indicate positively stained regions. Note the abundantly greater staining in the late CLA group. Abbreviations: PIR, piriform cortex; RF, rhinal fissure.

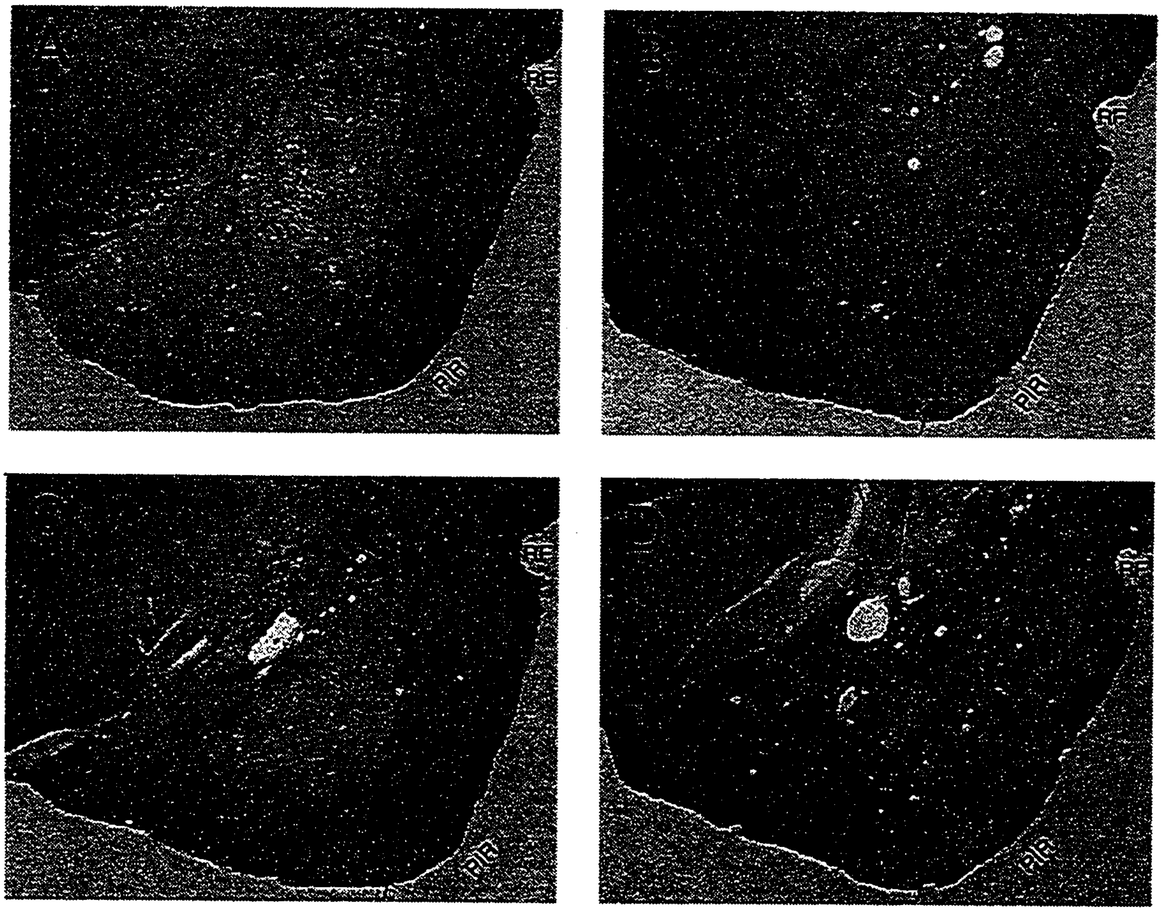

A
RF
RR
B
RF
RR
C
RF
RR
D
RF
RR

Figure 10. Mean (S.E.M.) counts of immuno-positive FosB/ΔFosB piriform cortical neurons ipsilateral and contralateral to the site of stimulation. Groups include non-stimulated implanted controls, amygdala (AM) kindled, early stage 5 seizure phase claustrum (early CLA) kindled, and late stage 5 seizure phase claustrum (late CLA) kindled. Single asterisk (*) denotes a group is significantly different from control ($p < 0.05$) and double asterisks (**) denotes the late CLA group is significantly different from all other groups ($p < 0.003$).

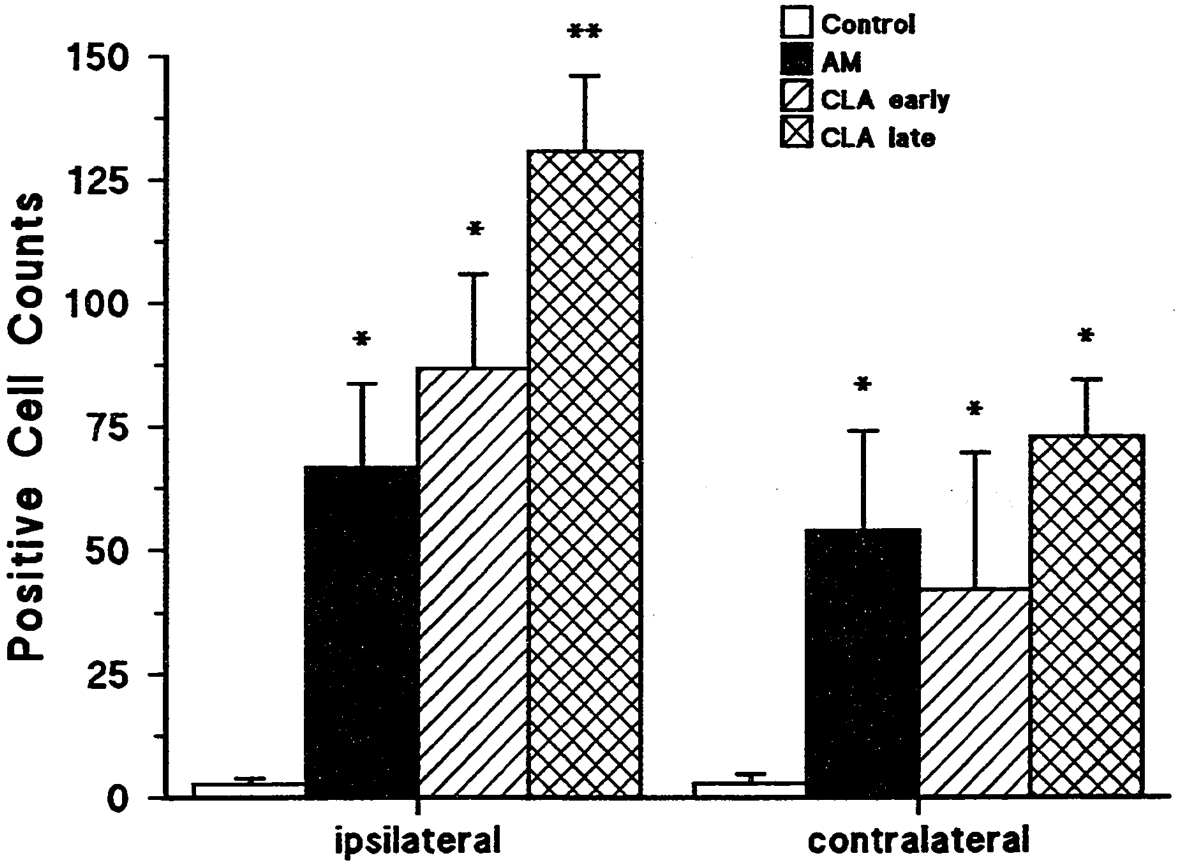

Control
AM
CLA early
CLA late
150
125
100
75
50
25
0
Positive Cell Counts
ipsilateral
contralateral
*
*
**
*
*
*

Figure 11. Representative photomicrographs of immuno-positive FosB/ΔFosB staining in the dentate gyrus (DG) of the hippocampus, ipsilateral to the site of stimulation. A: non-stimulated implanted control, B: amygdala (AM) kindled, C: early stage 5 seizure phase claustrum (early CLA) kindled, and D: late stage 5 seizure phase claustrum (late CLA) kindled. All rats were killed 2 hours after their last stage 5 seizure. Arrow heads indicate positively stained regions. Note the abundantly greater staining in the late CLA group.

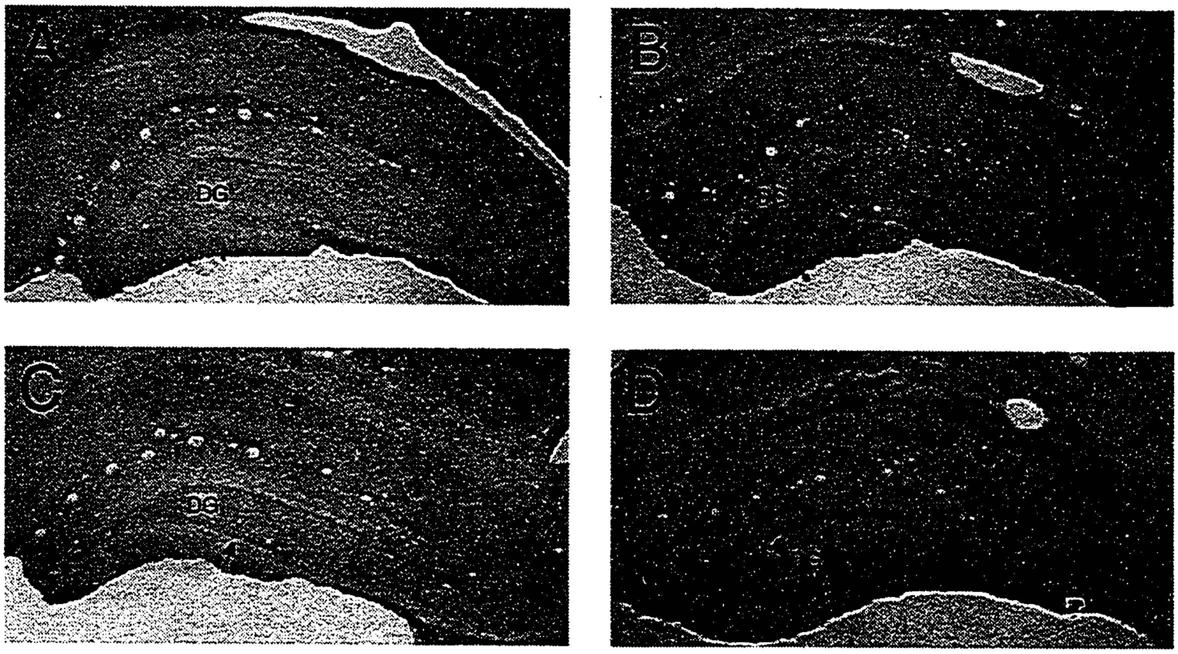
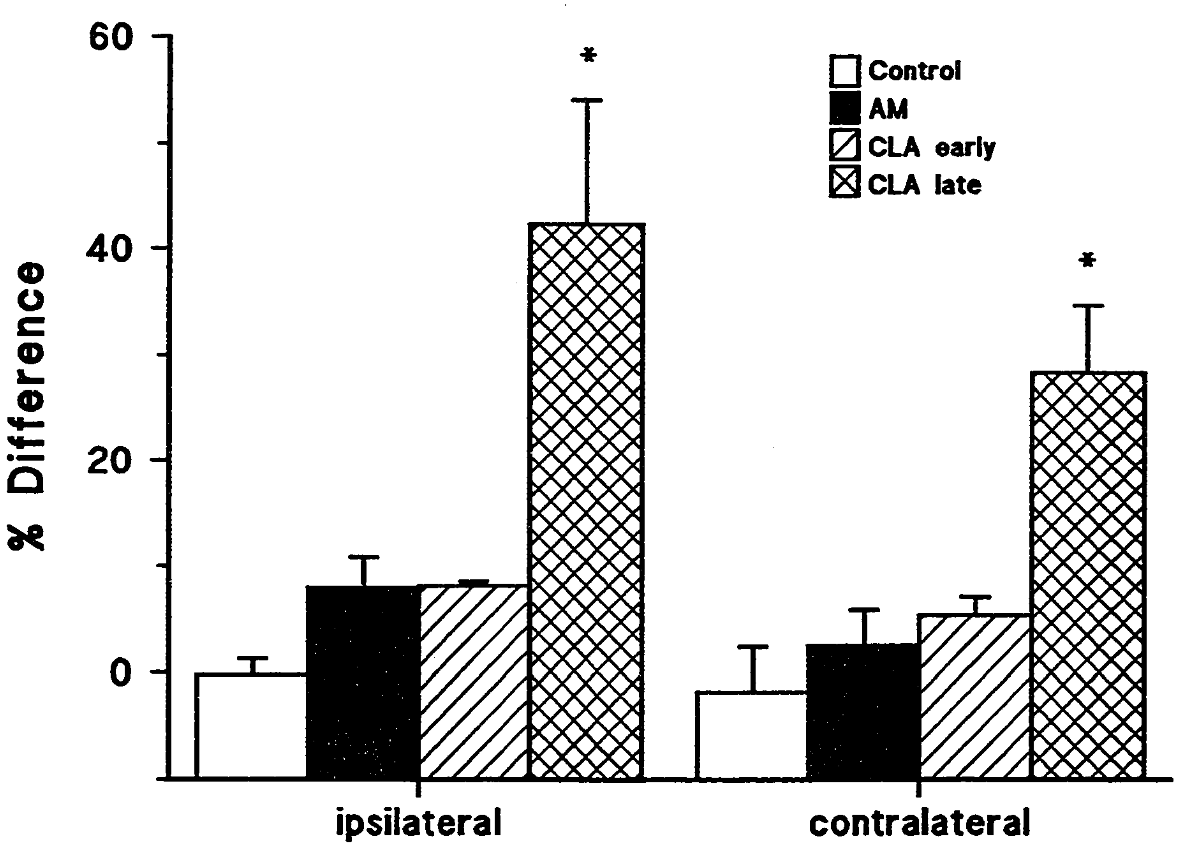

A
B
C
D
DG
DG
DG
DG

Figure 12. Mean (S.E.M.) optical density percent difference from background of immuno-positive FosB/ΔFosB dentate gyrus granule cells ipsilateral and contralateral to the site of stimulation. Groups include non-stimulated implanted controls, amygdala (AM) kindled, early stage 5 seizure phase claustrum (early CLA) kindled, and late stage 5 seizure phase claustrum (late CLA) kindled. Asterisk (*) denotes late CLA is significantly different from all other groups ($p < 0.005$).


60
40
20
0
% Difference
*
*
Control
AM
CLA early
CLA late
ipsilateral
contralateral

Figures 11 and 12). Note that staining densities were slightly higher, but not statistically significant, ipsilateral to the site of stimulation.

BDNF Profiles

Corresponding to previous published reports (Wetmore, Ernfors, Persson, & Olson, 1990), all rats demonstrated detectable BDNF mRNA expression in the DG, CLA, and in various cortical areas, including the PIR, endopiriform, INS, and PRH (see Figure 13). With all the kindling groups some of these structures appeared to exhibit more prominent expression. In the AM and late CLA groups additional expression was observed in the basolateral nucleus of the AM of some rats. Quantification of BDNF mRNA in the ventromedial (anterior) CLA revealed no mean differences in optical densities between any of the groups ($F(3,8) = 0.98$, $p = 0.45$). However, at the same coronal planes, the PIR region of the 3 kindled groups exhibited significantly greater mean densities than the control group ($F(3,8) = 18.32$, $p < 0.007$) (see Figures 13 and 14). In contrast, in the DG, only the AM and late CLA groups demonstrated significantly greater mean densities over the control group ($p < 0.05$) (see Figures 15 and 16). Although not statistically significant, there was a trend for the late CLA group to have even higher BDNF mRNA densities than the AM group in the DG (see Figure 16). Unlike the FosB/ΔFosB staining, no hemispheric differences were found with any of the structures quantified.

Discussion

Further distinctions between the two stage 5 phases of dorsolateral CLA kindling were uncovered with respect to FosB/ΔFosB protein and BDNF mRNA expressions. With FosB/ΔFosB expression, late phase CLA kindling activated a wider range of anatomical regions, with a greater intensity, than either early phase CLA kindling or even AM kindling. Specifically, late CLA kindling exhibited the most pronounced cortical expression, particularly the PIR, PRH, INS, and frontal cortices. Note that the early CLA exhibited the least amount of cortical staining. In the DG only the late CLA group exhibited robust FosB/ΔFosB staining. A different pattern of expression was observed

Figure 13. Representative photomicrographs from films exposed to radioactive probes showing BDNF mRNA expression in the piriform cortex and claustrum. Coronal sections correspond to approximately bregma +2.8 mm. A: non-stimulated implanted control, B: amygdala (AM) kindled, C: early stage 5 seizure phase claustrum (early CLA) kindled, and D: late stage 5 seizure phase claustrum (late CLA) kindled. All rats were killed 2 hours after their last stage 5 seizure. Note the similarities in BDNF expression between all kindled groups. The abbreviation PIR represents the piriform cortex.

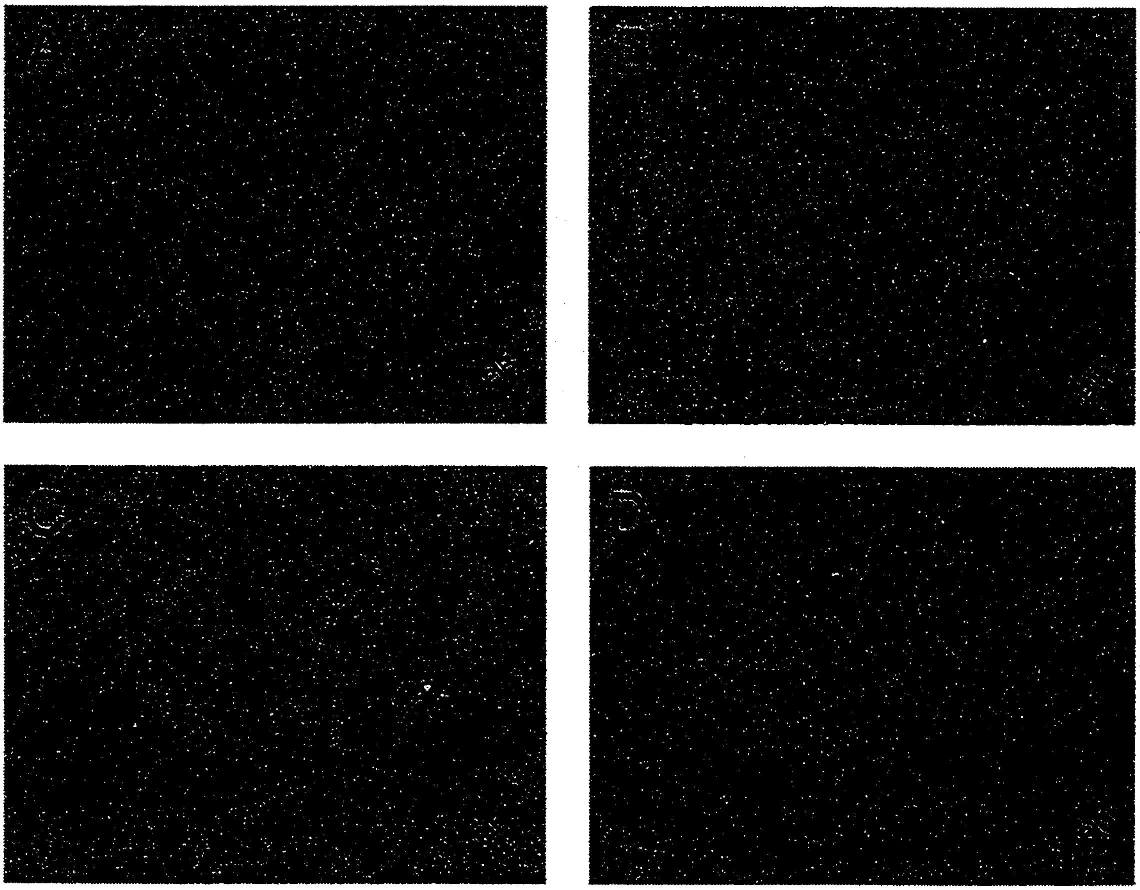

A
B
C
D

Figure 14. Mean (S.E.M.) spectrophotometric density percent values relative to implanted controls for BDNF mRNA from piriform cortex, both ipsilateral and contralateral to the site of kindling stimulation. Coronal sections sampled correspond between bregma +2.2 to +0.2 mm. Groups include non-stimulated implanted controls, amygdala (AM) kindled, early stage 5 seizure phase claustrum (early CLA) kindled, and late stage 5 seizure phase claustrum (late CLA) kindled. Asterisk (*) denotes a group is significantly different from AM group ($p < 0.05$).

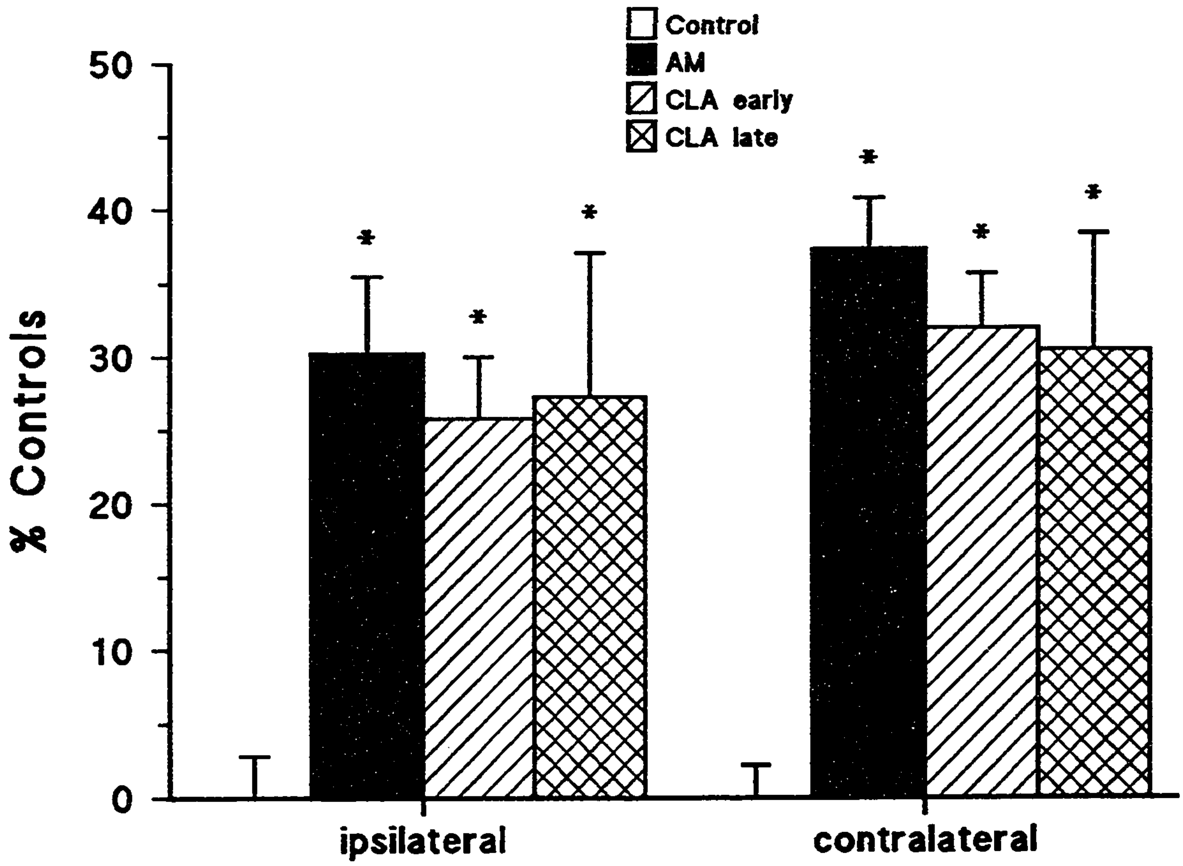

Control
AM
CLA early
CLA late
% Controls
0
10
20
30
40
50
ipsilateral
contralateral
*

Figure 15. Representative photomicrographs from films exposed to radioactive probes showing BDNF mRNA expression in the amygdala (AM), perirhinal cortex (PRH), piriform cortex (PIR), and dentate gyrus (DG) of the hippocampus. Coronal sections correspond to approximately bregma -2.8 mm. A: non-stimulated implanted control, B: amygdala (AM) kindled, C: early stage 5 phase seizure claustrum (early CLA) kindled, and D: late stage 5 seizure phase claustrum (late CLA) kindled. All rats were killed 2 hours after their last stage 5 seizure. Note the lack of BDNF expression in the dentate gyrus of the early CLA.

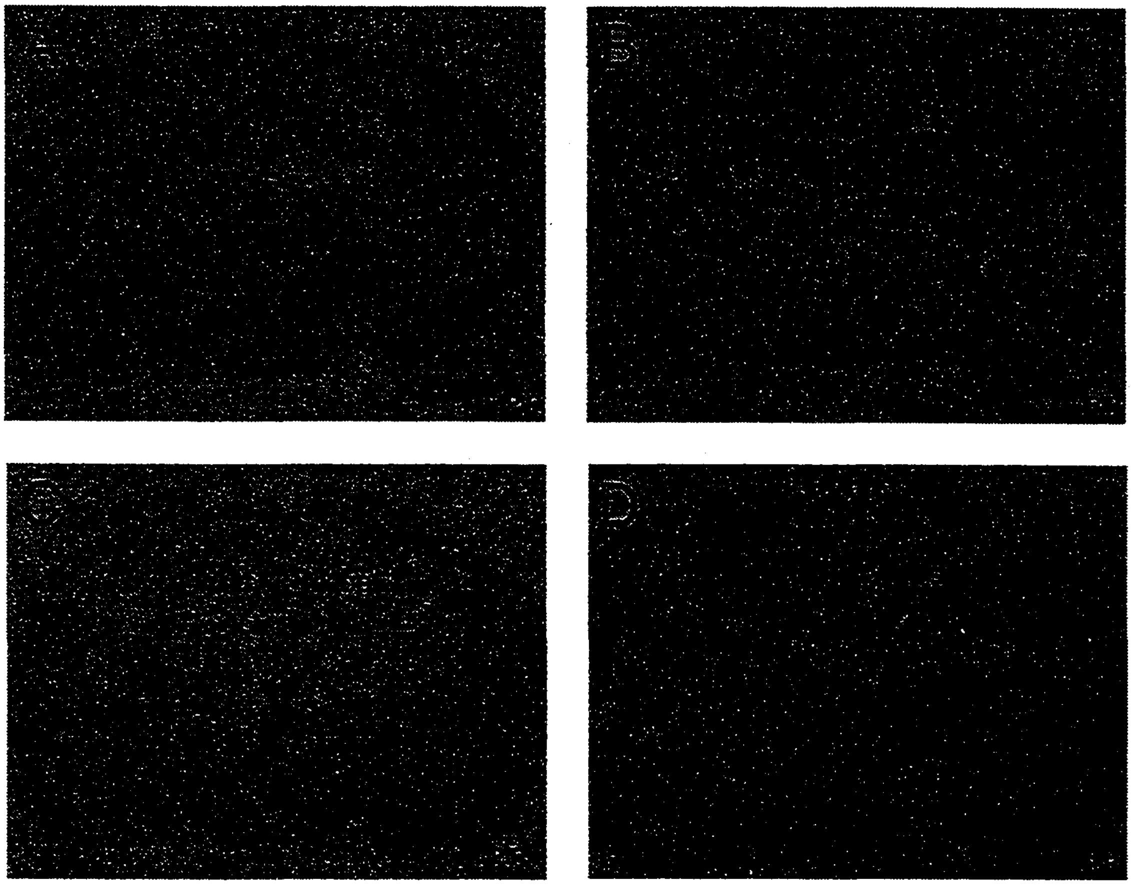
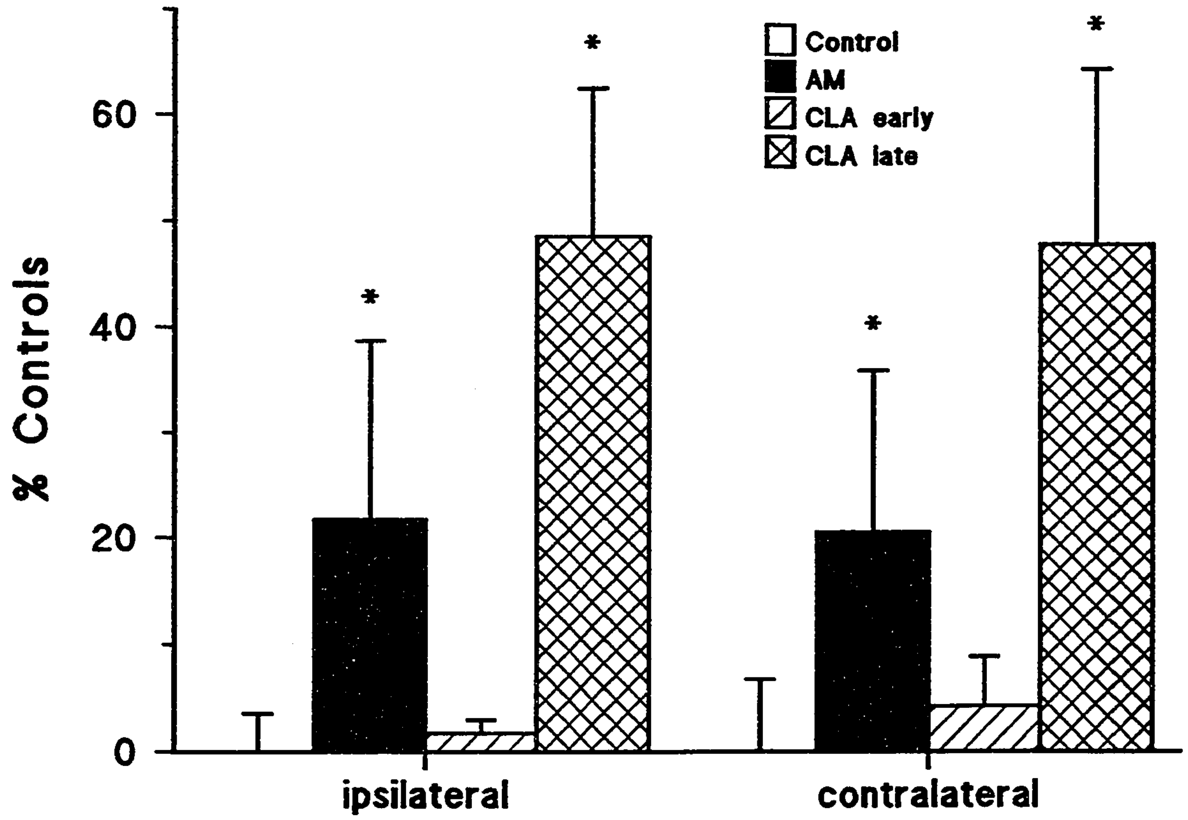

A
B
C
D

Figure 16. Mean (S.E.M.) spectrophotometric density percent values relative to implanted controls for BDNF mRNA from dentate gyrus, both ipsilateral and contralateral to the site of kindling stimulation. Coronal sections sampled correspond between bregma -2.6 to -4.2 mm. Groups include non-stimulated implanted controls, amygdala (AM) kindled, early stage 5 seizure phase claustrum (early CLA) kindled, and late stage 5 seizure phase claustrum (late CLA) kindled. Asterisk (*) denotes a group is significantly different from AM group ($p < 0.05$).


% Controls
0
20
40
60
ipsilateral
contralateral
☐ Control
■ AM
▨ CLA early
☒ CLA late
*
*
*
*

with BDNF mRNA distribution. For the most part all kindled groups, irrespective of the kindling site or the phase of stage 5 seizures, exhibited the same intensities in cortical expression. However, in the DG, the AM and late CLA kindled rats showed significantly stronger expression compared to the early CLA kindled rats. In summary, these FosB/ΔFosB and BDNF results suggest that late CLA kindling demonstrated the most extensive changes, followed by moderate changes with AM kindling, and least extensive changes with early CLA kindling.

Similar results in Fos protein induction were found in other studies with respect to the strong ipsilateral labelling in the cortical and subcortical regions, including the inconsistent hippocampal staining patterns (Burchfiel *et al.*, 1998, Chiasson *et al.*, 1995; Dragunow *et al.*, 1988; Ebert & Löscher, 1995b; Ferland *et al.*, 1998; Sato *et al.*, 1998) observed in this experiment. Because no differences were detected 2 hours after the last generalized seizure between the kindled groups, the Fos data did not provide for any distinctions between the different kindling treatments. This is probably due to the fact that Fos is primarily a marker for neuronal activity (e.g., Teskey *et al.*, 1991). Therefore, these data suggest that the same anatomical regions are being activated 2 hours after a stage 5 seizure, irrespective of the origin of the focus or even qualitative differences in the profile of the generalized convulsion.

This was not the case with FosB/ΔFosB staining. Differences in FosB/ΔFosB were detected at both cortical and subcortical levels between kindled groups. It is not certain whether the changes in FosB/ΔFosB expression were the result of simple neuronal activation, as with Fos, or whether they reflected true neuronal plasticity. There are no other published reports with FosB or ΔFosB expression and kindling to compare my results to. Examining the changes in the DG, only the late CLA group expressed dramatic increases. The other two kindled groups did not differ from the control. This expression pattern of FosB/ΔFosB is very similar to the "all-or-nothing" pattern observed with *c-fos*, whereby the DG will only demonstrate *c-fos* if AD durations exceed about 30 to 60 sec. in duration (Shin *et al.*, 1990). Since the late CLA group was the only group to average over 60 sec. of AD duration, FosB/ΔFosB expression in the DG may have been just a reflection of neuronal activity.

However, this may not be the case for cortical expression of FosB/ΔFosB. A recent study using electroconvulsive seizures found that ΔFosB was associated with plastic changes in the frontal cortex pyramidal neurons (Hiroi *et al.*, 1998). In that paper a link was found between increased ΔFosB expression and the upregulation of the glutamate NMDA Receptor-1, which is essential for the behavioural manifestation of motor convulsions in this ECS seizure model (McNamara, 1994). All kindled groups in my experiment exhibited increased cortical FosB/ΔFosB staining, which leads to the speculation that ΔFosB may regulate cortical motor seizure activity in kindling. Note that the antibody I used was incapable of distinguishing between the FosB and ΔFosB proteins. Similar to Fos, the FosB protein is only transiently upregulated for a few hours (see Chen, Kelz, Hope, Nakabeppu, & Nestler, 1997). Since rats in my experiment were killed 2 hours after their third stage 5 seizure, the antibody probably detected some as FosB. However, since ΔFosB proteins can persist for many days to months (Pennypacker *et al.*, 1995), I believe that the FosB/ΔFosB antibody was primarily detecting ΔFosB proteins that accumulated from the entire kindling stimulation period. In support of the assumption, kainic acid induced seizures can cause long-lasting expression of ΔFosB that have been shown to persist for months (Mandelzys *et al.*, 1997). We have found similar results with AM kindling (unpublished observations). To conclude, ΔFosB transcription factors may be a valid marker for neuronal plastic changes. In the present experiment I found that advanced CLA kindling potentially initiates many more plastic changes in the cortical motor substrates responsible for seizure generalization.

Most of the BDNF results from the present experiment correspond with the results of other published kindled reports. With AM kindling, expression of mRNA for BDNF has been shown to increase bilaterally in the PIR, PRH, and temporal cortices, and in the basolateral nucleus of the AM (Bengzon *et al.*, 1993; Ernfors *et al.*, 1991). Note that evoking kindled stage 5 seizures from many different structures, such as the hippocampus, will produce similar changes in BDNF expression (Bengzon *et al.*, 1993).

Similarly, in the present experiment, the late stage 5 phase CLA kindled rats exhibited BDNF mRNA profiles comparable to those of the AM kindled rats. The early stage 5 phase kindled rats did show similar cortical BDNF profiles, but they did not show similar subcortical profiles. In particular, the early CLA group displayed very little mRNA expression in the basolateral AM and in the DG. Similar to the *c-fos* results discussed above, previous reports have shown that the DG will not express BDNF if evoked AD durations are below 60 sec. (Bengzon *et al.*, 1993; Ernfors *et al.*, 1991). In fact, expression in the DG appears to be independent of kindling per se, since only a single stimulation is required to induce BDNF so long as the AD duration exceeds 60 sec. These results could explain the lack of DG BDNF expression in the early CLA rats, since their mean stage 5 AD durations were about 16 sec. These data again suggest that lack of expression in the DG may be a result of insufficient neuronal activation rather than plastic changes per se. It is difficult to ascertain the significance of this finding since it is not known exactly how BDNF and other neurotrophins mediate kindling plasticity. Some researchers have hypothesized that BDNF induces morphological changes in neurons responsible for the circuit re-development underlying kindling (see Reibel *et al.*, 1998), while others have speculated that neurotrophins act as a protective mechanism against the insult of seizures by countering cell death through neuronal stabilization. (Lindvall, Kokaia, Bengzon, Elmér, & Kokaia, 1994; Reibel *et al.*, 1998; Van der Zee *et al.*, 1995). The results of the present experiment further support the notion that the hippocampus is not crucial for establishment of kindled seizures, but instead may only reflect changes in neuronal activity from other regions.

EXPERIMENT 3

Alternating Kindling Between Claustrum and Amygdala

Much of the our understanding of the conceptual framework of kindling has been derived from the work with the kindling antagonism model (Burchfiel & Applegate, 1989a). Recall that kindling antagonism refers to the application of alternating electrical stimulation to two brain sites, resulting in the suppression of seizure generalization from one or both sites. An underlying assumption of kindling antagonism is that by establishing two seizure foci simultaneously, both sites are forced to compete for the *same* motor substrate(s) responsible for seizure generalization. By the delivery of alternating stimulation, only one of the kindled foci (dominant site) gains access to this motor substrate, which presumably denies the other focus (suppressed site) access to that same motor substrate and thereby arrests kindling at the suppressed site. Therefore, alternating kindling is a useful method for determining the hierarchy of anatomical associations to the motor substrates, such that a structure that suppress kindling from another site can be argued to have a greater intrinsic motor association.

The following experiment compared the propensity of various structures, all implicated as crucial mediators in kindling generalization, to exert kindling dominance over the AM. Alternating kindling was conducted between the AM and either the dorsolateral CLA, INS, PRH, or PIR.

Methods

Kindling and Groups

All rats carried 2 electrodes, one implanted into each hemisphere. One electrode was implanted into the AM and the other was implanted into either the contralateral CLA, INS, PRH, or PIR. Kindling stimulation was applied at 100 μA above the initial ADT for the particular site. In rats in which kindling antagonism was to be established, stimulation was alternated between the two sites such that no single site received two consecutive stimulations. The initial site of stimulation was randomly balanced between groups. Kindling stimulation was terminated only when 6 consecutive stage 5 convulsions were recorded from each site; in rats that exhibited suppressed kindling from one site, the stimulation was eventually terminated at a point well beyond the typical kindling range of the suppressed site. In control rats ADTs were sampled from both sites, but seizures were kindled at only one of these sites every other day (once per 48 hrs) until six generalized seizures were recorded. At the completion of kindling rats were perfused and electrode placements were assessed, resulting in 51 rats being placed into 1 of 9 kindled groups: CLA/AM (n=5), INS/AM (n=7), PRH/AM (n=7), PIR/AM (n=7), AM (n=8), CLA (n=5), INS (n=5), PRH (n=5), PIR (n=2).

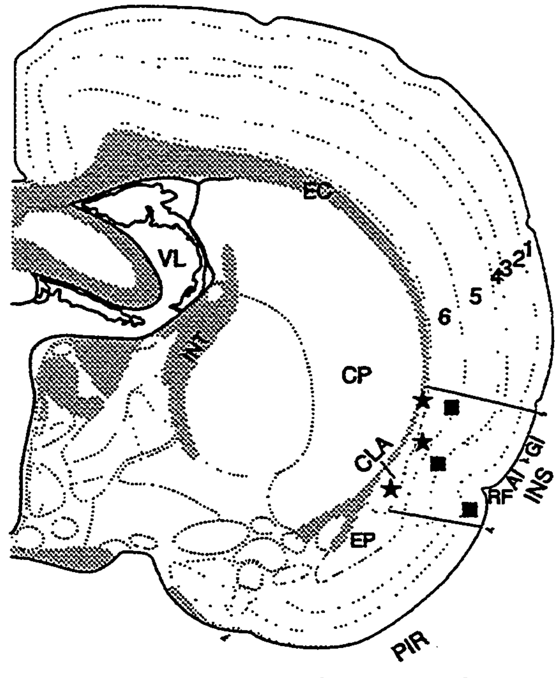
Criteria for Kindling Antagonism

The criteria for kindling antagonism were as follows: One of the alternately stimulated sites must express relatively normal kindling (dominant site), whereas the other site must express an arrest in kindling (suppressed site). Furthermore, at the suppressed site the progression to the first stage 5 seizure should be arrested such that no seizures (i.e., absolute antagonism) or only partial seizures below stage 3 (relative antagonism) develop during alternating stimulation with the dominant site (see Duchowny & Burchfiel, 1981). Finally, once the alternating stimulation with the dominant site is terminated, kindling from the suppressed site should progress normally from the stage at which the kindling was initially suspended. The generalized seizures eventually evoked from the suppressed site should appear identical to those evoked from the same site in single-site kindled rats. Rats that fail to exhibit antagonism should show normal kindling to stage 5 seizures from both sites, regardless of whether alternating or single-site stimulation is applied.

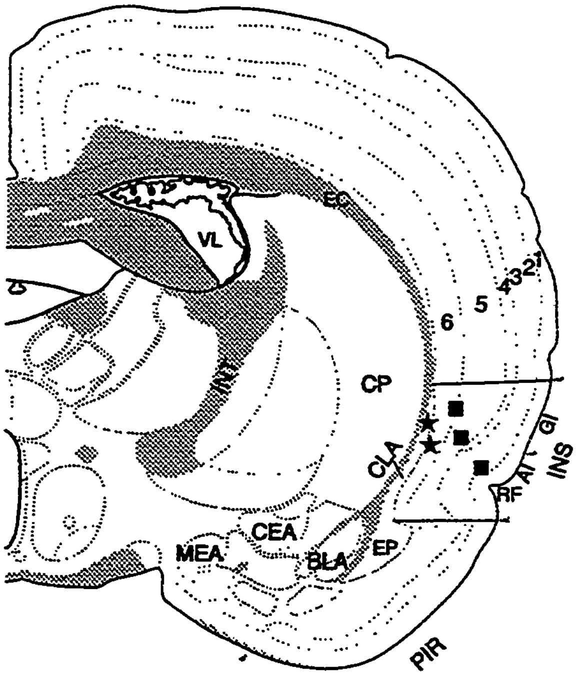
Results

Schematic locations of cortical and CLA electrode tips in each rat that received alternating stimulation with the AM are presented in Figure 17. Anatomical distinctions and boundaries were derived from the rat brain atlases of both Paxinos and Watson

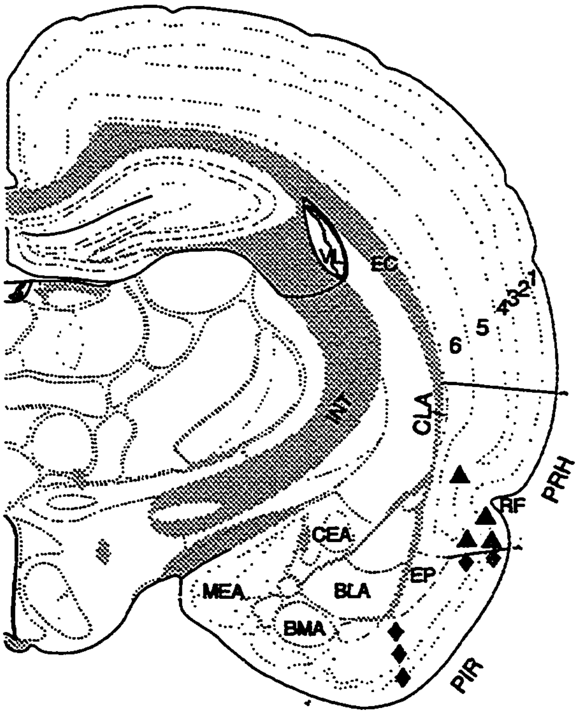
Figure 17. Schematic diagram of coronal sections of the rat brain, adapted from Swanson (1992), showing the location of cortical and claustrum (CLA) electrode tips in each rat that received alternating stimulation with the amygdala (AM). Note that two electrode tips overlapped; hence only 13 placements are shown. Solid stars (★) indicate CLA placements; solid squares (■) indicate placements in the insular cortex (INS); solid triangles (▲) indicate placements in the perirhinal cortex (PRH), and solid diamonds (♦) indicate placements in the piriform cortex (PIR). Abbreviations: AI, agranular insular cortex; BLA, basolateral nucleus of the amygdala; BMA, basomedial nucleus of the amygdala; CEA, central nucleus of the amygdala; CP, caudate-putamen; EP endopiriform nucleus; EC, external capsule; GI, granule insular cortex; INT, internal capsule; MEA, medial nucleus of the amygdala; PIR, piriform cortex; RF, rhinal fissure; VL, lateral ventricle.



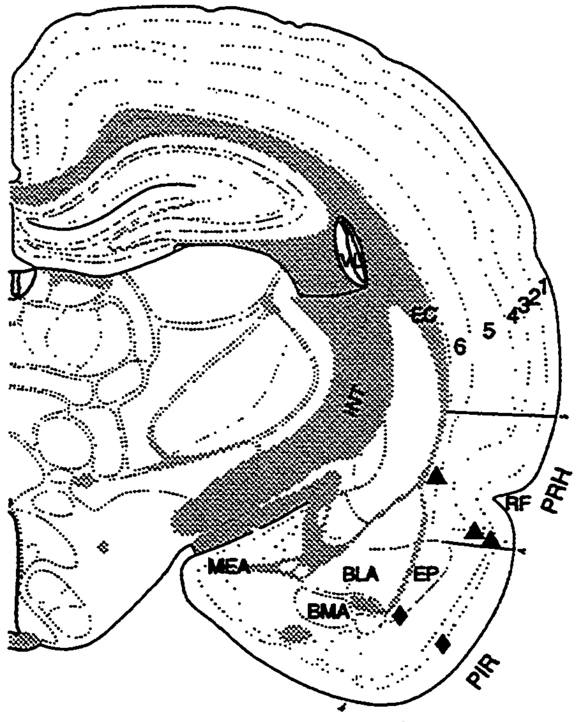
bregma -0.8



bregma -1.3



bregma -2.5

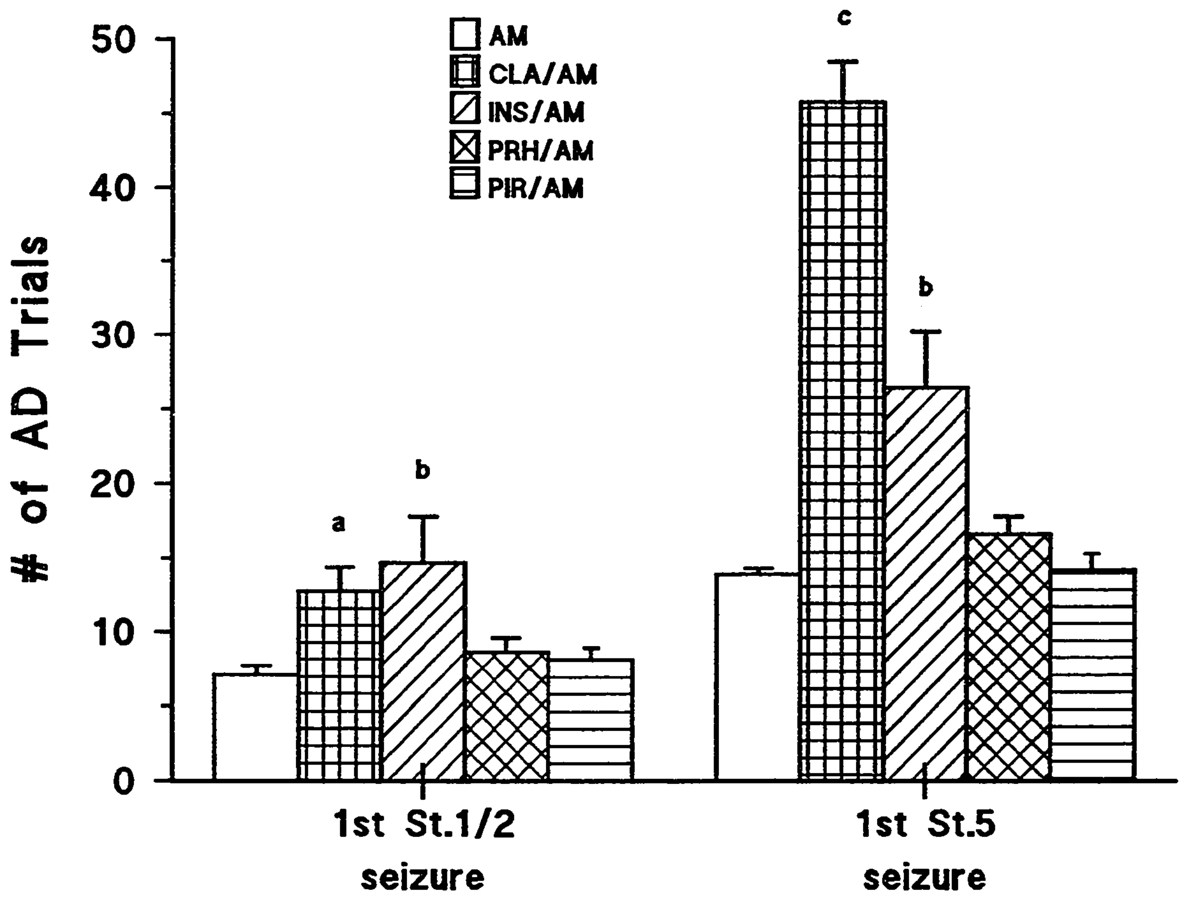


bregma -2.9

(1986) and Swanson (1992). All groups had approximately the same distribution of AM nuclei placements: 60% in the basolateral, 20% in the central, and 20% in the medial nucleus (electrode tips not shown). CLA placements were located in the dorsolateral aspect, adjacent to the deep cortical layers of the INS and PRH (approximately between bregma -0.8 to -1.8 mm). The INS placements were equally dispersed between the posterior granular and agranular regions (approximately between bregma -0.8 to -1.3 mm) and broadly distributed across the full range of laminar layers. The PRH placements were broadly distributed across the full range of laminar layers in the anterior portion of the cortex (approximately between bregma -2.0 to -3.3 mm). The PIR placements were distributed across all layers, including the endopiriform cortex (approximately between bregma -2.3 to -3.0 mm).

Distinctively different patterns emerged with respect to the development of kindling antagonism with alternating stimulation of the AM and either the CLA, INS, PRH, or PIR. The AM did not suppress kindling of any of the limbic cortical structures or CLA; likewise, the PRH and PIR did not suppress kindling of the AM. However, the CLA and most portions of the INS were capable of suppressing AM kindling. The arrest in AM kindling during alternating CLA and INS stimulation was reflected by the greater number of stimulations required to elicit the first partial and generalized seizures from the AM (see Figure 18). Note that in order for AM kindling to progress to generalized seizures, it was necessary to terminate CLA or INS stimulation. There was variation in the number of CLA or INS stimulations delivered before kindling ended and single-site kindling of the AM began (i.e., 12 to 40 consecutive stage 5 seizures were triggered from the CLA or INS before termination of CLA or INS kindling). As shown in Figure 18, alternating CLA or INS stimulation with AM stimulation resulted in a significantly greater number of mean stimulations to evoke the first stage 1 or 2 seizure from the AM than single-site AM kindling ($F(4,29) = 4.07$, $p < 0.01$). Furthermore, the alternating CLA/AM group required significantly more mean stimulations to develop the first stage 5 seizure from the AM than all other groups, including the INS/AM ($F(4,29) = 34.22$, $p < 0.0001$) (see Figure 18). The INS/AM group needed significantly more AM stimulations

Figure 18. Mean (±S.E.M.) number of kindling trials to the first stage 1 and stage 5 seizures from the amygdala (AM) with alternating stimulation of either the claustrum (CLA), insular cortex (INS), perirhinal cortex (PRH), or piriform cortex (PIR). (a) Significantly different from AM ($p < 0.01$); (b) Significantly different from AM, PRH/AM, and PIR/AM groups ($p < 0.01$); (c) Significantly different from all other groups ($p < 0.0001$).


AM
CLA/AM
INS/AM
PRH/AM
PIR/AM
# of AD Trials
50
40
30
20
10
0
a
b
c
b
1st St.1/2
seizure
1st St.5
seizure

to the first stage 5 seizure than the single-site AM control group ($p < 0.05$). These patterns were reflected in the cumulative AD durations required to reach the first stage 5 seizure ($F(4,29) = 26.60$, $p < 0.0001$), but not quite for the first stage 1 seizure, for the CLA/AM and INS/AM groups (Table 7).

Figure 19 illustrates the dramatic differences in AM kindling between a representative rat receiving alternating stimulation in the CLA and a rat receiving alternating stimulation in deep layer 6 of the PRH. The suppression of AM kindling exerted by the CLA or INS was always relative antagonism, as indicated by the observation that partial seizures (stages 1 or 2) were typically evoked from the AM during alternating stimulation (e.g., Figure 19A).

According to the criteria for kindling antagonism outlined in the methods section, the data support the notion that true arrest of AM kindling occurred with alternating CLA or INS kindling, as demonstrated in several ways. First, both the CLA and INS expressed normal kindling with alternating AM kindling. CLA/AM and INS/AM rats demonstrated the same kindling rates and convulsive profiles as their single-site kindled controls. Specifically, the stimulations trials ($F(3,18) = 1.47$, $p = 0.26$) and cumulative AD durations ($F(3,18) = 1.24$, $p = 0.32$) to the first stage 5, the latencies to forelimb clonus ($F(3,18) = 0.51$, $p = 0.68$), the duration of forelimb clonus ($F(3,18) = 0.90$, $p = 0.46$), and AD durations during clonus ($F(3,18) = 1.34$, $p = 0.29$) did not significantly differ from the single-site CLA or INS kindled groups (see Table 8). Second, rats expressing antagonism spent a significantly greater proportion of AM kindling trials in stages 0, 1 and 2 ($F(1,15) = 59.67$, $p < 0.0001$) than single-site AM controls, but did not differ in the trials spent in stages 3 and 4 ($F(1,15) = 0.37$, $p = 0.57$). Third, in 9 of 10 of the CLA and INS rats displaying kindling antagonism, generalized seizures were not triggered from the AM (up to 50 stimulations of the AM) until CLA or INS stimulation was terminated (e.g., Figure 19A). Fourth, after stimulation of the CLA or INS was terminated, the number of stimulations required for kindling of stage 5 seizures with AM stimulation was similar to the number of stimulations required for progression from stage 1 to stage 5 seizures in

Table 7.

Mean (± S.E.M.) kindling rate and convulsive profile parameters for amygdala kindling. Measurements include: total cumulative AD durations (ADD) to the first partial (stage 1 / 2) or generalized (stage 5) seizures; latency to forelimb clonus, duration of forelimb clonus, and AD duration over the first 2 stage 5 seizures. Kindled groups compared are the single-site AM control (AM), alternating stimulation of the claustrum and AM (CLA/AM), alternating stimulation of the insular cortex and AM (INS/AM), alternating stimulation of the perirhinal cortex and AM (PRH/AM), alternating stimulation of the piriform cortex and AM (PIR/AM).

Group	*n*	Cumulative ADDs to 1st stage 1 / 2	Cumulative ADDs to 1st stage 5	Latency to clonus - stage 5 (sec.)	Duration of clonus - stage 5 (sec.)	AD duration - stage 5 (sec.)
AM	8	172.3 ± 37.0	538 ± 50	32.8 ± 9.5	33.3 ± 5.7	81.1 ± 10.3
CLA/AM	5	380.2 ± 145.1	2372 ± 211[b]	29.3 ± 3.8	41.2 ± 9.3	79.2 ± 8.6
INS/AM	7	448.0 ± 83.6[a]	1166 ± 202[c]	36.4 ± 9.4	41.6 ± 6.3	90.1 ± 13.4
PRH/AM	7	273.7 ± 68.5	770 ± 109	46.6 ± 12.3	29.8 ± 4.3	100.5 ± 14.4
PIR/AM	7	165.7 ± 27.1	517 ± 96	27.2 ± 6.8	27.9 ± 4.0	73.7 ± 8.5

[a] Significantly different AM ($p < 0.04$).

[b] Significantly different from all other groups ($p < 0.0001$).

[c] Significantly different from INS/AM, PRH/AM, and AM groups ($p < 0.0001$).

Figure 19. Representative examples of kindling from individual rats receiving alternating stimulation of the amygdala (AM) and claustrum (CLA) or deep layer perirhinal cortex (PRH). A: Pattern of relative kindling antagonism with CLA stimulation where AM kindling is suppressed at partial stages; B: Lack of kindling antagonism with deep layer PRH stimulation. Inverted arrow indicates the termination of CLA stimulation.

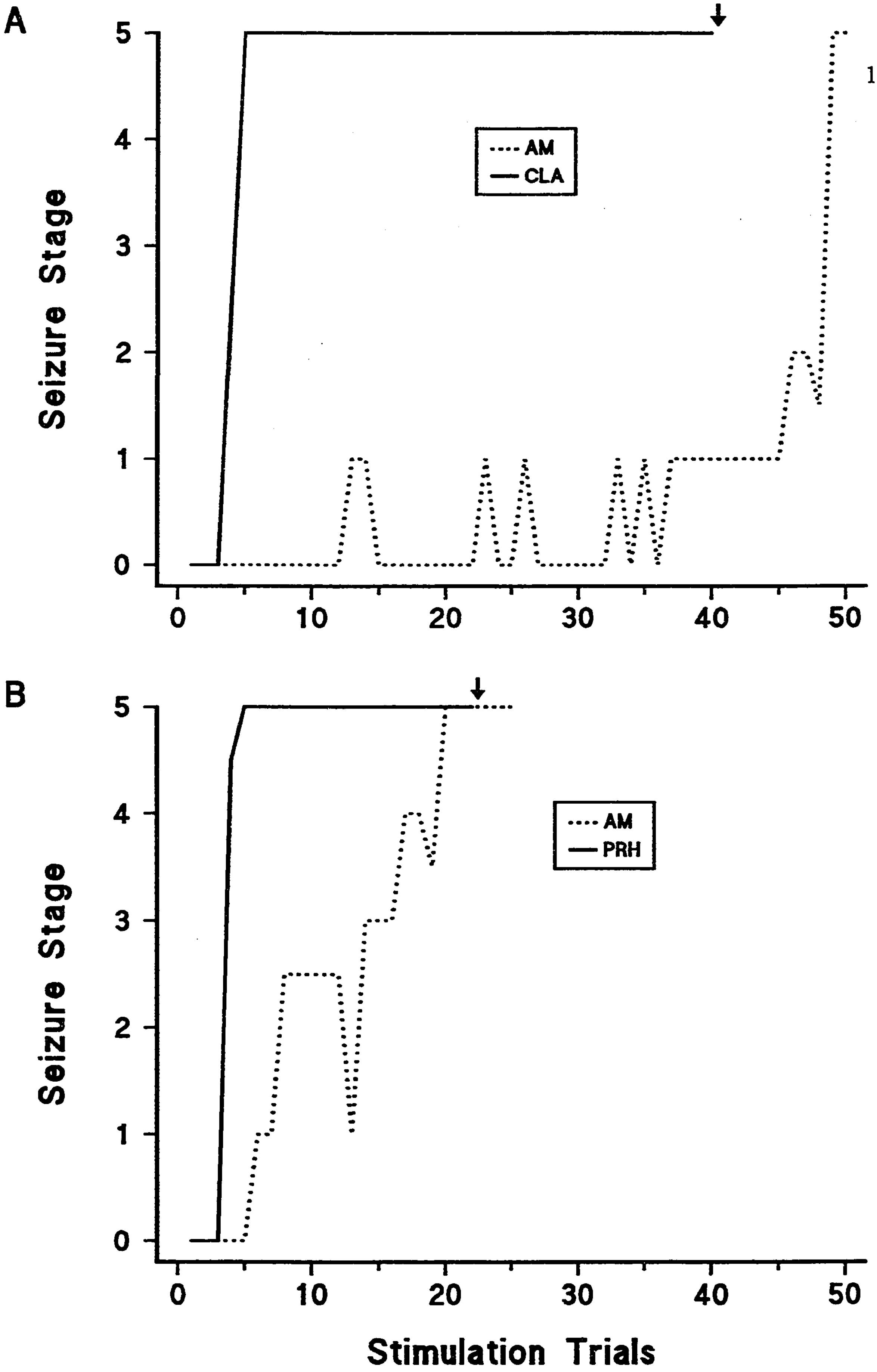

A
Seizure Stage
0
1
2
3
4
5
AM
CLA
0
10
20
30
40
50
B
Seizure Stage
0
1
2
3
4
5
AM
PRH
0
10
20
30
40
50
Stimulation Trials

Table 8.

Mean (± S.E.M.) kindling rate and convulsive profile parameters for claustrum and cortical kindling. Measurements include: the number of daily stimulations and the total cumulative after-discharge durations (ADD) to the first stage 5 seizure; latency to forelimb clonus, duration of forelimb clonus, and AD duration over the first 2 stage 5 seizures. Kindled groups compared are alternating stimulation of the claustrum and amygdala (CLA/AM) and its respective single-site stimulation control (CLA), alternating stimulation of the insular cortex and AM (INS/AM) and its respective single-site stimulation control (INS), alternating stimulation of the perirhinal cortex and AM (PRH/AM) and its respective single-site stimulation control (PRH), alternating stimulation of the piriform cortex and AM (PIR/AM) and its respective single-site stimulation control (PIR).

Group	*n*	1st stage 5 (# of trials)	Cumulative ADDs to 1st stage 5	Latency to clonus - stage 5 (sec.)	Duration of clonus - stage 5 (sec.)	AD duration - stage 5 (sec.)
CLA/AM	5	3.2 ± 0.6	32.8 ± 8.8	0.6 ± 0.4	11.2 ± 2.0	12.7 ± 2.1
CLA	5	3.0 ± 0.9	23.2 ± 7.6	0.5 ± 0.5	11.0 ± 0.7	11.4 ± 1.0
INS/AM	7	5.7 ± 1.3	67.3 ± 20.6	1.2 ± 0.6	14.9 ± 3.5	15.8 ± 3.7
INS	5	4.2 ± 1.1	74.4 ± 37.7	1.8 ± 1.4	16.7 ± 3.3	27.6 ± 12.2
PRH/AM	7	4.6 ± 0.9	75.9 ± 23.3	0.9 ± 0.6	22.7 ± 4.3	25.9 ± 5.5
PRH	5	4.0 ± 1.4	59.6 ± 36.6	0.2 ± 0.1	15.9 ± 1.9	19.3 ± 3.0
PIR/AM	7	9.9 ± 0.5[a]	272.4 ± 47.8[a]	10.7 ± 2.6[a]	26.6 ± 2.6[c]	56.3 ± 12.4[a]
PIR	2	9.5 ± 0.5[a]	187.5 ± 9.5[b]	4.0 ± 0.5	22.8 ± 1.8	42.3 ± 6.3

[a] Significantly different all other groups ($p < 0.002$).

[b] Significantly different from both CLA groups ($p < 0.0001$).

[c] Significantly different from both CLA groups and both INS groups ($p < 0.009$).

single-site AM controls (CLA: 6.3 ± 3.1 and INS: 7.6 ± 0.9 vs AM: 6.8 ± 0.6 stimulations, $F(2,13) = 0.24$, $p = 0.79$). Finally, the resulting stage 5 seizures from the suppressed AM were identical in latencies to forelimb clonus ($F(4,27) = 0.60$, $p = 0.66$), durations of forelimb clonus ($F(4,27) = 1.17$, $p = 0.34$) and AD durations during clonus ($F(4,27) = 0.78$, $p = 0.55$) as compared to single-site AM controls (see Table 7).

Further analysis was conducted on the data from the two groups that exhibited kindling antagonism of the AM. All of the CLA/AM kindled rats (5 of 5) and 5 of the 7 INS/AM kindled rats displayed suppressed AM kindling. As shown in Figure 17, all electrodes situated in either the CLA or deep layers 5 or 6 of the INS supported kindling antagonism, whereas electrodes located within the superficial layers 2 or 1 of the INS (i.e., in the remaining 2 INS rats) did not support kindling antagonism. Note that this deep layer effect was restricted to the INS, since placements in deep layers of the PC (3 of 7) did not support kindling antagonism. A separate analysis was performed on the data from the 5 CLA/AM and 5 INS/AM rats that expressed antagonism, and it was found that the AM suppression effect was significantly stronger with CLA kindling. More stimulation trials and cumulative AD durations were required to evoke the first stage 5 convulsion from the suppressed AM with alternating stimulation of the CLA (45.8 ± 2.7 stimulations, 2372 ± 211 sec.) than with stimulation of the INS (31.4 ± 3.0 stimulations, 1457 ± 102 sec.; $F(1,8) = 12.72$, $p < 0.008$ and $F(1,8) = 15.25$, $p < 0.005$, respectively).

Discussion

The present experiment has demonstrated that AM kindling can be arrested by alternating stimulations with the CLA and INS. Moreover, the capacity for establishing kindling antagonism appears very selective to the dorsolateral CLA and the adjacent deep layers of the INS, since neither the superficial layers of the INS nor any layers of the PRH or PIR were capable of suppressing AM kindling.

When stimulation of one site establishes antagonism of kindling from a second site it is assumed that the dominance of the first site reflects the failure of the suppressed site to kindle fully. The assumption is based on the premise that kindling antagonism involves the same neural mechanisms as standard single-site kindling, and that the delay in kindling from the suppressed site represents an actual arrest of normal kindling (Burchfiel & Applegate, 1989a; Kirkby *et al.*, 1995). My data support both these assumptions in two ways: First, the behavioural and electrographic characteristics exhibited at each similar seizure stage from either the CLA or INS or AM were virtually indistinguishable from characteristics observed in the respective single-site kindled controls. Second, CLA or INS kindling produced partial or relative antagonism (stages 1 to 2) of AM kindling, which did not proceed further until stimulation of the CLA or INS was discontinued, after which single-site kindling of the AM proceeded at a normal rate through the development of generalized seizures.

Recall that Burchfiel and Applegate (1989a) proposed that arrested seizure development with kindling antagonism reflected the inability of a site to open a transitional gate between two phases of kindling. Absolute antagonism refers to the inability to open the first (forebrain) transition gate and is reflected by the suppressed site exhibiting only stage 0 seizures. Relative antagonism refers to the inability to open the second (hindbrain/neocortical) transition gate and is reflected by the suppressed site exhibiting only stages 1 and 2 seizures. Since all suppressed AM kindling in this study was characterized by relative antagonism, it could be inferred that the CLA and INS were able to prevent the AM circuitry from entering the final late phase of kindling by preventing the opening of the second transition gate. Note that the kindling rates to stage 1 seizures was significantly delayed, but not absolutely blocked, suggesting that alternating CLA and INS were also capable of interrupting the AM circuitry from entering the middle phase of kindling.

The occurrence of kindling antagonism only in the CLA and INS and not in the PRH or PIR could reflect more intimate associations with the motor substrates important for seizure generalization in the former sites. It may be that the CLA and deep laminar INS regions represent an anatomically preferred route of access to the seizure substrates, that they are one step closer to the motor cortex than the other regions investigated in this experiment. The mechanism by which the CLA gains preferred access to the motor substrates is most probably related to the CLA's unique connectivity. The CLA has bilateral reciprocal connections with the motor cortex and the limbic system across the corpus callosum (Markowitsch *et al.*, 1984; Norita, 1977). Via these bilateral connections it is possible that a kindled epileptic focus established in the CLA would prevent the contralateral AM from propagating to the other hemisphere to establish a linkage with the seizure motor substrate(s). If the CLA is suppressing AM kindling in this manner then the further one moves anatomically from the CLA, such as to the more posterior regions of the PRH and PIR or more superficial cortical layers, less interference with kindling from the contralateral AM one might expect. This expectation is supported by the observation that there was somewhat of a gradient in the suppressive effects on AM kindling, such that alternating CLA kindling induced the slowest AM kindling rate, followed closely by the INS, with the PRH showing the next slowest AM kindling rates (albeit not statistically different), and lastly the PIR, the most anatomically removed structure from the CLA, displaying absolutely no suppressive effects on AM kindling.

EXPERIMENT 4

Claustrum Lesions and Amygdala Kindling

Wada and colleagues were the first to provide direct evidence that the CLA is important for both kindling and the propagation of kindled seizures from the AM. Unilateral CLA lesions slowed ipsilateral AM kindling in cats (Kudo & Wada, 1990; Wada & Kudo, 1997). Unilateral CLA lesions applied following the establishment of AM kindled seizures destabilized generalized seizures in cats and completely eliminating generalized seizures in baboons (Kudo & Wada, 1990; Wada & Kudo, 1997; Wada & Tsuchimochi, 1992, 1997).

The present experiment attempted to extend the analysis of the effects of CLA lesions to kindling in the rat. The effects on AM kindling of very small bilateral lesions, via radio-frequency (RF) current, to the both the ventromedial and dorsolateral CLA were examined. One group of rats received CLA lesions prior to AM kindling, and the other group received CLA lesions following the completion of AM kindling.

Methods

Groups and Surgery

All rats carried 6 electrodes, 2 bilateral kindling electrodes in the AM and 2 bilateral sets of lesion electrodes in the ventromedial and dorsolateral CLA. Lesion electrodes were constructed from the same material as the kindling electrodes (see general methods). The bilateral stereotaxic coordinates of the ventromedial CLA were 2.2 mm anterior from bregma, 3.4 mm lateral to midline with the electrode angled laterally at 13^0, and 5.4 mm ventral from dura; and the dorsolateral CLA coordinates were 1.2 mm anterior from bregma, 3.8 mm lateral to midline with the electrode angled laterally at 13^0, and 6.0 mm ventral from dura. Seizures were kindled from one AM site, and in some rats seizures were kindled from both sites to examine transfer, until a maximum of 3 consecutive stage 5 seizures were evoked. The initial site of stimulation was randomly balanced between groups. Twelve rats received CLA lesions prior to AM kindling, 6 rats received CLA lesions following AM kindling, and 6 control rats did not receive lesions.

Radio-Frequency Lesions

Rats were anaesthetized with pentobarbital (at least 40 mg/kg i.p.), and bilateral lesions were produced by brief application of a train of radio-frequency (RF) current (Radionics, Model RFG-4A, Burlington, Mass.) at 5 to 7 mA for 80 sec. via the 4 implanted lesion electrodes situated in the ventromedial and dorsolateral regions of the CLA. One group received RF current via all 4 lesion electrodes during the implantation surgery, and the other group received RF current via the 4 lesion electrodes after 3 consecutive stage 5 seizures were evoked from the AM. RF lesions are produced by high temperature cauterization around the tip of the electrode. RF lesions were chosen over electrolytic lesions because the RF current destroys tissue without inducing epileptiform effects and also cauterizes blood vessels in the area, thereby preventing uncontrolled bleeding and potential edema.

Lesion Quantification and Data Analysis

The total lesion area was determined for each of the following 8 coronal planes: bregma +1.0, +0.7, +0.2, -0.3, -0.4, -0.8, -1.3, and -1.8 mm (see Figure 20). From each individual coronal plane, a lesioned percentage area was calculated from the total area of the structure of interest. From these calculations the total lesioned volume of a structure was estimated by compiling all the percentage lesioned areas from each coronal plane (factoring in the distance between each of the 8 coronal planes). These lesioned percentage volumes were correlated with speed of amygdaloid kindling, and tests for statistical significance were conducted using one-tailed Pearson's correlation coefficients.

Results

Schematic illustrations of the minimal and maximal extent of RF lesions are presented in Figure 20. Note that many of the larger lesions consistently extended into parts of the INS and occasionally into the caudate nucleus and the parietal and endopiriform cortices. As shown in Figure 21, a mean of 61.5 ± 14.4% of the entire CLA

Figure 20. Minimal and maximal extent of RF lesions produced from the claustrum (CLA) and adjacent insular cortical (INS) regions in 18 rats. The 4 coronal plates were adapted from Swanson (1992). The dark shaded regions represent the minimal, and the hatched regions represent the maximal, extent of the lesions. Abbreviations: AI, agranular insular cortex; BLA, basolateral nucleus of the amygdala; CEA, central nucleus of the amygdala; CP, caudate-putamen; EP endopiriform nucleus; EC, external capsule; GI, granule insular cortex; INT, internal capsule; MEA, medial nucleus of the amygdala; PAR, parietal cortex; PIR, piriform cortex; RF, rhinal fissure; VL, lateral ventricle.

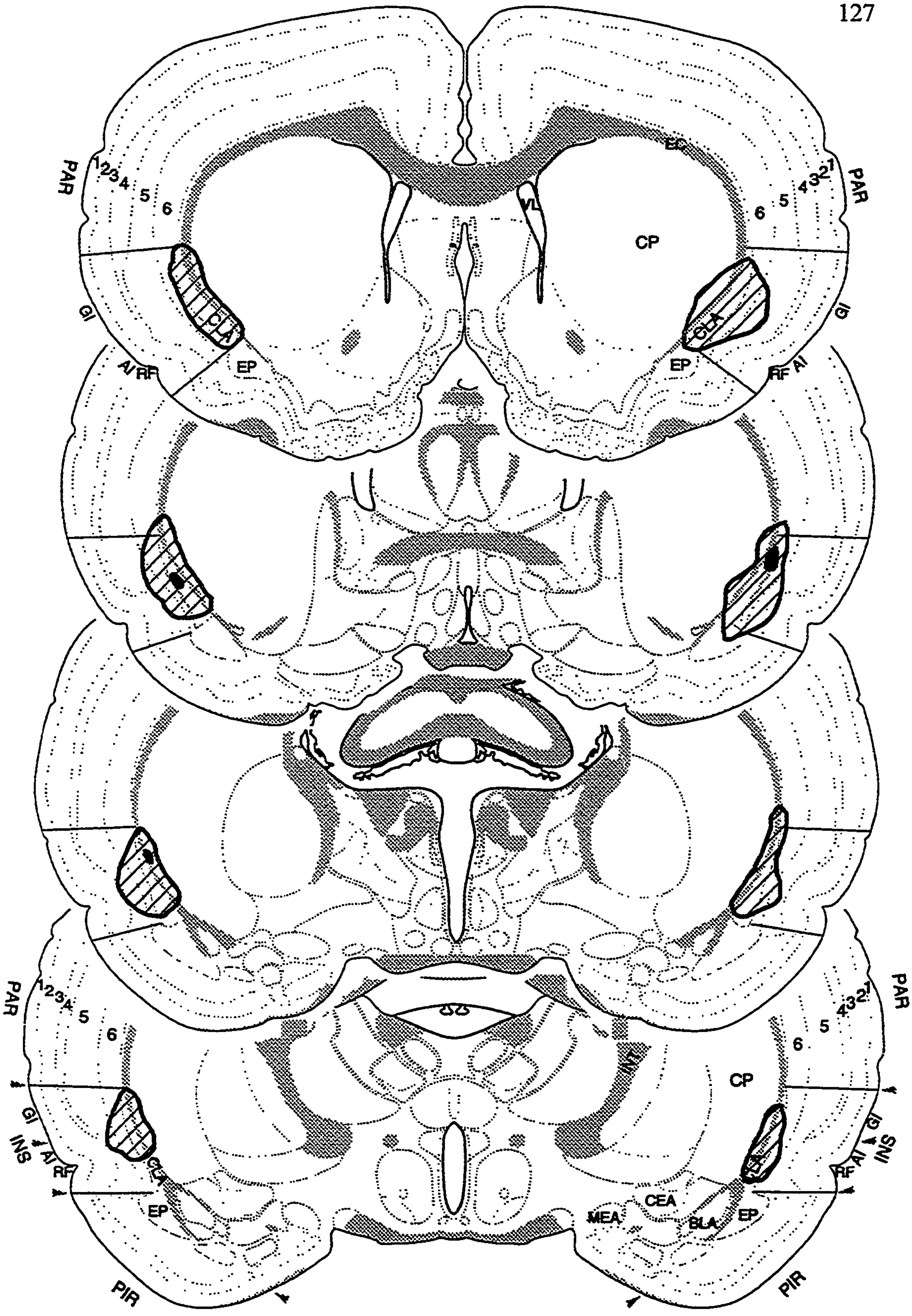
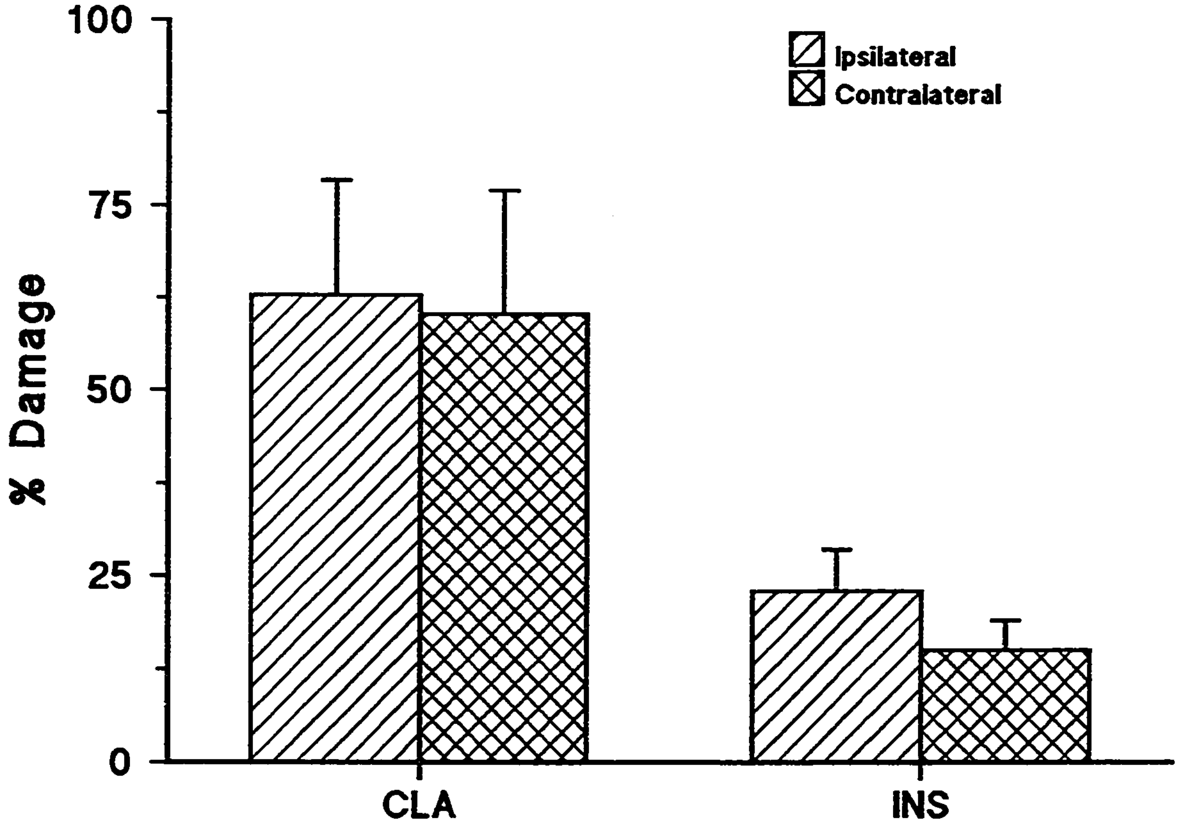

PAR
1 2 3 4 5 6
EC
VL
CP
GI
CLA
EP
AI/RF
PAR
1 2 3 4 5 6
GI
INS
AI
RF
CLA
EP
PIR
INT
CP
CEA
MEA
BLA
PIR

Figure 21. Mean percentage of volume damage in the claustrum (CLA) and insular cortex (INS). Values are expressed as percentage of structure destroyed in each hemisphere that is either ipsilateral or contralateral to the site of amygdaloid (AM) kindling. The lesion volume percentages were calculated by sampling from 8 coronal planes, ranging from bregma +1.0 to -1.8 mm.


100
75
50
25
0
% Damage
Ipsilateral
Contralateral
CLA
INS

was destroyed, and a mean 19.0 ± 4.2% of the entire INS was destroyed.

In both groups about 50% of the AM placements were located in the basolateral nucleus, and the remainder were distributed between the central and medial nucleus (electrode tips not shown).

CLA Lesions Prior to AM Kindling

No apparent gross behavioural deficits were observed in rats with small RF lesions. As shown in Table 9, CLA lesioned rats required significantly more mean stimulation trials to develop the first stage 5 seizure ($F(1,22) = 6.47$, $p < 0.02$; respectively). The delay in kindling was also reflected in the mean cumulative AD durations to the first stage 5 seizure ($F(1,22) = 7.16$, $p < 0.02$) (Table 9). However, no significant differences were found in the mean stimulation trials or cumulative AD durations to the first stage 1 seizure ($F(1,21) = 3.51$, $p = 0.08$; $F(1,21) = 1.37$, $p = 0.25$, respectively) (Table 9). No further differences were found between lesioned and non-lesioned groups with respect to mean initial and final ADTs (lesioned: 98.8 ± 14.8 vs. non-lesioned: 77.5 ± 16.7 μA, $F(1,22) = 0.90$, $p = 0.35$; lesioned: 95.0 ± 28.3 vs. non-lesioned: 85.0 ± 17.2 μA, $F(1,22) = 0.09$, $p = 0.77$, respectively) or in any of the other measures, including latency to onset of forelimb clonus ($F(1,22) = 0.24$, $p = 0.63$), forelimb clonus durations ($F(1,22) = 0.22$; $p = 0.64$), or AD durations during clonus ($F(1,22) = 0.79$, $p = 0.39$) (see Table 9).

A subset of rats from the CLA lesion and non-lesioned groups received kindling stimulation in the contralateral AM to test for transfer. All groups had developed 3 stage 5 seizures before stimulation was applied to the contralateral AM, 1 day following the last primary AM site stimulation. There were no significant differences in the mean number of daily stimulation trials required to evoke the first stage 5 seizure from the contralateral AM in the lesioned (8.1 ± 2.3) compared to the non-lesioned group (7.3 ± 0.5; $F(1,9) = 0.08$, $p = 0.78$).

It is apparent from Figures 20 and 21 that the lesions were not restricted to the CLA. Therefore, an analysis was conducted to determine whether damage to the CLA alone was responsible for the delay in AM kindling. To achieve this, the percentage of

Table 9.

Mean (± S.E.M.) kindling rate and seizure parameters for amygdaloid kindling following claustrum (CLA) lesion or no prior lesion (control). Measurements include: the number of daily stimulations and the total cumulative after-discharge durations (ADD) to the first stage 1 and 5 seizure; latency to forelimb clonus, duration of forelimb clonus, and AD duration over the first 3 stage 5 seizures.

Group	*n*	# of trials to 1st Stage 1	Cumulative ADDs to 1st stage 1 (sec.)	# of trials to 1st Stage 5	Cumulative ADDs to 1st stage 5 (sec.)	Latency to clonus - stage 5 (sec.)	Duration of clonus - stage 5 (sec.)	AD duration - stage 5 seizure (sec.)
CLA lesion	12	10.8 ± 1.8	162.2 ± 22.9	20.0 ± 2.7*	768.4 ± 91.5*	23.7 ± 3.1	38.1 ± 2.9	79.5 ± 6.5
control	12	6.9 ± 0.7	130.9 ± 12.2	12.6 ± 1.0	468.4 ± 61.1	26.7 ± 5.1	35.9 ± 3.6	70.6 ± 7.6

* Significantly different from non-lesion control group, $p < 0.02$

the total volume of CLA destroyed was correlated with the number of stimulation trials to the first 3 consecutive stage 5 seizures. As shown in the scatter plot of Figure 22, a significant negative correlation of -0.58 ($p < 0.03$) was detected between the percentage of CLA volume damage and the rate of AM kindling. Note that the negative correlation was the result of the larger CLA lesions, particularly those that exceed 50% structural damage, which show normal to accelerated AM kindling. It were the CLA lesions sizes below 50% damage that accounted for the delays in AM kindling (see Figure 22).

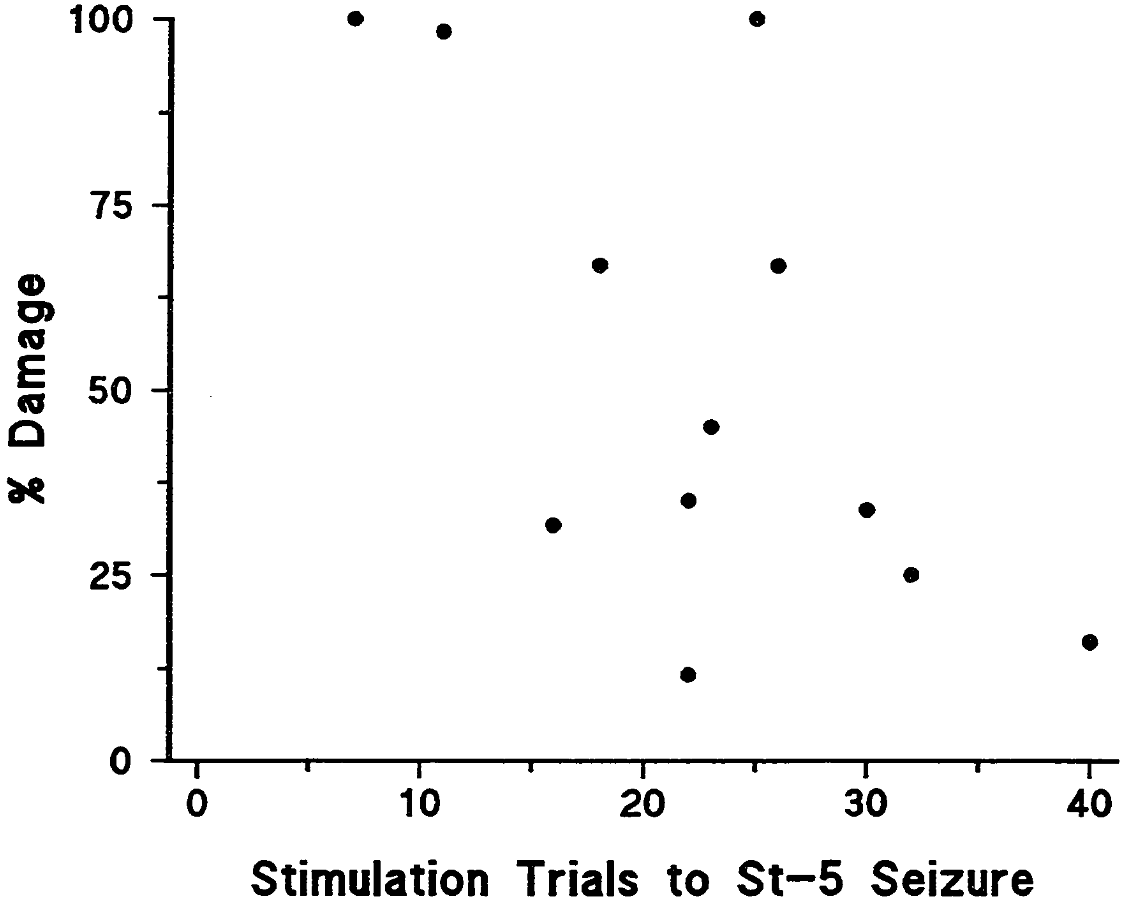
Subsequent analysis revealed that this significant correlation between CLA lesions and kindling rate was attributable to CLA damage ipsilateral to the site of AM stimulation ($r = -0.61$, $p < 0.02$). Furthermore, partitioning the lesion damage to ventromedial CLA (bregma +1.0 mm to -0.4 mm) and dorsolateral CLA (bregma -0.4 mm to -1.8 mm) revealed that damage only to the anterior ventromedial region, particularly ipsilateral to the AM stimulation, could significantly account for the delays in kindling (ventromedial: $r = -0.50$, $p < 0.05$; ipsilateral ventromedial: $r = -0.66$, $p < 0.01$). Note that the delay in AM kindling was related only to damage in the CLA, since damage to the INS ($r = -0.37$, $p = 0.12$) and general overall lesion size ($r = -0.16$, $p = 0.31$) did not significantly correlate with kindling.

CLA Lesions Following AM Kindling

Rats lesioned after the establishment of 3 consecutive stage 5 seizures required a mean of 1.3 ± 0.3 stimulation trials to evoke the first post-lesion stage 5 seizure from the AM. Furthermore, the post-lesion ADTs did not change for any of the rats. None of the convulsion parameters significantly differed over the first 3 stage 5 seizures between pre-lesion and post-lesion AM stage 5 seizures, including latency to forelimb clonus onset (pre-lesion: 15.6 ± 4.0 vs. post-lesion: 18.1 ± 3.8 sec, $F(1,10) = 0.21$, $p = 0.65$), forelimb clonus duration (pre-lesion: 31.2 ± 3.6 vs. post-lesion: 38.8 ± 5.5 sec., $F(1,10) = 1.37$, $p = 0.27$), and AD duration during clonus (pre-lesion: 56.1 ± 4.8 vs. post-lesion: 70.8 ± 5.1 sec., $F(1,10) = 4.40$, $p = 0.06$).

The overall lesion sizes did not significantly differ between the rats that received lesions before kindling (prior-lesion) and rats that received lesions after kindling

Figure 22. Scatter plot of the percentage of the total volume of claustrum (CLA) lesioned versus the number of amygdaloid (AM) kindling stimulation trials to the first 3 consecutive stage 5 seizures.


100
75
50
25
0
% Damage
0
10
20
30
40
Stimulation Trials to St-5 Seizure

(subsequent-lesion) (F(1,16) = 0.28, p = 0.61). Furthermore, the total percentage of CLA lesion volumes (prior-lesion: 61.5 ± 14.3% vs. subsequent-lesion: 41.1 ± 24.0%) and INS lesion volumes (prior-lesion: 18.9 ± 4.2% vs. subsequent-lesion: 13.3 ± 5.5%) did not differ significantly between groups (F(1,16) = 0.60, p = 0.45; F(1,16) = 0.65, p = 0.43, respectively). However, for the subsequent-lesion group, it was found that percentage volume of lesions in the left hemisphere was smaller for the CLA (prior-lesion: 52.9 ± 13.6% vs. subsequent-lesion: 4.0 ± 2.1%; F(1,16) = 6.25, $p < 0.03$), and for the INS (prior-lesion: 19.9 ± 5.5% vs. subsequent-lesion: 4.0 ± 2.4, F(1,16) = 3.82, p = 0.07). The reasons for the differences in lesion size in one hemisphere are unknown.

Discussion

The present experiment has demonstrated that AM kindling to stage 5 seizures can be delayed with selective lesions of the CLA (with about 60% of the structure damaged). Specifically, the delay appeared to be mediated by ipsilateral CLA lesions, in the anterior ventromedial region. However, once AM kindling was established, CLA lesions had no effect on the expression of generalized seizures.

Interestingly, there was a negative relation between the speed of kindling and the size of the CLA lesion, such that smaller lesions resulted in greater delays in kindling. At first glance this relation appears counterintuitive, in that one would expect larger lesions to produce greater interference with kindling. One possible explanation for this effect may be related to the occurrence of incidental damage to the INS. The larger CLA lesions invariably damaged portions of the neighbouring INS. Since it was shown that INS damage alone could not account for the delayed kindling, it may be that the INS damage actually *countered* or *bypassed* the suppressive effects on kindling of damaging the CLA. When the lesion was small and restricted to only the CLA, AM kindling was delayed. When the lesion was larger and extended into INS, AM kindling was not impaired. Note that this negative effect with INS lesion contradicts earlier reports that found significant delays in AM kindling with INS lesions (Corcoran *et al.*, 1976a). However, in that study the lesions were much larger and damage extended into the orbital cortex and into the anterior portions of the PRH.

This interpretation suggests that the CLA is distinct from the INS in its seizure propagating properties. Furthermore, the CLA may represent a structure that is further downstream than the INS in the seizure generalization circuit. More precisely, the CLA might represent a critical point of access to motor seizure generalization (i.e., the second transitional kindling gate). The AM may have multiple avenues by which to access the motor generalization substrates; however, its preferred route (i.e., the path of least resistance) may be the CLA circuitry via the INS circuitry. Supposing that plastic changes occur that "commit" AM epileptiform activity to accessing the CLA circuity, then selective lesioning of the CLA would force the AM to "retreat" and find an alternative route to the motor substrates. This "retreat" would be reflected in the delayed, but not blocked, AM kindling rates after lesions restricted to the CLA. However, when *both* the INS and CLA are damaged, the AM epileptiform activity may not advance far enough to commit to the CLA circuitry since it is hindered by the INS damage. The AM epileptiform activity may then easily commit to another avenue to access motor substrates for seizure generalization.

The delay in AM kindling after CLA lesions corresponds to the results of Wada and Kudo's reports with kindling in cats (Kudo & Wada, 1990; Wada & Kudo, 1997). They found that unilateral CLA lesions in the anterior (ventromedial) region were capable of delaying kindling from the ipsilateral AM. I discovered a similar effect, but with bilateral CLA lesions; however, the primary effect was related to the lesion that was ipsilateral to the site of kindling, and located in the ventromedial portion of the CLA. Wada reported that the delay in AM kindling in cats was primarily due to the number of stimulations required to reach stage 4 seizures (in cats this involves tonic extension of contralateral forepaw and contralateral head turning and circling). In my experiment, the delay of kindling in rats was attributed the number of stimulations to reach the first stage 5 seizure. It is difficult to directly compare our results, because the cat exhibits a more complex pattern of kindling.

In contrast to my results, Wada and colleagues also reported that unilateral CLA lesions applied after the establishment of kindling could destabilize or even completely block the expression of AM generalized seizures (Kudo & Wada, 1990; Wada & Kudo, 1997; Wada & Tsuchimochi, 1992, 1997). There are various factors that could account for these differences. First, different species were used. Since Wada has reported differences between cats and baboons in the effects of CLA lesions, it is conceivable that there may also be differences in rats. Second, bilateral ventromedial and dorsolateral CLA lesions were employed in my experiment, whereas mostly unilateral ventromedial CLA lesions were employed in Wada's experiments (Kudo & Wada, 1990; Wada & Kudo, 1997). Wada has reported that bilateral CLA lesions *do not* affect subsequent AM kindling, whereas unilateral lesions do. His findings could explain my inability to influence established AM kindling with bilateral CLA lesions. Finally, Wada reported more extensive damage with his lesions, including structures such as the globus pallidus, putamen, internal capsule, and prepiriform cortex (Kudo & Wada, 1990; Wada & Kudo, 1997).

GENERAL DISCUSSION

The Involvement of the Claustrum in Kindling

Experiment 1 and 2 employed correlative techniques to assess the CLA's participation in kindling. In Experiment 1 various electrographic and behavioural characteristics were documented with kindling from the dorsolateral CLA. The profile of CLA kindling was compared with profiles from the AM and the two cortical structures that lay laterally adjacent to the CLA, the INS and PRH. CLA kindling was found to exhibit more pronounced epileptogenic properties than AM kindling. Specifically, the CLA exhibited dramatically quicker progression to seizure generalization. Moreover, extended generalized seizures kindled from the CLA exhibited almost instantaneous onsets to limb convulsions that were more vigourous and sustained than seizures from the AM. Unlike the AM, CLA kindling shared many more characteristics with INS and PRH kindling. Specifically, these three sites exhibited a similar two phase development of generalized seizures, rapid progression to seizure generalization, quick onset to limb convulsions, and similar patterns and development of evoked AD spike characteristics. However, CLA kindling did exhibit marginally quicker progression to seizure generalization than PRH kindling, and CLA seizures included more vigorous and sustained limb convulsions.

Experiment 2 revealed further dissociations between CLA and AM kindling by examining changes in molecular products linked with neuronal plasticity. With extensive dorsolateral CLA kindling to the late stage 5 seizure phase, greater FosB/ΔFosB protein expression occurred over a wider range of anatomical regions and with a greater intensity than with AM kindling. Similarly, extensive CLA kindling led to relatively more intense expression of BDNF mRNA than AM kindling; however, this difference was primarily observed in the DG of the hippocampus. Overall, it appears that with extensive CLA kindling, many more far-reaching plastic alterations occur that correspond with the enhanced epileptogenic properties observed in Experiment 1.

Experiment 3 and 4 employed intervenient techniques to assess the CLA's participation in kindling. Experiment 3 tested the ability of the dorsolateral CLA to exert kindling dominance over the contralateral AM via the alternating stimulation paradigm. Kindling antagonism from the CLA was compared to the antagonistic properties of the INS, PRH, and PIR. It was found that only the CLA and the adjacent deep layers of the INS were capable of suppressing AM kindling, suggesting that these regions possess greater epileptogenic properties. In Experiment 4 the effects of combined bilateral ventromedial and dorsolateral CLA lesions were examined on AM kindling. It was determined that small restrictive lesions to the CLA could delay, but not prevent, the kindling of AM generalized seizures. The delay was principally mediated by CLA lesions in the ipsilateral hemisphere and in the anterior ventromedial region. These results suggest that the CLA participates in the establishment of limbic seizure generalization.

Is the Claustrum Distinct from the Adjacent Cortical Regions?

Recall that one of the primary objectives of my research project was to determine whether the epileptogenic properties of the dorsolateral CLA are distinct from those of the deep laminar layers of the PRH. In the process of addressing that question, I discovered that the INS also demonstrated powerful epileptogenic properties. Since many of the deep laminar layers of the PRH and INS merge with the dorsolateral CLA (Minciacchi *et al.*, 1986), it was important to address the relation between the CLA with the PRH and INS.

The Claustrum versus the Perirhinal Cortex

It has been proposed that the PRH is the critical structure for the amplification and expression of kindled limbic generalized seizures (Applegate *et al.*, 1998; Kelly & McIntyre, 1996; Sato *et al.*, 1998). Some of the strongest evidence of this notion is the PRH's rapid rate of progression to motor seizure generalization (McIntyre et al., 1993; Kelly & McIntyre, 1996). In Experiment 1 I showed that seizures kindled from the CLA developed more rapidly and were more vigorous than those kindled from the PRH. These differences indicate that the CLA may have more intimate associations than the PRH with the motor substrates responsible for seizure generalization. This hypothesis is further supported by the demonstration in Experiment 3 that CLA kindling, but not PRH kindling, is capable of antagonizing AM seizure development. The CLA presumably is capable of preventing the AM's access to the generalized motor substrates.

Another argument for the PRH's crucial role in kindling is its anatomical connectivity. The PRH has many interconnections with limbic structures that have been implicated in the establishment of the middle and late stages of kindling, including the AM and PIR (Burwell *et al.*, 1995; Stefanacci, Suzuki, & Amaral, 1996), and with many cortical areas that may mediate motor seizure expression (Burwell *et al.*, 1995; McIntyre *et al.*, 1996). However, the same argument can be applied to the CLA, in that many of the same interconnections exist between these subcortical and cortical structures (e.g., Clasa *et al.*, 1992; Markowitsch *et al.*, 1984; Norita, 1977; Sloniewski, 1986b; Witter *et al.*, 1988). Further, there is evidence that the CLA has more widespread connectivity between hemispheres and along the rostal-caudal axis (Sadowski *et al.*, 1997; Minciacchi *et al.*, 1986; Sloniewski, 1986b; Witter *et al.*, 1988) than the PRH. However, the exact functional organization of the CLA and PRH with respect to seizure propagation is mostly conjectural, since little anatomical information is available about the rat as compared to some other species (e.g., Burwell *et al.*, 1995; Kowiański, 1999; Minciacchi *et al.*, 1986).

McIntyre and colleagues have contended that the deep laminar layers of the PRH, particularly layer 5, are the most crucial for mediating limbic epileptogenic properties. Evidence that supports this hypothesis includes: (1) PRH layer 5 is the main source of projection fibers from the PRH to the frontal cortex (McIntyre *et al.*, 1996); (2) deep layers of the PRH exhibit the fastest kindling rates (Felstead *et al.*, 1995); (3) PRH layer 5 neurons exhibit strong intrinsic bursting properties (Kelly & McIntyre, 1996); and (4) infusion of KCL into the deep PRH layers degrades contralateral generalized seizures evoked from the AM (Ferland *et al.*, 1998). As was previously argued, it may be that some of these attributes of the deep PRH layer are actually mediated by the adjacent CLA. For instance, Experiment 1 demonstrated that with my stimulation parameters the deep layers of the PRH were slower to kindle than the CLA. I did not conduct any other experiments that directly tested the CLA involvement with the other points listed above.

The Claustrum versus the Insular Cortex

Very little is known about the role of the INS in kindling. As was previously mentioned, bilateral frontal cortical lesions that included the INS retarded AM kindling (Corcoran *et al.*, 1976a), infusions of NMDA antagonists into the INS blocked the expression of AM kindled seizures (Holmes *et al.*, 1992), and strong Fos labelling occurred in the INS with contralateral PRH kindling (Ferland *et al.*, 1998).

The results of my experiments further implicate the INS in kindling. In Experiment 1 the INS exhibited kindling profiles similar to the CLA and PRH. It is interesting to note that INS kindling rates were slower than the CLA and faster than the PRH; however, the firmness of this interpretation is limited by the lack of sufficient statistical power. Since suspended rats were not kindled in the INS it is not known how the motor seizure profiles would compare to that of the CLA or PRH. Based on the kindling rates, one might predict that kindled INS generalized seizures would lie somewhere between the PRH and CLA in convulsive intensity. Experiment 3 more strongly implicated the INS in possessing intimate associations with the motor substrates for generalization, since the deep laminar layers INS were capable of antagonizing AM seizure development.

There were notable distinctions between the kindling properties of the CLA and the INS. Experiment 1 showed that the vigorous motor seizure profiles of the CLA could be maintained from foci sites that were far removed from the INS, suggesting strongly that CLA seizure expression cannot be attributed to the INS. In Experiment 3, even though both the INS and CLA were capable of exerting kindling dominance over the AM, the CLA exhibited a much more powerful antagonistic effect than the INS. Finally, in Experiment 4 lesions that extended past the CLA into the INS appeared to counter the suppressive effects on AM kindling that were seen when lesions were restricted exclusively to the CLA. This finding provides more convincing evidence that the INS is distinct from the CLA in its contributions to epileptogenesis.

To summarize, my experiments strongly implicate the unique contribution of the CLA in mediating kindling. Specifically, most of my results support the hypothesis that the CLA has very close associations with the motor substrates responsible for seizure generalization. Moreover, the CLA appears to be more closely linked to these motor substrates than the PRH or INS. However, my results do not preclude the possibility that all three of these structures participate in the expression of limbic generalized seizures. For example, McIntyre *et al.* (1996) have demonstrated strong anatomical connections of the PRH (particularly the deep laminar layers) with both the CLA and the INS. Furthermore, similar regions of the frontal motor cortex project extensively to the PRH, INS, and CLA (McIntyre *et al.*, 1996). In addition, Fos studies demonstrate robust activation in both the PRH and INS with kindling from various sites (Ferland *et al.*, 1998; Sato *et al.*, 1998).

The next section will attempt to further address the hierarchial relation between the CLA, INS, and PRH with respect to the transitional gating hypothesis of kindling.

The Claustrum and the Transitional Gating Hypothesis of Kindling

According to the gating hypothesis, kindling can be conceptualized as involving three distinct sequential phases or neuronal states that are separated by two crucial gates (Burchfiel & Applegate, 1989a) (see Figure 1). Each kindling phase represents discrete transitions from one state of neural organization to another. In order for kindling to progress from one phase of seizure generalization to the next, a gate must be opened. During the early phase only nonconvulsive (stage 0) seizures are expressed, and seizure propagation is restricted to the focal area. Neuronal reorganization is transient and independent of the kindling process. The middle phase of kindling is characterized by partial (stages 1 and 2) seizures and is entered when AD activity generated in the early kindling phase overcomes the forebrain (first) transition gate. Neural organization at this phase is permanent and is common to all limbic ADs, regardless of where they were originally evoked. It is the transition into the middle phase that accounts for differences in the progression of kindling. The late phase of kindling is characterized by fully generalized (stages 3, 4, and 5) seizures and is entered when AD activity generated in the middle kindling phase opens the hindbrain/neocortical (second) transition gate. Neural organization in this phase is common to all ADs, regardless of where they were originally evoked. The late phase is typically considered the end point of kindling.

The Claustrum and the Second Hindbrain/Neocortical Gate

Experiment 3 utilized the kindling antagonism paradigm to directly assess the CLA's relation to the transitional gates separating the kindling phases. Recall that the CLA was capable of arresting contralateral AM kindling at the partial seizure stages. Burchfiel and Applegate (1989a) argued that suppression of kindling at the partial stages reflects an inability of the second hindbrain/neocortical gate to be opened, and thereby denies the suppressed site access to the substrates of generalized motor seizures. Therefore in my study, the dominance of CLA kindling over AM kindling implies that the CLA has more intimate associations with, or even possibly encompasses, the second transitional gate.

The results of Experiment 4 further support the hypothesis that the CLA has close associations with the hindbrain/neocortical gate. Lesions exclusively confined to the CLA delayed, but did not completely suppress, the onset of AM generalized seizures. The delay in kindling was attributable principally to the increased number of stimulations required to reach the first generalized seizure (i.e., moving from the middle to the late phase of kindling). This delay in kindling may reflect the inability of AM seizure propagation to open the second transitional gate. However, unlike the antagonism effect, lesions of the CLA were not able to completely arrest AM kindling. Evidence suggests that the delay in kindling was not necessarily the result of different seizure circuits being established, since AM generalized seizures evoked in CLA lesioned rats exhibited profiles virtually identical to those in nonlesioned rats. Note that I detected no delays in partial AM seizure kindling with the CLA lesions, indicating that the first transitional gate was not effected.

Further insights into the relation between the CLA and the second transitional gate were provided by Experiments 1 and 2. In Experiment 1 two phases of stage 5 seizure development were identified with CLA kindling. This unusual pattern of two phase kindling can be effectively resolved by the transitional gating hypothesis of kindling. In many ways early stage 5 phase CLA kindling exhibits characteristics of late phase kindling of the transitional gating hypothesis. For instance, partial stage 1 and 2 seizures are never observed with CLA stimulation. Furthermore, the number of CLA kindling stimulations required to evoke the first stage 4 or 5 seizure (i.e., 1 to 3 stimulations) are virtually identical to the number of stimulations required by most limbic sites to progress from stage 3 to stage 5 seizures (Burchfiel *et al.*, 1998; Le Gal La Salle, 1981; Löscher *et al.*, 1998; Sato & Nakashima, 1975). Moreover, early CLA kindling shares many characteristics with anterior neocortical kindling, including high ADTs, short AD durations, forced motor responses during stimulation, immediate onset of clonus with stimulation, and short clonus durations (Burnham, 1975; McIntyre, 1979; Racine, 1975). Recall that the anterior neocortical areas are thought to house some of the motor substrates of seizure generalization (e.g., Corcoran *et al.*, 1976a; Kelly & McIntyre, 1996; Racine, 1972b; Wada *et al.*, 1975). Consequently the evidence points to the conclusion that early CLA kindling actually *bypasses* the first forebrain gate, such that kindling "begins" in the middle phase and proceeds immediately to the motor seizure substrates of the late phase by opening the second transitional gate. Only with continued CLA stimulation does the first transitional gate eventually open (but from the "inside"). This reversed opening of the first transitional gate is reflected by the progression from early to late stage 5 phase CLA kindling. Opening of the first gate represents access to forebrain seizure circuitry, which would explain why late phase CLA kindling shares many properties with AM kindling.

Results from Experiment 2 further reinforce the notion that early stage 5 phase CLA kindling reflects the opening of only the second transitional gate. With early CLA kindling there was predominantly cortical expression, with very little subcortical forebrain staining, for either FosB/ΔFosB or BDNF. These results imply that seizure related activity occurred primarily in cortical regions, some of which contain the motor substrates for seizure generalization. The pattern of early CLA kindling expression is consistent with the idea that only the second transitional gate was transversed. Consequently, with late phase CLA and AM kindling, strong FosB/ΔFosB and BDNF expression was observed in *both* cortical and forebrain structures, indicating that both transitional gates had to be opened.

The Perirhinal and Insular Cortices and the Second Hindbrain/Neocortical Gate

The INS and PRH share many attributes of kindling with the CLA. Because of these similarities, the INS and PRH also appear to be intimately associated with the second transitional gate. For example, the deep layers of the INS were capable of arresting AM kindling at partial seizure stages, and both the INS and PRH exhibit the two stage 5 phase kindling.

However, some subtle differences exist between the INS, PRH, and CLA with respect to their relation to the second transitional gate. For example, the rate of kindling is significantly slower in the PRH as compared to the CLA, which can be attributed to fact that partial seizures are occasionally evoked before generalized seizures develop with PRH stimulation. The activation of partial seizures implies that PRH stimulation can intermittently activate forebrain circuitry. Furthermore, with alternating stimulation the PRH was incapable of arresting AM kindling, but it did delay it slightly. Finally, small lesions to the INS appeared to counter the suppressive effects of selective CLA lesions on AM kindling. The effect of INS lesions was argued to reflect the fact that the INS is one step removed from the second transitional gate (see discussion of Experiment 4). Taken together, these results strongly suggest that the INS and PRH are not as intimately associated with the hindbrain/neocortical gate as the CLA.

To conclude, my results strongly implicate the CLA as the best candidate to accommodate the second hindbrain/neocortical gate, or at least as having the most intimate associations with it. I would argue that the INS cortex would have the next closest association with the second transitional gate, followed by the PRH. I propose that the association the INS and PRH have to the hindbrain/neocortical gate of the CLA is analogous to the association the AM has to the forebrain gate of the PIR.

The Claustrum and Seizure Propagation

There is much debate as to whether seizure propagation in epilepsy and animal seizure models follow selective and restricted pathways, or whether epileptiform activity propagates more diffusely and less discriminantly throughout the brain (e.g., Bertram, 1995; Mirski, 1993).

At first glance, my CLA lesion data seem to indicate that seizure propagation is a diffuse phenomenon. CLA lesions were capable only of disrupting focal AM kindling, but not blocking it entirely. Two explanations could account for this effect: (1) the CLA is the critical nodal structure for seizure propagation, but insufficient amounts of the structure were destroyed to allow for the complete arrest of kindling, or (2) seizure discharge can propagate easily through a variety of different avenues. The kindling antagonism data argue for the former explanation. The fact that AM kindling could be completely arrested at a particular seizure stage and could not find an alternative route to motor seizure generalization speaks strongly to discrete rather than diffuse propagation routes.

Perhaps the intricacies of seizure propagation can be best explained with an analogy of automobiles using roads to drive downtown. Suppose that roads represent circuits or potential propagation pathways and the automobiles represent the epileptiform AD. A vehicle driving (propagating epileptiform activity) towards downtown (motor substrates for seizure generalization) can find multiple routes (neuronal circuits) to reach its destination. Some routes are more direct and easily accessible, such as the wide open expanse of a eight lane freeway, whereas other routes are less direct and more difficult to access, such as winding back-country gravel lanes. However, all routes can eventually lead downtown. Therefore, seizure propagation can be conceptualized as representing both a selective process (the preferred major freeways) and also a diffuse process (alternative back roads). Given the choice, AD propagation will take the most direct and accessible route to motor generalization, but if these routes are disrupted or blocked, less direct and accessible alternative routes may be used.

The automobile metaphor can be extended to explain the transitional gating hypothesis of kindling. The first forebrain transition gates of kindling (i.e., PIR) can be represented as an exclusive on-ramp that allows access to the freeway leading to downtown motor generalization. The second hindbrain/neocortical gate (i.e., CLA) can be represented by a toll booth along the same freeway. The transitional gating hypothesis of kindling states that all automobiles take the same accessible freeway route, which implies that they must use the on-ramp and pass through the toll booth to get to downtown. This analogy would predict that how quickly an individual (kindled focus) can get to downtown is a function of: (1) how near an individual lives to major freeway, and (2) how near an individual lives to downtown. Recall that the rate of kindling is primarily a function of the number of stimulations required to open the first transitional gate (Burchfiel & Applegate, 1989a). Therefore, structures that kindle more slowly (e.g., DG) may reside in the far corners of limbic suburbia and require more time and effort to reach the freeway on-ramp (first transitional gate), whereas structures that kindle more quickly (e.g., AM) may reside relatively closer to the freeway, which affords them quick and easy access to the freeway on-ramp. Once the automobiles enter the freeway via the on-ramp, the time it takes for to them to reach the toll booth (second transitional gate) is relatively the same for all automobiles, irrespective of their point of origin. This explains why neural organization in the middle and early kindling phases is common to all ADs (Burchfiel & Applegate, 1989a), and why the number of stimulations to seizure stages 3 to 5 is similar for all limbic structures (Burchfiel *et al.*, 1998).

Metaphors aside, having a clear understanding of the pathways of seizure propagation is useful for determining the effective epileptic zones that need to be targeted for clinical treatment. My research, and the research of others, suggest that the traditional view of targeting the epileptic foci for treatment may not necessarily be the best solution. Instead, my data suggest that targeting the transitional gate structures may be more effective in truly arresting temporal lobe seizure disorders.

Conclusions and Future Directions

Evidence has been presented that implicates the CLA in kindling. Specifically, the CLA has been proposed to represent the crucial mediator that allows limbic epileptiform activity access to the motor substrates responsible for seizure generalization. Further studies are needed to elucidate how the CLA achieves this gating role. Some of this research should focus on changes in neuronal excitation and inhibition, cellular death, and sprouting in the CLA with limbic kindling. One important line of research to pursue is the manipulation some of the molecular changes associated with limbic and CLA kindling. For example, it would be interesting to employ transgenic and antisense technology to block the expression of ΔFosB and its associated effector the NMDA Receptor 1 (see Hiroi *et al.*, 1998, McNamara, 1994) in the CLA and its targets and determine the effects on kindling from other limbic sites. Finally, the kindling properties of the ventromedial CLA need to be more systematically studied, since this region was shown to be important in regulating generalized seizure disruption.

To a lesser degree, the INS and PRH were also implicated in mediating progression to motor seizures. Future experiments need to focus on differentiating the epileptogenic properties of these two structures. In particular, the INS should be examined for its convulsive patterns, morphological changes, and excitation/inhibition changes associated with kindling.

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APPENDIX A

List of Abbreviations

AD	afterdischarge
ADT	afterdischarge threshold
AM	amygdala
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	brain derived neurotrophic factor
CA1	*cornu Ammon* 1
CA3	*cornu Ammon* 3
CLA	claustrum
DG	dentate gyrus
EEG	electroencephalogram
GABA	γ-aminobutyric acid
GFAP	glia-fibrillary-acidic-protein
IIS	interictal spiking
IPSP	inhibitory postsynaptic potential
MDA	maximal dentate activation
mRNA	messenger ribonucleic acid
MTLE	mesial temporal lobe epilepsy
NA	noradrenaline
NGF	nerve growth factor
NMDA	*N*-methyl-D-aspartate
NT-3	neurotrophin-3
PIR	piriform cortex
PRH	perirhinal cortex