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CONTENTS

SHORT INTERVAL ELECTRICAL AMYGDALA KINDLING IN INFANT RATS

The Paradigm and Its Application to the Study of Age-Specific Convulsants

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1. ABSTRACT

Kindling is a powerful paradigm for investigating seizure generation, propagation and generalization. Kindling has been extensively utilized as a model of limbic seizures in the adult rat, and its reproducibility and precision are also particularly useful for the study of epilepsy in the developing brain. In the juvenile rat (>15 days), the refractory period between stimuli is much shorter than in the adult, potentially reflecting the increased excitability of the immature brain during this developmental period. "Rapid" or short-interval amygdala kindling of juvenile rats has been characterized and studied extensively.

The neuropeptide, corticotropin releasing hormone (CRH), produces limbic seizures in adult rodents, with a latency of 7–9 hours. The temporal and behavioral similarities between CRH-induced seizures and electrical amygdala kindling have suggested a common mechanism. In the infant rat (7–14 days), intracerebroventricular (icv) administration of picomolar doses of CRH produces amygdala-origin prolonged seizures with a very short latency (two minutes). The goals of studies described in this report were: a) to determine whether the rapid amygdala kindling paradigm could be applied to infant rats. b) to characterize the behavioral and electrical parameters of the kindling paradigm at this age. c) to study the interaction of CRH-induced seizures and amygdala kindling during infancy in the rat.

Using the short-interval-kindling method, Stage 5 behavioral seizures were achieved even in 7-day-old pups. However, the progression of behavioral kindling was different from that of older rats, and the correlation between electrographic after-discharges and behavioral stages was inversely related to age. Reliable, progressive amygdala afterdis-

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charges were difficult to ascertain in many animals prior to postnatal day 9. Spontaneous seizures occurred relatively frequently in younger age groups. Administration of a specific blocker of CRH receptors either icv or into the amygdala did not alter the rate of kindling development. Once stage 5 seizures were achieved, blocking CRH-receptors did not affect the expression of these seizures.

In conclusion, electrical amygdala kindling using short inter-stimulus intervals is a reliable and reproducible paradigm in rats during the second postnatal week, suggesting significant functional maturity of the amygdala-limbic circuitry at this age. The data provide no evidence for a mechanistic interaction between amygdala kindling and amygdala-origin CRH-induced seizures in the developing rat.

2. INTRODUCTION

Kindling has been proposed as a measure of excitability of the involved neuronal circuits[1–3]. Since the seminal manuscript by Goddard[1], the kindling paradigm has proven immensely useful as a model of epilepsy and of neuronal plasticity in adult rats[2,3]. Kindling-evoked afterdischarges may reflect the susceptibility of an interconnected group of neurons to generate synchronized, potentially epileptic activity[3]. The rate of progression of behavioral kindling stages from focal, through unilateral, to generalized phenomena provides a measure of both inhibitory and excitatory mechanisms in seizure propagation[2–4].

The amygdala kindling paradigm has been extensively utilized in immature rats, older than 15 days[4–8]. In this age group, the "refractory period" is much shorter than that in adult rats[4,6,9]. Therefore, immature rats are capable of developing afterdischarges and generalized seizures following several hours of stimulation at intervals of 15 minutes. This forms the basis for the short interval or "rapid" amygdala kindling paradigm. In a series of studies[5,6,8], Moshe et al. characterized the electrical amygdala kindling model in the immature rat. They determined that the minimal current required for generation of afterdischarges (afterdischarge threshold) in 15-day-old rats was higher than in older animals.[5] In their hands, only 75% of rats at that age could be kindled. Other authors[10] concluded that 10- and 14-day old rats could not be kindled consistently.

A number of experimental approaches suggest that during infancy (the second week of life) the rat brain is highly susceptible to induction of seizures.[11–13] The precise mechanisms underlying this observation are not fully understood. The rapid kindling model, using stimulus intervals of 15 minutes, results in a fully kindled brain within several hours. This should permit assessment of susceptibility to seizure-induction on a specific postnatal day, which is critical during a period of rapid brain growth and maturation.[14] Aside from providing a powerful tool for the study of seizure-susceptibility in the developing brain in general, kindling may be particularly useful in understanding specific issues of developmental epilepsy[5,14]. For example, it may provide insight into the mechanisms of the age-specific potency and rapidity of the "natural" convulsant peptide, corticotropin releasing hormone (CRH)[15,16].

CRH has been shown to excite neurons both in vivo and in vitro[17–23]. The peptide produces neuronal depolarization in CA1 and CA3 hippocampal pyramidal cells in the slice preparation[17–19]. CRH administered into the cerebral ventricles (icv) of mature rats causes epileptiform discharges in the amygdala[20,21], which spread to the hippocampus. The latency to the onset of these discharges is one to three hours, and over 3–7 hours, behavioral and electrographic seizures develop. The doses needed for seizure generation in adult rats are 1.5 to 15 nanomole[20,21]. We have reported that CRH is a far more rapid-acting and potent convulsant in the neonatal (first postnatal week) and infant rat (second postnatal

week)[24–27]. The latency to seizures onset is less than two minutes, and convulsant doses are as low as 0.075 nanomole.

The long latency of CRH induced seizures in adult rodents, and their behavioral resemblance to kindling, suggested a potential participation of endogenous CRH, present in high concentration in several amygdala nuclei, in the kindling process[20,21,28]. The interaction of synthetic CRH administration with the development and expression of kindled seizures in the immature rat have not been studied. This report focuses on the response of infant rats to repeated amygdala stimulation at short (15 minute) intervals[29]. We demonstrate the progressive behavioral and electrical kindling stages, and the achievement of stage 5 seizures in infant rats. Rate of kindling and the onset and incidence of spontaneous seizures are discussed, as well as the application of the model to evaluate the mechanisms of CRH-induced seizures.

3. MATERIALS AND METHODS

3.1. Animals

Pregnancy-timed, Sprague-Dawley derived rats were obtained from Zivic-Miller, (Zelionple, PA). They were housed under a 12-hour light/dark cycle, and fed ad libitum. Delivery times were monitored and were accurate to within 12 hours, and the day of birth was considered day zero. The pups were kept with the mothers, and litters were culled to 12 pups. Infant rats were subjected to surgery 24–48 hours prior to kindling (24 hours for the younger rats, 48 hours for 9 days and older)[24,25,29]. Kindling was carried out starting between 9:00 and 9:30 am, to avoid potential diurnal variation in the rate of kindling development[28]. All experiments were carried out in normothermic shielded chambers[24,25,30] and were approved by the institutional Animal Care Committee.

3.2. Surgical Procedures

Electrodes were implanted under halothane anaesthesia[31], using an infant-rat stereotaxic apparatus, as previously described[24,25,29]. Bipolar twisted wire electrodes, (Plastics One, Roanoke, VA) with a wire diameter of 0.1–0.15 mm and vertical inter-tip distance of 0.5–1.0 mm) were inserted through a burr-hole and aimed at the basolateral nucleus of the amygdala. Electrodes were anchored to the skull with an acrylic cement "cap" attached also to one or two screws. The coordinates for the basolateral amygdala nucleus are age dependent, and have been published elsewhere.[29] Subsequent to each experiment, an electrolytic lesion (5–15 mAmp, 5–10 seconds) was generated, and electrode placement was verified. Animals were decapitated; brains were removed onto dry ice and blocked. Sequential 20 micron coronal sections were stained with cresyl violet.

For infusion of CRH or of the CRH antagonist, a stainless steel cannula was inserted into the cerebral lateral ventricle (icv) at the time of electrode placement[24,25]. For infusions and recording from the central nucleus of the amygdala, an electrode/cannula (315G; MS 303/2, Plastics One, VA) directed to the ACE replaced both electrode and icv cannula.

3.3. Administration of CRH and a CRH Antagonist

Alpha-helical CRH-(9–41), (4–6 μg) and CRH itself (0.3 nanomole) were administered icv via the chronic cannula in 1–2 microliters using a micro-infusion pump. Control animals were given saline/dye vehicle. Cannula placement and presence of dye in third ventricle were verified for each animal.

3.4. Short Interval Kindling Technique

The kindling paradigm was modified from Haas[8] as described[29]. Briefly, the kindling stimulus consisted of a one-second train of 60 Hz monophasic current, or a 3-second train of 400 μA peak-to-peak current, generated by an A-M isolated pulse stimulator (model 2100) and visualized using a Tektronix 5111a oscilloscope. Baseline EEGs were recorded for five minutes, as well as two-minute or longer samples immediately after each stimulation. Pups were stimulated at 15-minute intervals. Since infant rats (7–12 days) displayed a unique sequence of kindling-induced behaviors, a kindling scale was generated for them, based on the one defined by Moshe's group[4,8] for older pups (Table 1). The rate of kindling development was assessed by measuring afterdischarges duration after each stimulation, and the number of stimulations needed for the achievement of each kindling stage.

The presence of "spontaneous" seizures was determined as well. The latter were defined as stage 4 or 5 behaviors occurring, de novo, more than 4 minutes after the most recent stimulation. Rats with spontaneous seizures were usually stimulated once subsequent to the initial seizure, in an attempt to assess the length of the refractory period between the seizure and subsequent stimulation. These rats were then observed for several hours without further stimulation. All pups with "spontaneous" seizures continued to manifest them intermittently until the end of the experiment.

3.5. Analysis of the Rapid Kindling Experiments

Of 51 infant rats, correct placement of electrodes was achieved in 41 (nine 7-day, nine 8-day, ten 9-day, five 10- and eight 12-day-old). Only animals with correct placement were included in the analysis. Animals were combined into three age groups: 7–8 days (n = 18), 9–10 days (n = 15) and 12 days old (n = 8). Pooled data are presented as mean values ± standard error of the mean (SEM). Significance of difference among groups was analyzed using Mann-Whitney's rank sum test.

3.6. Interactions of CRH and Rapid Kindling: Experimental Design and Analysis

3.6.1. Does Acute Pretreatment with CRH Antagonist into the Cerebral Ventricles Alter the Rate of Kindling Development? Rats aged 10–13 days (n=17) were infused with CRH antagonist 15 minutes prior to initiation of kindling. The time of administration and the dose were appropriate for blocking CRH receptors as determined by prevention of CRH induced seizures[24].

Table 1. Stages of kindling-induced behaviors in infant rats

Stage	Days 7–9	Days 11–12
0	Behavior arrest	Behavior arrest
1	Head bob/facial movement	Head bob/facial movement
2	"Chewing"/neck flexion	Neck flexion "chewing"
3	Vigorous lick/limb rotation	Unilateral clonus/body flexion
3.5	Unilateral clonus*	Alternating clonus
4	Rearing (rare)	Forepaw rotation/bilateral clonus/rearing
5	Tonic extension	Loss of balance/extension

*Alternating clonus was seen rarely in 7-day-old rats.
(Adapted from ref. 29, with permission.)

3.6.2. Does Acute Pretreatment with CRH Antagonist into the Amygdala Alter the Rate of Kindling Development? 11-day-old pups (n=6) were infused with alpha-helical CRH-(9–41) via a double lumen electrode/cannula 15 minutes prior to kindling.

3.6.3. Does Chronic Pretreatment with a CRH Receptor Blocker Alter Kindling Development? Pups (n=4) were implanted with osmotic pumps on postnatal days 9–10, and kindled on postnatal day 12.

3.6.4. Does CRH Antagonist Alter the Expression of Kindled Seizures? CRH receptor blocker (4 μg) was administered to rat pups (n=6) once afterdischarge duration was 60 seconds.

3.6.5. Does "Chronic" Pretreatment with CRH Facilitate Kindling? Rat pups were given CRH (0.3 nanomole) icv four times on postnatal days 9–12, and were kindled on postnatal day 12. They were compared to sham-infused controls.

3.6.6. Does Acute Pre-Administration of CRH Facilitate Kindling? Rat pups were infused with CRH immediately prior to the first kindling stimulus. Since the behavioral seizures induced by CRH resemble those of kindling (Table 2), the CRH infused group was compared to controls only for EEG parameters (duration of afterdischarges).

For all these experiments, control and experimental groups with verified electrode and cannula placement were compared for the number of stimulations needed to achieve afterdischarges longer than 60 seconds, for the progression of behavioral kindling stages and for the number of stimulation needed to achieve stage 5 seizures.

4. RESULTS

The spectrum of progressive, stimulation-induced behaviors was age-dependent. In 7–9-day-old rats, as in those aged 10–12 days, behavior arrest and head/face movements were observed initially. Subsequent stages in the youngest group (7–9 days) consisted of tonic neck-flexion or forelimb rotation, followed by forelimb clonus (Table 1). Further, alternating clonus, prominent in 10–12-day-old pups (as well as in older rats[8]) was infrequent. The behavioral seizures observed after CRH closely resembled the sequence of kindling stages (Table 2), suggesting common propagation pathways.

Inter-animal variability was greater in 7–9-day-old rats than in older pups (Table 3); the number of stimulations required for each behavioral stage differed substantially. Overall, 7–8-day-old animals progressed faster to stage 3 (4.94 ± 0.5 stimulations; n =18) than

Table 2. Comparison of CRH- and kindling-induced behavioral stages in infant rats

	Behavior	
Stage	Kindling	CRH Seizures
0	Behavior arrest	—
1	Head bob, facial movement	Jaw myoclonus
2	"Chewing"/neck flexion	Licking, chewing
3	Unilateral clonus	Forepaw clonus
3.5	Alternating clonus	"Swimming"
4	Limb rotation, bilateral clonus	—
5	Loss of balance, extension	Loss of balance

Table 3. Rate of kindling in infant rats per number of stimulations for each stage of kindling

Age (days)	n	Behavioral stage				
		1	2	3	4[#]	5
7–8	18	1.3 ± 0.1	2.9 ± 0.4	4.9 ± 0.5	8.2 ± 0.7	11.7 ± 0.6
9–10	15	1.3 ± 0.1	2.3 ± 0.3	5.8 ± 0.6	11.3 ± 1.0[*]	17.2 ± 0.7[*]
12	8	1.1 ± 0.1	3.1 ± 0.5	5.6 ± 0.8	11.5 ± 1.1[*]	20.4 ± 0.5[*]

Values are mean ± s.e.m. *Significantly different than 7–8 days ($p < 0.005$). [#]Stage 3.5 or stage 4 in the 7–8-day-old group.

10–12-day-old pups (5.7 ± 0.6 stimulations: n = 23). Kindling rate of the younger group to behavioral stage 4 was rapid; a mean of 8.2 stimulations at 7–8 days versus 11.5 stimulations in 12-day-old pups ($p < 0.005$, and Table 3).

The current of 400 μA was above threshold for the 9–12-day-old rats, and resulted in after-discharges. In younger rats, using a longer train duration (suggested by E. W. Lothman, personal communication) yielded discernible afterdischarges in most pups, though with a more variable contour (Figure 1). 9-day-old rats tended to have longer after discharge duration after each stimulation than 12-day-old ones (Figure 1).

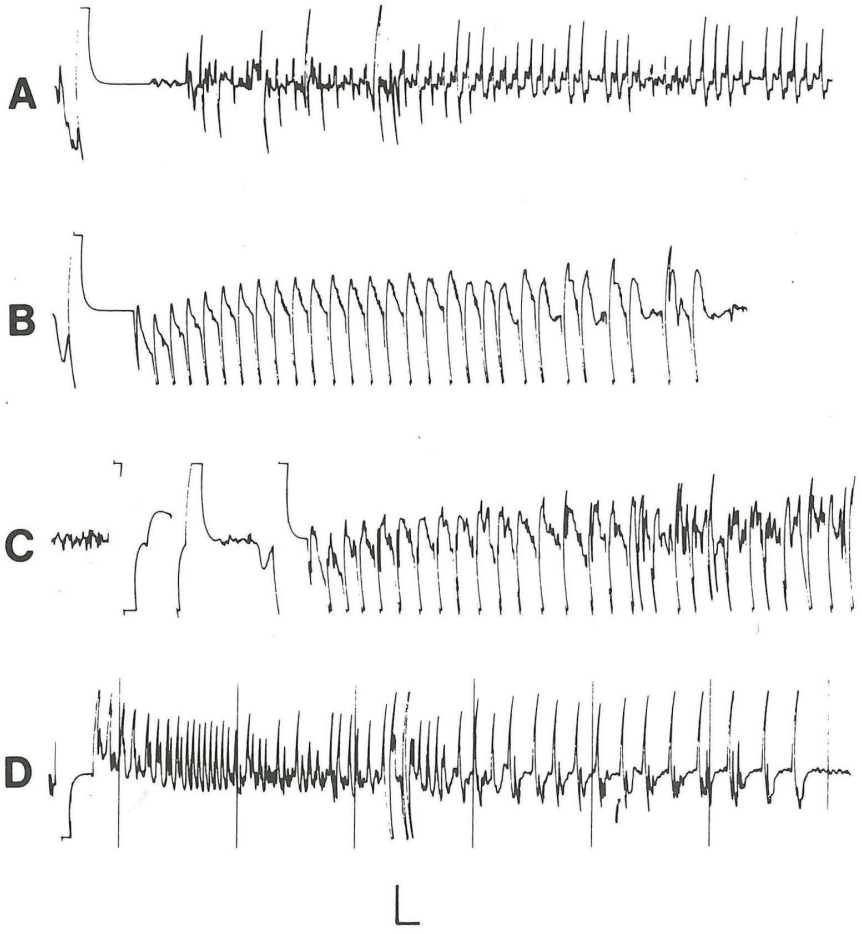

Figure 1. Afterdischarges in 7–12-day-old infant rats subjected to short-interval stimulation. A. 7-day-old rat, subsequent to the fifth stimulation. B. 9-day-old rat, fifth stimulation. C. 10-day-old rat, 19th stimulation. D. 12-day-old rat, 17th stimulation. Horizontal bar = 1 sec. Vertical bar = 10 uV in A, 20 uV in B, C, 40 uV in D. (From ref 29, with permission.).

Table 4. "Spontaneous" seizures during kindling in infant rats

Age (days)	Number of rats with seizures (%)	Median stimulation number at onset (range)
7–8	9/18 (50)	11 (5–12)
9–10	7/14 (50)	17 (15–19)
12	2/8 (25)	19,19

From ref. 29, with permission.

Spontaneous seizures were observed with an earlier onset and a higher incidence in the youngest animals (Table 4). The incidence of such seizures was 50% in 7–8-day-old animals. They occurred subsequent to kindling stage 3.5–5, and persisted until the termination of the experiment (2–3 hours).

None of the manipulations of CRH-induced neurotransmission, either chronically or acutely, altered the rate of kindling development. For example, pre-treatment with CRH immediately prior to kindling did not decrease the number of stimulations needed for achievement of afterdischarge duration of 60 seconds (7 stimulations in both control and experimental groups). Further, pre-treatment with CRH antagonist did not suppress the expression of kindled seizures: i.e., when given after afterdischarge duration reached 60 seconds, the antagonist did not prevent further increases in afterdischarge duration, or the achievement of stage 5 seizures. The number of experimental animals per group was too small to entirely exclude the possibility of a small (10–25%) effect of CRH or the antagonist on the parameters measured. The significance of such potential, relatively low-magnitude effects in view of substantial inter-animal variability in the rate of kindling in the developing rat, is questionable.

5. DISCUSSION

These studies demonstrate that the rapid kindling paradigm can be used in the infant rat, during the second postnatal week. The model is a powerful tool with several advan-

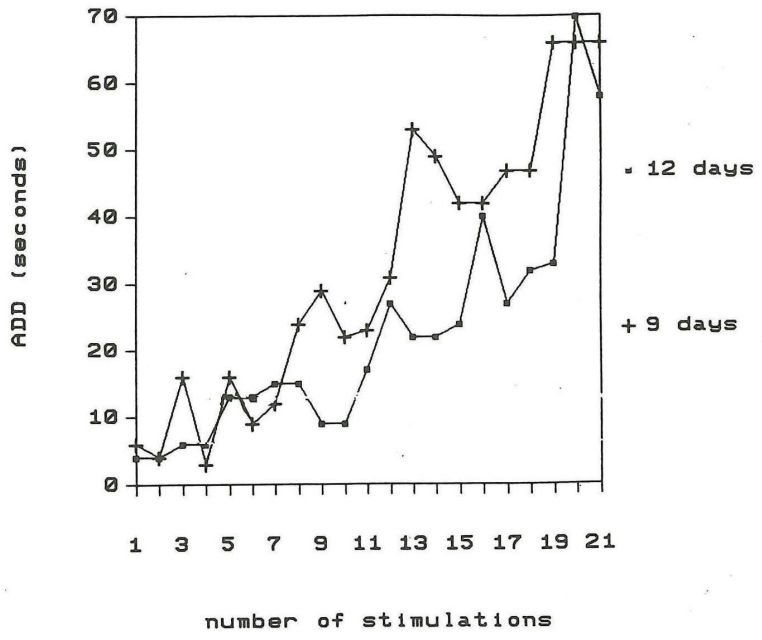

Figure 2. Correlation of afterdischarge duration and stimulation number in 9- and 12-day-old infant rats. See text for details of kindling paradigm (from ref. 29, with permission).

tages: 1) It is a measure of susceptibility to seizure generation at a specific, single age-point, which is of paramount importance in studying rapidly developing infant rats. 2) It provides a well characterized, reproducible model, with several quantitative parameters, permitting comparison of control and experimental groups. 3) Amygdala kindling relies on limbic mechanisms of seizure propagation—which is useful for studies of limbic or "temporal lobe epilepsy" models during development. 4) The paradigm is also age-specific, and is thus attractive for study of age-specific convulsants with overlapping neuroanatomic substrates, such as CRH-induced seizures.

Both amygdala[5,6,8,32,33] and hippocampal[7,34] rapid kindling paradigms have been well characterized in "suckling" or "weanling" rats (older than 15 days). Haas et al. devised a behavioral scale to account for the different progression of stimulation induced seizures in the immature brain[8]. Amygdala kindling has been used to assess the effect of anatomical, metabolic and hormonal[35] inputs on seizure susceptibility in the immature rat[4,32,33]. We demonstrate that this experimental model can be extended to rats during the second postnatal week (infant rats). Gilbert and Cain[10] were unable to induce amygdala kindling in 10-day-old rats, and only "weak stage 3" response in 14-day-old rats. The authors used kindling parameters similar to ours, but applied stimuli at 2 hour intervals, which may account for their results. Further, the afterdischarge threshold is higher in younger rats[5]. Eliciting afterdischarges at this age depends on a composite of the magnitude and the duration of the stimulating current (unpublished observations, and E.W. Lothman, personal communication). We utilized a current of 400 μA, which is suprathreshold for the 15-day-old rat. The ability to obtain afterdischarges in 7–8-day-old was enhanced by increasing train duration to 3–4 seconds. These pups progressed to stage 5 seizures.

Fully kindled rats (subsequent to three or more stage 5 seizures) may develop spontaneous seizures[9]. The likelihood of such events increases with the number of stimulations[36]. Spontaneous seizures occurred with high frequency in the kindled infant rats, and required relatively few stimulations. Furthermore, even within the limited spectrum of ages studied, the frequency of spontaneous seizures correlated inversely to age. This is consistent with an increased excitability and susceptibility to seizures of the immature brain[4,6,11–13,33].

The infant rapid amygdala kindling process, as opposed to amygdala kindling in the adult, was not influenced by alteration of CRH-mediated neurotransmission. Ehlers[20] described the long latency to the onset of seizures induced by CRH in mature rodents. A single administration of CRH produced a sequence of behaviors similar to the behavioral stages of kindling. It was hypothesized that CRH could be a kindling stimulus for the development of limbic seizures[20,21,28]. Weiss et al., using mature male Sprague-Dawley rats, studied the effect of CRH on the development of kindling[21]. Large doses of CRH were given daily for five days, followed by daily electrical kindling. On the first two days, CRH resulted in behavioral seizures and in afterdischarge-like activity on amygdala EEG. CRH effect diminished after the third and fourth injections, and no seizures occurred after the fifth. Pre-administration of CRH, however, significantly accelerated the development of stage 3 seizures (after 8.2 stimulations versus 16.1 in vehicle-treated rats). Afterdischarge duration throughout the kindling process was significantly longer in CRH pre-treated rats. The data were interpreted to suggest a role for endogenous CRH in limbic excitability, and a mechanistic interaction with the kindling process.

In the infant, administration of a single, low dose (e.g., 0.15 nanomole) of CRH results in limbic seizures with behavioral progression similar to the stages of kindling[24,25] (Table 2). It was therefore not possible to study the alteration of behavioral stages of rapid kindling by pre-administration of CRH. However, no effect of CRH on afterdischarge du-

ration was evident. Blocking of CRH receptors with doses of alpha-helical CRH which are sufficient to prevent CRH-induced seizures, altered neither the behavioral nor the EEG (afterdischarge duration) aspects of kindling development. Although lack of effect does not exclude a synergistic interaction between rapid kindling and CRH in the infant rat, our data do not indicate the presence of such interaction.

The large doses of CRH administered in adult rat studies can be expected to act peripherally, on CRH receptors in the pituitary[15,16], elevating the levels of plasma ACTH and glucocorticoids. Glucocorticoids clearly augment kindling development: Indeed, the Lewis rat strain which is deficient in CRH production kindles more slowly, an effect largely reversible by corticosterone[28]. Therefore, the apparent interaction of CRH and kindling in the adult may be due to elevated plasma glucocorticoids[21,28]. The low doses of CRH producing seizures in the infant rat do not increase plasma cortcosterone[24]. Seizures, including those produced by CRH, are stressful, and elevate plasma corticosterone transiently. Glucocorticoid receptors are present in the amygdala and hippocampus of the infant rat[37], yet, as discussed above, neither CRH nor a CRH receptor blocker altered the rate of kindling development.

In summary, rapid electrical amygdala kindling over several hours is possible during the second postnatal week in the rat. The short refractory period and the high frequency of "spontaneous" seizures are consistent with enhanced excitability of limbic circuitry during this developmental stage. Brain development in the 7–12-day-old rat roughly corresponds to that of the newborn and infant human. This experimental paradigm should thus permit further study of mechanisms of epilepsy development during this critical and highly vulnerable age.

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