Kindling, Unit Discharge Patterns and Neural Plasticity

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SUMMARY: *Two approaches to the study of the kindling phenomenon were discussed: 1) an attempt to identify the pattern of neural activity required to produce the changes underlying kindling and 2) an investigation into the nature of those changes. Three experiments were reported that used the neocortical transcallosal system as a monosynaptic model system in which to study possible synaptic mechanisms of the kindling effect. Experiment I showed an increase in the transcallosal evoked potential following neocortical kindling. Experiment II showed an increase in the strength of the transcallosal evoked cell discharge following neocortical kindling. Experiment III reported the results of an histological examination of neocortical tissue in kindled and non-kindled animals using the Golgi-Cox technique. Spine density, spine dimension and branching were measured for pyramidal cell apical dendrites. No differences were found between primary and secondary (contralateral) foci or between kindled and non-kindled animals.*

RÉSUMÉ: *Deux approches à l'étude du phénomène de "kindling" sont présentées: 1) une étude du pattern d'activité neurale requis pour la production des modifications présentes dans le "kindling"; 2) une investigation de la nature de ces modifications. Trois expériences relatées utilisent le système transcalleux neocortical comme modèle monosynaptique de l'effet "kindling". Ces expériences indiquent une augmentation du potential évoqué transcalleux après "kindling" neocortical, une augmentation dans la force de la décharge cellulaire évoquée de façon transcalleuse par kindling neocortical et un étude histologique par technique Golgi-Cox du tissu neocortical d'animaux ayant reçu, ou non, le kindling. Aucune différence ne fut mise en évidence en ce qui concerne la densité, la dimension ou les ebranchements des épines dendritiques apicales des cellules pyramidales.*

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This work was supported by NRC grant A8629.

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INTRODUCTION

Except for neural development and learning, the kindling phenomenon may be the most robust example of neural plasticity in the mammalian nervous system. The changes in brain function produced by repeated electrical stimulation are numerous, easily detectable, and appear to be permanent (Goddard et al., 1969; Racine, 1972a, b). Among the electrographic and behavioral changes produced by the kindling treatment are reductions in epileptiform afterdischarge (AD) threshold (Racine, 1972a; Tress and Herberg, 1972) increases in AD duration (Delgado and Sevillano, 1961; Racine, 1972b; Tanaka, 1972; Morrell, 1973), increases in primary and secondary foci AD spike amplitudes (Racine, 1972b; Racine, 1975) increases in the complexity of the AD spike waveform (Racine, 1972b), increases in the AD spike frequency (Racine, 1972b), and the development of convulsive responses (Delgado and Sevillano, 1961; Goddard et al., 1969; Racine, 1972b; Morrell, 1973; Racine, 1975). Other changes in behavior can also be produced by repeated electrical stimulation of the amygdala (Adamec, 1973; also, see Goddard, 1972).

Several experiments have shown that the changes in neural response underlying many of these developments are not restricted to the primary (stimulated) focus. After completion of amygdaloid kindling, for example, fewer stimulations are required in secondary limbic sites to develop generalized seizures (Goddard et al., 1969; Burnham, 1971; Racine, 1972b), even after removal of the "kindled" primary focus (Racine, 1972b). These "transfer" experiments established that transsynaptic changes in neural response were developing as a result of the

kindling treatment. It has not yet been determined, however, that the kindling effect is a result of changes at the synaptic level. Nevertheless, in view of the fact that many examples of synaptic plasticity have been demonstrated it seems reasonable at this stage to concentrate on the synapse in our investigation of the kindling phenomenon. There are a number of features of the kindling effect which are consistent with the hypothesis that synaptic transmission is facilitated between neural structures as a result of kindling. We have already mentioned the transfer experiments showing that some type of transsynaptic changes are taking place. The increase in the amplitude of secondary site AD spikes also suggests a possible increase in the strength of synaptic transmission. These secondary site spikes are initially clearly evoked by the efferent volleys originating from the primary focus. Even after completion of kindling, when reactive self-sustaining discharges are being recruited in some of the secondary foci, the first few seconds of secondary site AD presumably consist of evoked spikes.

If there is a permanent change in synaptic transmission then it should affect any activity involving those synapses, including non-epileptiform activity. It was demonstrated by Racine et al. (1972) that potentials evoked in secondary sites by test pulses applied to the primary (kindled) focus are increased in amplitude following kindling. All components of potentials evoked in the hippocampus, preoptic area, ventromedial nucleus of the hypothalamus and frontal pole by amygdala stimulation were increased in amplitude following amygdaloid kindling. The late components were most strongly affected suggesting, again, a synaptic mechanism. Subsequent experiments (Racine, unpublished) have shown that the same changes are produced in the responses (including monosynaptic responses) triggered by test pulses applied to the pathways between the primary and secondary foci after kindling in the primary focus. These findings would seem to eliminate the possibility that the increase in amplitude of responses evoked in the secondary site is simply due to an increase in output from the primary focus. On the other hand, responses evoked within the pathways between primary and secondary foci by primary focus stimulation are also increased in amplitude after kindling. This finding suggests an increase in output from the primary focus. We are now trying to determine, more precisely, the relative magnitude of these EP amplitude increases. The possibility that cholinergic circuits are involved in the kindling effect is indicated by the fact that atropine retards amygdaloid kindling (Arnold et al., 1973). It is not yet known, however, if the neural changes that underlie amygdaloid kindling take place within cholinergic circuits or if these circuits simply play a supportive role in seizure development.

If changes in synaptic transmission underlie the kindling phenomenon, and if, as we hope, these changes may also underlie other more normal physiological processes (e.g. learning), then it is important to consider the pattern of activity required to produce these changes. Racine (1972b) determined that it was necessary to trigger epileptiform discharges in order to develop most of the kindling effects. Stimulation that was below AD threshold did not appear to facilitate subsequent kindling with suprathreshold stimulation. If ADs were required to produce the kindling effects then we felt it was necessary to determine the pattern of cell discharge which occurred during an AD. There was considerable data in the literature dealing with this question (see, for example, Jasper et al., 1969). We were dealing primarily with amygdaloid kindling, however, a structure and a phenomenon not well studied at the unit level, so we felt that it was necessary to determine the nature of the unit response within the amygdala during an AD triggered by stimulation of the amygdala. We have been conducting a series of single cell recording experiments in both subcortical and cortical primary and secondary foci, but our initial observations were from amygdaloid primary foci. These experiments indicated that the primary site unit responses, during the first few discharges, consisted of 50 msec. bursts of action potentials with an intra-burst frequency of about 300/sec. and a burst frequency of about 2/sec. The burst frequency and the intra-burst action potential frequency increased with repeated stimulation. These experiments are not complete but it appears that the intra-burst frequency moves toward 500-750/sec., the burst duration towards 75 msec., and the burst frequency towards 4/sec.

We used the discharge pattern obtained during the initial experiments (50 msec. 300/sec.) to establish the parameters for a stimulation experiment. There was no guarantee, of course, that stimulation which mimicked the single cell discharge pattern would in turn trigger similar discharges when applied to the amygdala, but the results were encouraging. We found that the AD-mimicking pattern of stimulation produced a very strong recruiting response in secondary sites when applied to the amygdala and was effective in the triggering of "first trial" generalized convulsions if run continuously for several minutes (Racine et al., 1973). Subsequently, we have been successful recording cell responses within the stimulated focus as the stimulation is applied to that focus and we have seen several cells following the 300/sec. stimulation (at least one action potential between each stimulus artifact), so at least some of the cells are responding appropriately to the stimulation. If one may generalize from the stimulation pattern back to the AD pattern on which it was based, then one of the striking features of an epileptiform discharge is that it is ideally suited for the production of a strong potentiation effect in secondary foci. It seemed a likely possibility that the potentiation characteristics of amygdaloid ADs might be critical to the kindling effect in view of recent findings of Bliss and Gardner-Medwin (1972) that long term potentiation can be produced in

https://doi.org/10.1017/S0317167100020540 Published online by Cambridge University Press

the dentate gyrus by repeated tetanic (potentiating) stimulation of the perforant pathway. Bliss and Gardner-Medwin measured the potential evoked within the granule cell layer by test pulses applied to the perforant path. These potentials were increased in amplitude for long periods of time following repeated tetanization of the perforant path.

Consequently, we ran several experiments in an attempt to determine the importance of potentiation effects in the kindling phenomenon (Racine et al., 1975). Groups of animals were stimulated in the amygdala with various patterns of stimulation which did or did not trigger potentiated responses. These patterns included recruiting stimulation (10/sec.) and higher frequency "tetanic" stimulation (300/sec.) The stimulation was spaced and repeated. We found that preexposure to stimulation patterns producing a large potentiation effect facilitated subsequent kindling and produced a permanent increase in the amplitude of test potentials evoked in secondary sites by single pulses applied to the primary site. Stimulation patterns which resulted in the same amount of total stimulation, but did not produce potentiation effects, had no effect on subsequent kindling or on test evoked responses. If the single cell discharges underlying the potentiated responses are examined, high frequency bursts of action potentials, not unlike those found during epileptiform discharges, are found (Racine, unpublished observations).

Most of this work has been done on amygdaloid kindling, which probably serves as a good model for most limbic kindling. One of the difficulties presented by amygdaloid kindling, however, is that it is difficult to identify a monosynaptic system which would facilitate investigation of synaptic alterations. The amygdalo-fugal tracts are either somewhat difficult to isolate from adjacent pathways (e.g. the stria terminalis) or diffuse (e.g. the amygdalo-fugal pathway), and the terminations of those fibers have not yet been precisely determined. In an attempt to develop an experimental preparation that provided some of the features we were looking for, we began to look at the neocortical transcallosal system. The neocortex shows a different pattern of kindling than most limbic structures. Weak motor seizures accompany the first AD evoked in the anterior neocortex. These convulsive responses increase in strength with cortical kindling, but they always appear different from subcortically triggered convulsions (Racine, 1975), at least until generalization to subcortical areas is achieved (Burnham, 1971). Whereas kindled convulsions triggered by amygdala stimulation usually involve forelimb clonus and rearing, neocortically triggered convulsions usually involve a response approaching an extension with the animal flat on his back or side. Forelimbs may be undergoing an extension although more frequently they will still be showing clonus. The neocortical discharges tend to remain short even after very strong convulsions have developed (Racine, 1975). They will become long, however, after generalization to subcortical structures. Generalization to subcortical structures can be facilitated by prekindling of subcortical structures (Burnham, 1971), or by increasing the duration of kindling stimulation, the intensity of kindling stimulations, the interval between kindling stimulations, or the number of kindling stimulations (Burnham and Racine, in preparation).

Neocortical areas, then, also demonstrate the kindling effect. Several features of the neocortical system made it ideal, from our point of view, for the study of kindling mechanisms: 1) The transcallosal response is monosynaptic, strong, remarkably uniform from animal to animal, (Racine, personal observation; Curtis, 1940), the pathway is large and readily accessible, and the terminations easily identified (Heimer et al., 1967). 2) The lack of growth in duration of the neocortical AD suggests a less mobile epileptic pacemaker providing, perhaps, a simpler seizure system with which to work. 3) Although the neocortical AD does not show much growth in duration, the increase in amplitude of AD spikes in the contralateral cortex is as great as that found in secondary limbic sites after limbic kindling if only the first 10 sec. of discharge is analyzed (Racine, 1975). 4) Although it is now clear that subcortical areas are involved in learning, historically the neocortex has always been the structure of choice when investigating more complex forms of learning. 5) Many experiments have been published demonstrating structural plasticity in the neocortex (Valverde, 1967, 1971; Cragg, 1967; West and Greenough, 1972; Møllgaard et al., 1971; Greenough and Volkmar, 1973; Ruttledge, 1974).

The experiments reported below examine the effect of neocortical kindling on the transcallosal response and describe our first attempts to determine whether or not structural changes are produced in cortical cells by the kindling treatment.

Experiment I

As described above, the monosynaptic transcallosal potential evoked in an anterior neocortical area by stimuli applied to the homologous contralateral site provides a suitable response with which to investigate changes in neural function. In the following experiment we measured changes in the transcortical response, in both directions, following neocortical kindling. This experiment is a second replication and each of the 2 preceding experiments (unpublished) yielded significant increases in the transcallosal response as a result of cortical kindling. There was, however, a confounding variable in the previous experiments. The transcortical response is clearly affected by motor behavior (Fig. 1). It increases in amplitude when the animal is motionless or showing what Vanderwolf (1971) calls "type II" behaviors (eating, drinking, grooming, etc.), and decreases in amplitude when the animal is showing "type I" behavior (walking, rearing, etc.). Similar relationships between behavior and brain activity were reported by Vanderwolf (1971) for spontaneous hippocampal rhythms and by Schwartz-

baum and Kreinick (1973) for the amplitude of visual evoked responses.

The spontaneous motor behavior of kindled vs control animals in our previous experiments on the transcallosal response did not appear to be different, but we had not monitored the behavior very closely. The following experiment is a replication of the previous experiments except that motor behavior was closely monitored during the sessions in which the transcortical evoked responses were measured.

METHOD

Twelve male hooded rats, 275-325 gms. from Canadian Breeding Farms, St. Constant, Quebec, were used in this experiment. Twisted bipolar stimulating/recording electrodes made from .01 in teflon coated nichrome wire were implanted bilaterally into area 2 (Krief, 1946) of the neocortex. Coordinates for this and following experiments were 1.0 mm anterior to Bregma, 3.5 mm lateral to the midline, and 2.3 mm below the surface of the skull. All subjects were handled for 4 days following surgery. On day 5, biphasic 0.5 msec. square wave pulses were applied to the right cortex at a frequency of 0.5/sec. while the potentials evoked in the contralateral cortex were amplified and recorded on magnetic tape. Fifty potentials were evoked, recorded and subsequently averaged with the aid of a PDP 8e computer. Identical

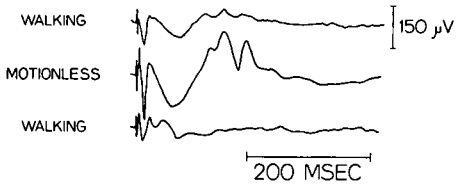

Figure 1—The effect of behavior on the amplitude of the transcortical evoked potential. Stimulation pulses (50) were applied to neocortical area 2 in the right hemisphere while the potentials evoked in the homologous contralateral site were recorded and averaged. Potentials were measured during a period when the animal was walking almost continuously and then during a period when the animal was sitting still and again when the animal was walking.

pulses were then applied to the left cortex while potentials evoked in the right cortex were recorded. A description of ongoing motor behavior was also recorded for each animal during each session.

Six of these rats were then randomly selected to be kindled by stimulation applied to the right cortex while the remaining 6 control rats received identical treatment (e.g. stimulating/recording leads were attached) except that stimulation was not applied. Kindling stimulation consisted of 2 sec. of biphasic one msec. pulses at a frequency of 60 cps. Thresholds for AD were roughly determined by adding 100 μ A to the starting current of 100 μ A and reapplying the stimulation every 2 min. until an AD was triggered. If AD was triggered at 100 μ A then that current level was used for stimulation; in other cases stimulation intensity was adjusted to 25% above threshold. Kindling stimulations were then applied once every 48 hrs. for a total of 20 stimulations.

Evoked potentials were remeasured in all rats 48 hrs. after the 20th stimulation.

RESULTS

The electrographic and motor seizure responses, evoked by cortical stimulation, developed as described by Racine (1975). Motor responses during the initial discharges consisted of weak head movements or weak forelimb clonus. As the convulsive responses became stronger, the rats began to show some body movements. The head and trunk would typically turn slightly to the right and rotate clockwise. This response became stronger until the subjects were in a prone position. At this point there was considerable variability in the form of the response. Some rats showed a response approaching tonic extension while others showed partial extension of the upper torso while still showing forelimb clonus and, in some cases, hindlimb clonus. The electrographic developments were also as previously described. The initial AD durations ranged from 2 sec. to 10 sec. with a mean of 6.5 sec., while the final durations ranged from 8 sec. to 19 sec. with a mean of 11.2 sec. AD spike amplitudes also increased, particularly in the contralateral cortex. Initial spike amplitudes in the contralateral cortex ranged from 90 μ V to 975 μ V with a mean of 357 μ V, while the final amplitudes ranged from 600 μ V to 1575 μ V with a mean of 1095 μ V.

As expected, cortical kindling also produced a significant ($p < .01$) increase in the amplitude of potentials evoked in the contralateral cortex by test pulses applied to the primary focus. The evoked potential changes are illustrated in Fig. 2. Potentials evoked in the primary focus by stimulation of the secondary focus were also significantly altered when compared to control rats ($p < .01$).

All components of the secondary site EP, including the monosynaptic transcallosal response, were clearly altered (Fig. 2).

As described above, in the 2 pilot experiments that preceded this experiment we noticed that there was a relationship between the amplitude of the late components of the cortical EP and motor behavior. When the subject was quiet or immobile the amplitudes were large, and when the subject was active, particularly when walking, the amplitudes were small. In the present experiment we attempted to run the EP test during similar activity states and to record the activity as the potentials were being recorded. Also, the control subjects were treated in exactly the same way as experimental subjects during the kindling treatment (except that no stimulation was applied) so the same amount of handling was received by all subjects. Nevertheless, there was a difference in behavior between the kindled and non-kindled rats. The kindled rats were, in fact, more active than control animals which should tend to make the transcortical responses smaller rather than larger. We ran a second series of evoked potentials on the kindled animals during periods when they were relatively quiet. As expected, the already large amplitude transcortical potentials in the kindled animals again were increased in amplitude. It must be kept

in mind, however, that the experimental subjects were stimulated and this treatment may have led to a number of conditioned alterations in the central state of the rat when placed in the experimental situation. These changes in central state (arousal, etc.), rather than the changes in neural function underlying kindling, could have caused the changes in the amplitude of evoked responses. This is a control problem which confronts many studies of this type. The transcallosal response, however, appeared to be less sensitive to changes in behavior than the late components and we believe that the changes produced in that response are truly related to the kindling treatment.

Experiment II

The following experiment was designed to determine the changes in the transcallosal single cell response following neocortical kindling.

METHOD

Sixteen male hooded rats, 300-350 gms, were used in this experiment. Bipolar stimulating electrodes were implanted into area 2 of the right neocortex. The electrode wire and coordinates were the same as in Experiment I. The skull overlying the homologous site in the contralateral hemisphere was covered with bone wax and left exposed via an access hole in the acrylic head assembly. A screw inserted posterior to Lambda served as ground while a screw over the olfactory bulb served as a reference electrode. Holes were also placed in the acrylic head assembly to serve later as anchor points for the ear bars. Half the rats were randomly selected to serve as experimental subjects and were kindled in the cortex. Kindling procedures were the same as those used in Experiment I. The remaining subjects served as controls and were only handled. Forty-eight hrs. following the completion of the kindling (or handling) treatments the animals were paralyzed with an intraperitoneal injection of 4 mg/kg succinylcholine. They were then placed in the stereotaxic apparatus, with the earbars inserted into the prepared holes in the acrylic cap, and respirated via a face mask (Roberts and Wright, 1974). A modified tooth bar, which was inserted from the side rather than the front, insured the correct head angle. Paralysis was maintained with a continuous infusion of .0035 ml/min. of succinylcholine into the leg muscle. Heart rate and body temperature were monitored continuously.

The bone wax was removed from the exposed skull and predrilled hole overlying the contralateral cortex. The dura was carefully removed, and a sodium chloride (90% saturated) filled micropipette, with a 2-3 μ inside tip diameter, was lowered into the cortex until a stable cell response was found. Biphasic 0.2 msec. square wave pulses at an intensity of 800 μ A peak to peak were then applied to the right neocortical area 2. Responses evoked in the contralateral site were split into low frequency (field potentials) and high frequency (action potentials) components and recorded on magnetic tape. Fifty responses were evoked and recorded for each cell.

Post stimulus histograms were constructed for each cell. In addition, individual responses were photographed from an oscilloscope screen. The responses evoked in kindled subjects were then compared to those evoked in non-kindled subjects. Particular attention was paid to the transcallosal response which occurred within the first 0-15 msec. after the stimulus.

RESULTS

One of the experimental animals pulled off his electrode assembly, one failed to show seizure responses and in 3 of the 6 remaining experimental animals we were not able to obtain cell responses. This left 3 experimental animals from which we were able to record responses from 17 cells. A total of 52 cells were sampled from the control subjects. Even though the experimental group was small, the transcallosal responses evoked in the kindled animals were significantly stronger than those evoked in the control animals

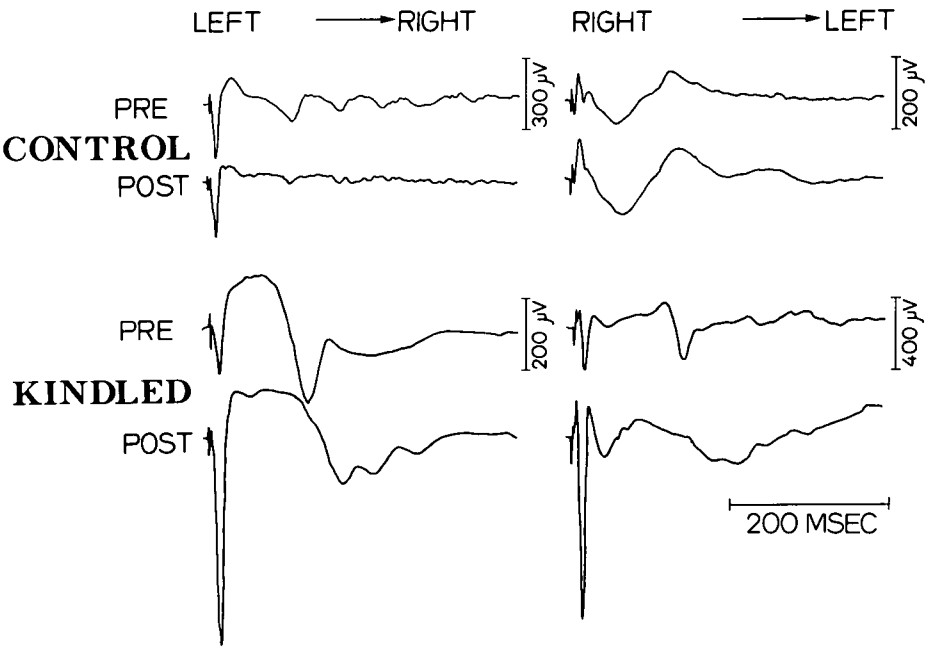

Figure 2—The effect of kindling on the transcortical evoked potential. Potentials were recorded in area 2 of the right hemisphere (primary focus) as stimulation pulses were applied to the left hemisphere (LEFT→RIGHT) and in the left hemisphere as stimulation pulses were applied to area 2 of the right hemisphere (RIGHT→LEFT). The responses were measured before (PRE) and after (POST) a 20 day period of handling (CONTROL) or a 20 day period of kindling (KINDLED).

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($p < .01$; Fig. 3). The number of action potentials accompanying the late components (150 to 450 msec.) did not appear to be increased in the kindled preparation. There was, however, a tendency for a cyclic pattern of discharge to appear in the late components of the kindled compared to the non-kindled rats (Fig. 3). Except for the increase in strength of the response in kindled animals, the responses were remarkably consistent from cell to cell and from animal to animal. For all cells recorded, the transcallosal response consisted of an increase in cell firing. This was followed, again in all cells, by 50 to 150 msec. of inhibition followed in turn by an excitatory "rebound". The excitatory rebound sometimes took the form of a brief rhythmic discharge at about 20 burst per sec. (Fig. 3).

Experiment III

Experiments I and II indicate that there may be a facilitation of synaptic transmission between cortical areas resulting in an increase in the strength of the transcallosal evoked potential and evoked cell discharge. An increase in the strength of the synaptic response could be achieved by a number of different mechanisms. The presynaptic terminals, for example, could increase in size, the synaptic contact area could increase, the available transmitter stores could be increased, and so on. Some of these possible mechanisms involve structural changes which might be detectable with the use of currently available histological techniques. There have been many recent demonstrations of structural plasticity in the mammalian central nervous system. Axonal sprouting and synaptic terminal development have been demonstrated after tissue damage (Raisman, 1969; Moore, Björklund and Stenevi, 1974). Reversible reductions in branching and spine density of apical dendrites of striate cortex pyramidal cells have been reported after light deprivation in mice and rats (Coleman and Riesen, 1968; Valverde, 1967, 1971; Scheibel and Scheibel, 1970; Cragg, 1967). Increases in dendrite branching (Holloway, 1966; Grennough and Volkmar, 1973) spine density (Globus et al., 1973), and synaptic contact area (Møllgaard et al., 1971; West and Greenough, 1972) have been reported following exposure to enriched environments. Ruttledge et al. (1974) have recently reported that repeated electrical stimulation of the suprasylvian gyrus in the cat resulted in an increase in the branching and spine density of layer 3 pyramidal cell dendrites in the contralateral homologous focus. Although their experiment paired cortical stimulation with foot shock, control animals that received only brain stimulation showed many of the same changes in neural structure. If electrical stimulation of the cortex produced struc-

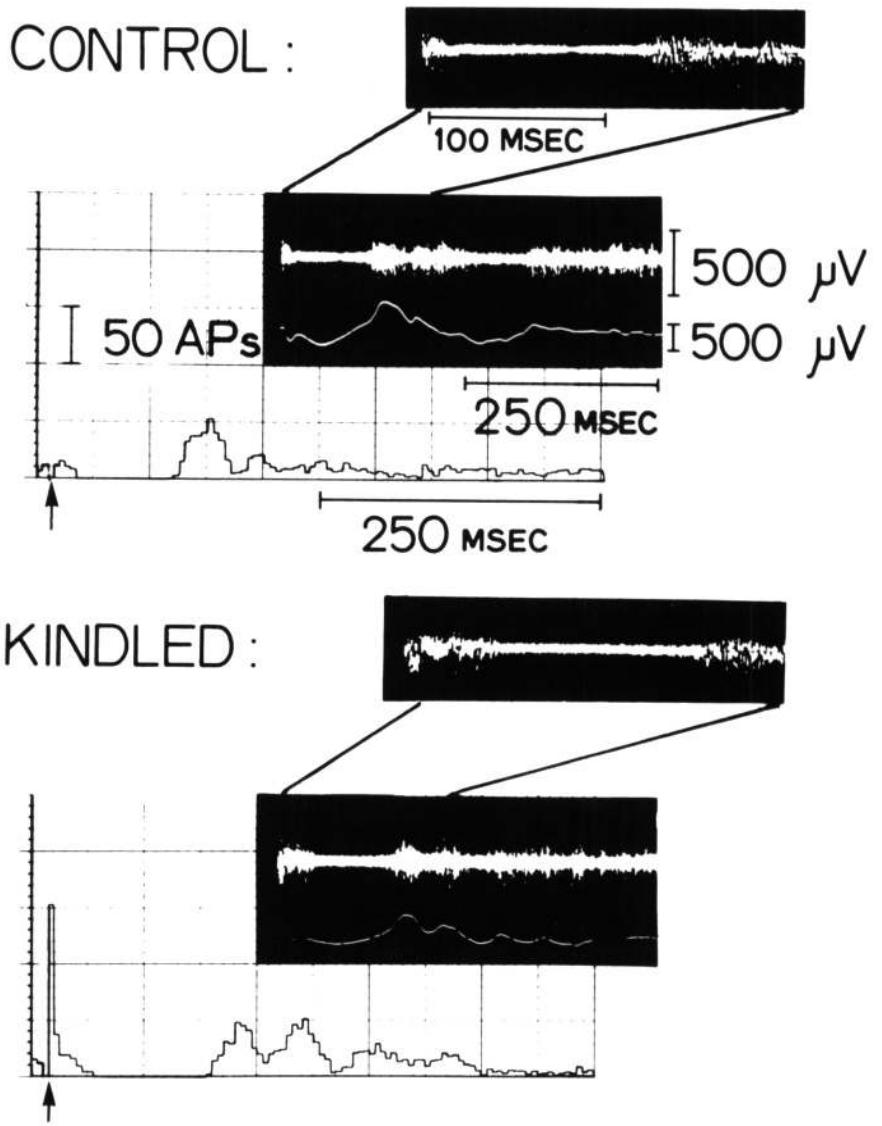

Figure 3—The effect of kindling on the transcortical evoked single cell response. Action potentials were recorded in area 2 of the left hemisphere as stimulation pulses were applied to area 2 of the right hemisphere (primary focus). The responses were measured after a 20 day period of handling (CONTROL) or a 20 day period of kindling (KINDLED). A post-stimulus histogram representing 50 samples is shown for each cell as well as 2 photographs of the response to one stimulus. Each bin represents 5 msec. and the 3rd bin (containing the stimulus artifact) has been erased. One photograph is taken at a slow sweep speed (500 msec.) and the other at a fast sweep speed (200 msec.) in order to illustrate the short and long latency responses. The field potential is shown with the slow trace. Both cells were located at 1.5 mm below the surface of the cortex.

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tural changes in the experiments of Ruttledge et al., then it seemed likely that structural changes must also be produced in our cortical kindling preparation (which showed a definite facilitation of the transcallosal response). We decided to use the Golgi-Cox technique to examine cortical cells in kindled as compared to non-kindled rats.

METHOD

Sixteen male hooded rats, 300-350 gms, were used in this experiment. Bipolar electrodes were implanted into the anterior neocortex in both hemispheres. Electrode wire and placements were the same as those used in Experiments I and II. Transcortical evoked responses were first measured in all subjects using the procedures described in Experiment I. Eight animals were then randomly selected to undergo the kindling treatment as described in Experiments I and II. The remaining 8 control animals were handled in exactly the same way as the experimentals except that stimulations were not applied.

Upon completion of 20 trials of stimulation, EPs were remeasured in all animals. The animals were then allowed 2 days without stimulation or handling after which the brains were prepared for histological examination. A modified Golgi-Cox technique was used to stain the cells (see appendix I). After embedding in epoxy resin, the tissue was sectioned on a sledge microtome at 120 μ.

Spine density

The slides were coded and all measurements were done without knowledge of the animal or group from which the tissue was taken. Cells were selected from areas immediately medial, lateral, anterior and posterior to the primary and secondary foci electrode tracks. Within these areas both layer 3 and layer 5 pyramidal cells were examined. Eight cells were sampled from each coronal section. In a section through the electrode track, for example, a layer 3 and a layer 5 pyramidal cell were selected from the tissue medial to primary focus track, lateral to the primary focus track, and medial and lateral to the secondary focus track. Spine counts were made on 50 μ lengths of primary oblique dendritic branches of layer 5 apical dendrites that were located at least 250 μ dorsal to the soma. For the layer 3 pyramidal cells the counts were taken primarily from 50 μ lengths of the terminal branches of the apical dendrites. In total, 320 sections were examined resulting in dendritic spine counts from 2,560 cells.

Branching

Branching was measured on layer 3 pyramidal cells medial to each electrode track. A cell was selected within a 250 μ wide region medial to the track and the number of apical dendrite branches on that cell measured. The branches were also recorded as primary, secondary or tertiary. Eight cells were measured

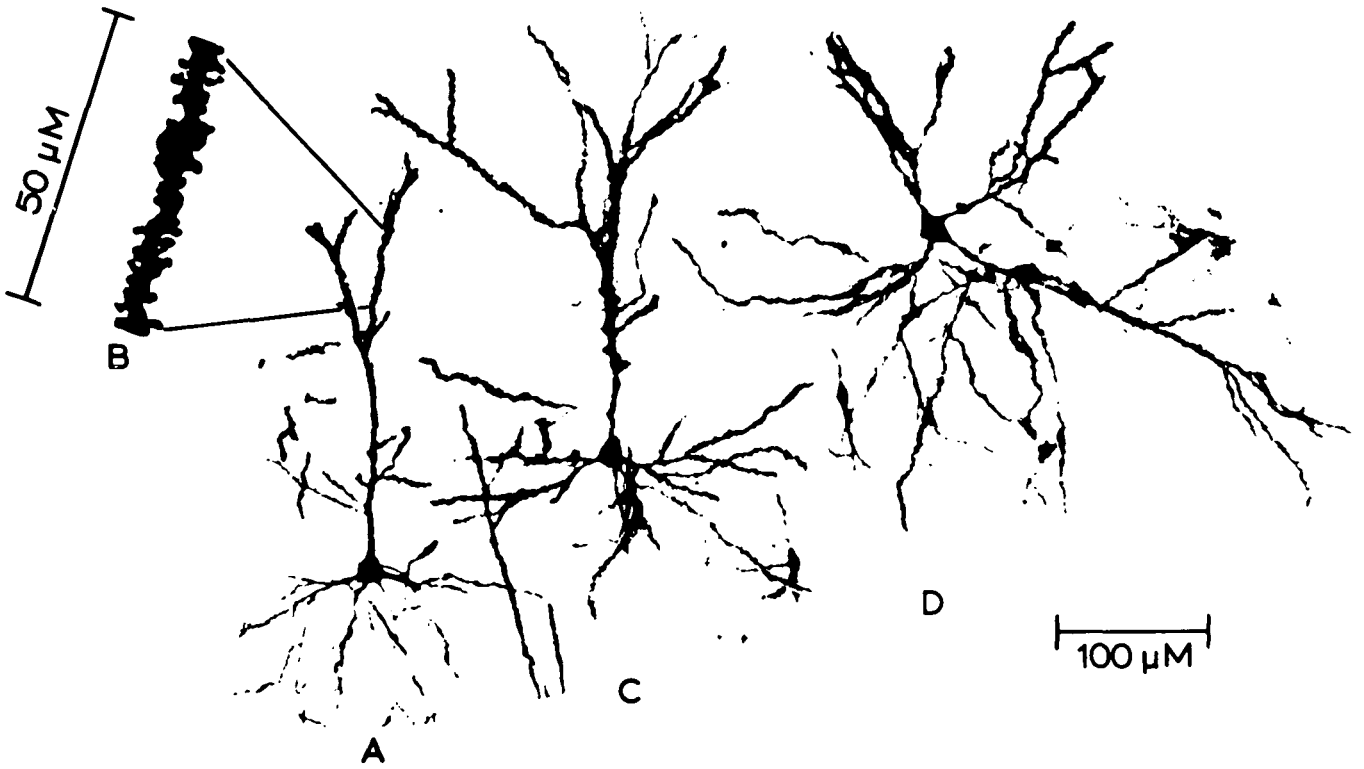

Figure 4—The 3 cell types that were examined most closely in Experiment 3. A: A layer 3 pyramidal cell. B: A 50 μ m segment from a terminal branch of cell "A" showing the dendritic spines. C: A pyramidal cell often seen in the lower half of layer 2 usually more heavily branched than layer 3 cells. D: A large stellate cell found in layer 2.

https://doi.org/10.1017/S0317167100020540 Published online by Cambridge University Press

from each focus for each animal resulting in a total of 256 cells.

Spine dimensions

The length and thickness of the largest spines (those most likely in profile) were measured on terminal branches of layer 3 pyramidal cell dendrites. These cells were selected from the tissue medial to the primary and secondary foci tracks. Eight cells were measured from each focus for each animal, and 2 spines were sampled from each cell, resulting in a total of 256 cells or 512 spines.

Other measures

The slides were then decoded and examined for differences in other characteristics (e.g., general differences in spine dimension not detected with the above measures, extent of branching under pial surface, spine density and branching of layer 2 pyramidal and "star-pyramidal" cells, etc.).

The counts for kindled animals were then compared to those taken in non-kindled animals.

RESULTS

Kindling progressed normally and the EP amplitudes were significantly increased in kindled animals ($p < .01$).

The staining was successful for all animals. The spine staining was somewhat weak for layer 5 cells but was very clear for layer 3 cells (Fig. 4). None of the measures of cell morphology, in fact, showed any differences between kindled and non-kindled subjects. Table 1 shows the range and means for the spine counts, spine dimensions and branching from each area and Fig. 4 shows a typical layer 3 pyramidal cell as well as 2 types of layer 2 cell that were examined. Layer 1 did not usually stain very well. When this region was stained, however, we found no differences between kindled and non-kindled subjects.

DISCUSSION

Much of our work has focused on 2 main themes: 1) What pattern of neural activity is required for producing the neural changes underlying kindling, and 2) what are those changes? Some progress has been made on the first problem and the main findings can be summarized by the following 5 points: 1) the epileptiform discharge must be repeatedly triggered during the course of a standard kindling experiment in order to produce the changes underlying kindling. 2) The unit discharge pattern during epileptiform discharges consist of high frequency bursts. 3) If this unit discharge pattern is mimicked with stimulation parameters that are then applied to the amygdala, a strong potentiation effect is produced in the responses

TABLE I

SPINE DENSITY (50 μ m)

	Primary Focus				Contralateral Focus			
	Anterior	Medial	Lateral	Posterior	Anterior	Medial	Lateral	Posterior
Control $\overline{X}$ Layer 3 r	49.3 46.2 - 51.9	54.5 48.0 - 60.5	52.1 49.9 - 55.2	49.5 46.5 - 57.5	49.1 47.4 - 51.3	52.5 49.1 - 53.5	50.9 48.3 - 51.6	49.6 48.5 - 50.8
Control $\overline{X}$ Layer 5 r	44.4 41.6 - 47.4	45.8 41.9 - 51.6	45.3 43.0 - 46.5	43.6 40.0 - 48.5	44.4 43.1 - 46.0	44.4 41.5 - 47.9	43.5 40.3 - 46.5	42.4 39.8 - 44.2
Kindled $\overline{X}$ Layer 3 r	50.8 48.0 - 54.5	54.1 46.8 - 56.5	51.1 47.6 - 55.0	48.3 45.8 - 49.0	48.2 45.3 - 52.3	53.5 47.3 - 58.6	49.8 46.8 - 51.9	46.4 44.3 - 49.0
Kindled $\overline{X}$ Layer 5 r	46.2 42.3 - 49.5	44.4 38.7 - 46.6	42.8 41.0 - 47.3	45.9 43.6 - 51.1	44.8 41.1 - 51.8	44.4 40.8 - 50.9	42.8 38.3 - 48.0	43.3 36.8 - 46.8

SPINE DIMENSIONS (μ m)

	Primary Focus			Contralateral Focus		
	Length	Stalk width	Head width	Length	Stalk width	Head width
Control $\overline{X}$ Layer 3 r (medial)	2.8 2.6 - 2.9	.38 .22 - .56	1.1 .84 - 1.3	2.8 2.6 - 3.1	.36 .25 - .61	.95 .79 - 1.2
Kindled $\overline{X}$ Layer 3 r (medial)	2.5 2.0 - 3.0	.33 .30 - .37	1.0 .95 - 1.2	2.8 2.5 - 3.0	.33 .24 - .46	.99 .80 - 1.3

BRANCHING

	Primary Focus			Contralateral Focus		
	Primary	Secondary	Tertiary	Primary	Secondary	Tertiary
Control $\overline{X}$ Layer 3 r (medial)	6.2 5.4 - 6.8	2.2 1.5 - 3.3	.35 .13 - .63	6.3 5.9 - 7.0	2.4 1.4 - 3.3	.28 0 - .50
Kindled $\overline{X}$ Layer 3 r (medial)	6.0 5.1 - 7.0	2.7 1.8 - 3.5	.22 0 - .38	6.1 5.5 - 6.5	2.6 1.5 - 3.5	.29 0 - .50

https://doi.org/10.1017/S0317167100020540 Published online by Cambridge University Press

evoked in secondary sites. 4) The cell responses triggered by these potentiating trains of stimulation also consist of high frequency bursts of action potentials. 5) If patterns of stimulation that produce potentiation on responses evoked in secondary sites are repeatedly applied to the amygdala, subsequent kindling will be facilitated and test evoked potentials will be increased in amplitude. These findings suggest that the high frequencies of cell discharge (or the massive depolarization underlying that discharge) triggered during ADs and during potentiating stimulation (e.g., post-tetanic potentiation) may be critical to the kindling effect. We are following up on this hypothesis in our current research.

Less progress has been made in our attempts to determine the nature of the neural changes underlying the kindling effect. In the introduction we reviewed data which supports the hypothesis that synaptic transmission is permanently facilitated between neural structures as a result of the kindling treatment. Unfortunately, several other hypotheses also could explain the data described. One mechanism which will have to be carefully considered is an increase in the output of the cells within the foci affected by kindling. There is no question that there is an increase in output from the primary focus during amygdaloid kindling; we have seen it in many animals. We do not yet know if this increase in output is enough to account for all of the increase in the amplitude of AD spikes evoked in secondary sites. We are now trying to determine whether or not amygdaloid and cortical kindling also results in an increase in output when single pulses are applied to the kindled focus. Preliminary data suggest that there may be such an increase. Also, we have already described experiments in the introduction which show increases in the amplitude of responses evoked within pathways between primary and secondary sites, by primary site stimulation, following kindling. We are currently using the transcallosal system to study the relative importance of increased output (from the primary focus) compared to increased responsiveness within the secondary site. Other hypotheses, of course, could also account for all the data. There could, for example, be a reduction in tonic levels of inhibition. Nevertheless, we intend to continue our search for possible synaptic mechanisms and we hope to eliminate some of the alternative hypotheses in future work.

In the experiments reported in this paper we selected the transcallosal system of the neocortex as a monosynaptic model system in which to investigate the neural changes underlying kindling. We first tested the effects of neocortical kindling on the response evoked in the contralateral homologous focus by test pulses applied to the primary (kindled) focus. We measured both gross potentials (Exp. I) and single cell responses (Exp. II) evoked in the contralateral focus, and we found that kindling increased the strength of these responses. In Experiment III we investigated the possibility that certain structural changes might underly the kindling effect. We measured the branching, spine density and spine dimensions of the apical dendrites of layer 3 and 5 pyramidal cells. On every measure taken, there were clearly no differences between the primary and secondary foci or between kindled and non-kindled animals. We are now analyzing the data from an electron microscopy experiment on cortical tissue in an attempt to evaluate the possible development of smaller magnitude structural changes (e.g., growth of presynaptic terminals). In view of the failure of Goddard (personal communication) to demonstrate such changes after amygdaloid kindling, however, we are not very optimistic about the prospects of finding them in the cortex.

If we assume that gross structural changes are not involved in the kindling effect, and that the kindling mechanism is based on synaptic facilitation, there are still many possible mechanisms to be investigated. It may be worthwhile at this stage to concentrate on the transmitters. There are many techniques available to measure concentrations, activities and distribution of transmitter substances. Another possibility is an increase in the number of receptive sites in the subsynaptic membrane. This mechanism seems less likely in view of the widely held assumption that there is a large safety margin in favor of receptor sites (Paton and Waud, 1967). Nevertheless, it might be worthwhile to use the labelling techniques now available for cholinergic receptor sites (e.g., Azcurra and DeRobertis, 1967) and apply them to kindled and non-kindled preparations. In any case, the use of a monosynaptic preparation, whether it be the callosal system or the perforant path system (Bliss and Gardner-Medwin, 1973; Douglas and Goddard, 1975) or another system, should facilitate the investigation of kindling mechanisms.

DISCUSSION

Dr. Pinel: What degree of kindling did you use in these animals before you did the Golgi analysis? *Dr. Racine:* In all of these cortical experiments I have talked about, all of the animals received 20 days' stimulation, that is, 20 afterdischarges separated by 48 hours. The discharges were evoked by 2 seconds of stimulation. *Dr. Pinel:* We're trying to do a similar kind of study. We have used a different kind of approach which I would like you to comment on. That is, we thought the best way to start to look for changes of this sort, especially after hearing of some of the failures that Dr. Goddard has had in seeing obvious changes, was to carry out the kindling as long as possible. We find, for example, that kindling does not stop when you get a full classified seizure as you describe it. If you keep stimulating you get further changes in the motor seizure pattern which eventually culminate in the development of spontaneous seizures and we are now in the midst of our Golgi analysis, but we have a series of spontaneous animals that we are trying to work at. Maybe from your approach and that of Dr. Goddard, you may not be interested in our preparation as you might view it as being pathological because you might be interested in kindling from being a model of learning. However, you might be able to get some hints from this kind of study as to the kinds of changes that might occur with less extreme forms of kindling. *Dr. Racine:* Yes, that's true, but the point you make that there may be two different preparations is a valid one, I think, and I think it is an important one. I do still like to think of the kindling model as a potential learning model, although I am very interested in epilepsy as well. Your animals, if I remember correctly, are animals that have been stimulated for a period of about seven months. Dr. Burnham mentioned something that I don't think anyone has mentioned before, or at least I haven't heard it: that is, what is the kindling model a model of? In many cases of kindling we have seen several

experiments which show, at least so far, that if there is no lesion there is no damage that is even detectable with an electronmicroscopy. I think the kindling model is a model of the changes that take place outside of the damaged focus. If you have a damaged focus, that is acting essentially like the stimulating electrode that is producing an airtight focus of some kind which is triggering massive discharges in the cells around that focus that in turn evoke volleys which are bombarding these secondary sites. Very likely, that bombarding of secondary sites is producing responses very similar to those that are produced in the kindling phenomenon, so that you have two different things going on: 1) the triggering of abnormal responses in a lesioned area, and 2) the changes produced by that abnormal response in secondary sites. It is possible that if that abnormal response in the secondary site may also be damaged, you may have another mechanism involved there as well. Initially, you may have a response which is based on relatively normal physiological changes, but if that is continued with massive input for a long period of time you may produce degenerative changes and that becomes more like the lesioned sites. *Dr. Goddard:* One of the striking things about the cortex when we are talking about models of learning or epilepsy is the development outside the focus. The modifications that might be expected anatomically or physiologically are that first of all, at least in the rat, and I don't know of any other work in other animals, the posterior cortex does not kindle, whereas the anterior cortex does. In all of the studies on the early rearing conditions of rats the modification of the cells in the visual system make a lot of very well documented plasticity. All of our best evidence of plasticity in the brain comes from the infant visual cortex and yet if you try to stimulate this in the adult you cannot kindle it, which is possibly a problem. All of those experiments also show that there is a critical period. There is something about the visual system which makes it highly plastic early in life and after that it does not change anymore so that if you set up an abnormal environment for the animal you get an abnormal visual cortex and you cannot even reverse it later in life with years of normal visual experience. I would like to know if anybody has tried kindling the posterior cortex in an infant rat or whether or not anybody has other evidence about posterior cortex instability to kindle. *Dr. Morrell:* I think that Dr. Goddard's point about the critical period is very valid and a good one to keep in mind, but the other factor which may bear upon it is simply the density of connections outward. For instance, the primary visual cortex has no callosal connections. It only has connections to the association cortex immediately surrounding it. These have massive connections and it may be that the association cortex is more easily kindled, just as it is much easier to establish a mirror focus in the association cortex. You never see a mirror focus from primary motor cortex or any of the primary sensory fields; it doesn't occur. *Dr. Racine:* I have a comment on Dr. Goddard's original statement. For one thing, it is not quite true that the posterior cortex does not kindle. It is true that you don't get convulsions but if you look at the electrographic response the developments are very similar to those seen in the anterior cortex. You have a slight increase in the duration but you also have an increase in the amplitude of spikes evoked in the contralateral site that are equally large to those evoked in the anterior neocortex. Also, we recently have done a pilot experiment, not a completed experiment but we have enough data to be certain that the effect is real, testing the responses to visual input before and after kindling in the posterior neocortex. These animals are never convulsing animals but they are developing electrographically. The responses to flashes presented to these animals also increased in amplitude after posterior neocortex kindling. *Dr. Burnham:* Once more to Dr. Goddard's point about the posterior cortex. When you say it doesn't kindle, what you mean is that we don't see generalized seizures as a result of kindling it but remember we didn't see generalized seizures, for a long time, when we were stimulating the anterior cortex either. It was probably because we were stimulating it at too short an interval and I'll predict that if we continue working with it we will see generalized seizures from the posterior cortex too. My second point, as to what is kindling good for? Having a certain amount of clinical reference at this point, what I can say is that one thing kindling is good for is it is a marvelously convenient and useful model of focal epilepsy, something which we have been lacking for a long time. What I really wanted to say was this: Dr. Racine has told us essentially that when you stimulate at spaced intervals you don't see potentiation and you don't see changes in evoked potentials. When you stimulate in burst-like series, you do. We have been thinking of kindling as a fairly direct analog of the old fashioned idea that use or disuse of neural circuits determines the stamping-in of pathways. What does this finding that some sorts of use do and some sorts of use don't lead to changes in evoked potentials do to our whole outlook on kindling as a learning analog? Do we feel that it is a bad learning analog, or do we assume that the normal business of the nervous system is done in tetanic-like bursts? *Dr. Racine:* I don't really worry about questions quite that finely tuned about kindling as a learning model. Clearly, the patterns of response triggered during kindling are extremely complex and I am sure that you could probably satisfy almost any pattern you wanted to satisfy if you looked hard enough during the course of a kindling experiment, so the fact that there is a lot of decreased use involved doesn't bother me too much. But you can probably make a case for the pattern, the pattern being very important as well. I really don't worry too much about that at this stage. *Dr. Pisa:* I would like to ask whether the pattern of stimulation that you have to use in order to induce afterdischarge across your kindling session must be exactly the same as you used at the beginning of the session or whether even subtle changes in the pattern of your stimulation, such as frequency or duration perhaps, can affect the rate at which the stimulation in the following does or does not produce the afterdischarges. I am asking this because if the kindling phenomenon is thought of as having some relation to learning one would think that if you change the pattern of stimulation, the rate at which the afterdischarge elicited should change too. *Dr. Racine:* What you have to keep in mind is that it is the discharges which are producing these effects, not simulations. As long as the discharge is triggered then it will progress normally in its own way. It is possible to change the rate or progression of kindling if you alter the discharge and you can do that a number of ways. You can run stimulation concurrently with the discharge and you make the discharge longer. If you kindle with one frequency you can trigger with different frequencies of input. You can see that the optimum frequency for triggering a convulsion in kindled animals is about 60 Hz, maybe a little higher. The probability of triggering a convulsion when you are at or near the threshold is highest, and that frequency drops off to fairly low probabilities at 300 Hz or at 25 Hz and nothing at 10 Hz. There are three curves there. One group of rats was kindled at 25 Hz, one group at 60 Hz, and one group at 150 Hz. There are absolutely no differences whatever. There is no tuning. It could be that if we were to ask the question in a more sophisticated way about what the cells are doing, you might get at your kind of question. There may be something but this is the only direct test that I know of with your question and the answer was certainly a resounding no.

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APPENDIX 1:

Modified Golgi-Cox Technique (For Adult Rats)

1. Lethal dose of Nembutal.
2. Perfuse through the heart with physiological saline.
3. Remove brain and cut into 5 mm. thick coronal slabs.
4. Place on gauze net in freshly made fixing solution* inside 50 ml. dark glass bottles.
5. Store in dark for 50 days at room temperature.

*Fixing Solution

5% potassium dichromate	200 ml.
5% mercuric chloride	200 ml.
5% potassium chromate	160 ml.
distilled water	400 ml.

6. Wash in tap water.
7. Place in impregnating solution** — in 50 ml. bottles for 24 hrs.

**Impregnating Solution

lithium hydroxide	0.5 g.
potassium nitrate	15.0 g.
distilled water	100. ml.

8. Wash in solution of distilled water (500 ml.) and acetic acid (1 ml.) — 3 changes, 6 hrs. each.
9. Wash in abundant tap water — 2 hours (not running water)
10. Dehydrate:

40% alcohol	6 hrs.
60% alcohol	6 hrs.
80% alcohol	6 hrs.
90% alcohol	6 hrs.
absolute alcohol (3 changes)	2 hrs. apart

11. Infiltration:
    propylene oxide (2 changes) 2-3 hrs. each
12. Embedding:
    50 propylene oxide — 50 epoxy solution*** — overnight (15-18 hours.)
    pure epoxy solution — 8-10 hrs.
    pure epoxy solution — block cured in oven at 60°C. overnight.

***Epoxy solution

Epon resin	20 parts (volume)
DDSA	20 parts
MNA	8 parts
DMP-30	0.5 parts
Dibutyl phthalate	15 parts

13. Allow blocks to cool. Then section on sledge microtome at 120 μ.
14. Mount sections in same epoxy mixture on glass slides under cover slips.
15. Cure mounting epoxy in 60°C. oven overnight.

https://doi.org/10.1017/S0317167100020540 Published online by Cambridge University Press