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Benzodiazepine receptor increases after repeated seizures: Evidence for localization to dentate granule cells

(kindling/epilepsy/drug receptor/hippocampal formation/granule cell)

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ABSTRACT **Repeated seizures, whether induced by kindling or electroshock, result in increased numbers of benzodiazepine receptors in hippocampal formation membranes. We sought to determine the cellular constituents containing the receptor increases. Binding studies of microdissected samples localized the receptor increases to fascia dentata. [^{3}H]Flunitrazepam autoradiographic studies showed increases of silver grain density over the granule cell and molecular layers of fascia dentata but not in other regions of hippocampal formation. Destruction of granule cells by colchicine or neonatal x-irradiation was associated with marked decrease of benzodiazepine receptor binding. Together, these results provide evidence for localization of the receptor increases to the somata and dendritic tree of the granule cells. We suggest that this cellular localization may provide a clue to the network of altered neural circuitry underlying amygdala kindling.**

The benzodiazepines are effective anticonvulsants in both animals and man (1, 2). Recent investigations have identified binding sites in brain membranes that likely represent the receptors mediating the pharmacologic action of these drugs (3). We previously showed that repeated kindled ** seizures result in increased numbers of benzodiazepine receptors bilaterally in the hippocampal formation but not in neocortex (6).

The basic mechanisms underlying the kindling phenomenon are obscure (7). The responsible neuronal modifications appear to involve a selected network of neural circuits in multiple brain regions (8, 9). Identification of the precise spatial distribution of the altered neural circuits is essential to understanding the underlying cellular and molecular mechanisms.

The heterogeneous regional distribution of the benzodiazepine receptor increases raised the possibility of a discrete cellular localization within hippocampal formation. We present evidence supporting localization of the benzodiazepine receptor increases to the dentate granule cells. This may provide a clue to the network of altered neural circuitry underlying amygdala kindling.

METHODS

Kindling. Adult male Sprague–Dawley rats (Charles River Breeding Laboratories) weighing at least 200 g were used. A bipolar electrode was stereotaxically implanted in the basolateral nucleus of the right amygdala under pentobarbital anesthesia as described (6). After a postoperative recovery period of at least 7 days, stimulations from a Grass S88 stimulator (400- to 900-μA biphasic square-wave pulses, 1-msec duration, 60 Hz for 1 sec) were administered at daily intervals. Electroencephalograms were recorded before and after each stimulation with a Grass model 78 polygraph. Kindling developed as described (6). Experimental animals experienced 18–23 stimulation-induced seizures. Each animal experienced at least one class 5 seizure consisting of rearing and falling. The number of stimulations required to elicit the first class 5 seizure was 13.1 ± 1.4 (mean ± SEM). Control animals underwent electrode implantation but were not stimulated. Electrode placements were not histologically verified because the stimulated amygdala was used for other experiments.

Electroshock Seizures. These seizures were induced by administering 110 V (60 Hz) for 1 sec through earclips at daily intervals, 5 days per week. These seizures usually consisted of tonic extension of all four limbs followed by clonic contractions and subsequent postictal unresponsiveness. Control animals were connected to earclips but received no current.

Dissection and Membrane Preparation. Paired control and experimental animals were killed by decapitation 24 hr after the last experimental manipulation. After removal by blunt dissection, the hippocampal formations were sectioned at 600-μm intervals with a McIlwain tissue chopper in a plane perpendicular to the longitudinal axis. By using a dissecting microscope and a scalpel, the sections were carefully separated into fascia dentata and hippocampal gyrus. The dentate hilus was included in the hippocampal gyrus sample.

The microdissected samples were pooled separately and homogenized in 1 ml of cold 0.32 M sucrose/1 mM Tris citrate buffer, pH 7.1, in a Teflon and glass homogenizer. The supernatant was removed by centrifugation at 43,500 × *g* for 20 min, and the pellet was suspended in 2 ml of cold 1 mM Tris citrate buffer, pH 7.1, with a Polytron (setting 6 for 30 sec) and then stored at −80°C. The samples were thawed on the day of assay. Aliqouts were removed for protein determination according to the method of Lowry *et al.* (10).

Benzodiazepine Receptor Binding. Binding was measured with the radioligand [^{3}H]flunitrazepam (specific activity, 86.4 Ci per mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear). Specific binding was defined as the difference in total

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Abbreviation: B_{max}, maximal binding value.

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** Kindling is an animal model of epilepsy induced by electrical stimulation of the brain (4, 5). The term kindling refers to the phenomenon whereby repeated periodic initially subconvulsive electrical stimuli induce progressive intensification of stimulus-induced seizure activity, culminating in a generalized clonic motor seizure. Once established, this enhanced sensitivity to electrical stimulation is permanent. The amygdala is a sensitive region in that relatively few stimulation episodes are required to induce kindling.

Table 1. [^{3}H]Flunitrazepam binding in fascia dentata and hippocampal gyrus membranes after repeated seizures

	Fascia dentata	Hippocampal gyrus
Control vs.	551 ± 27	353 ± 6
electroshock	735 ± 33*	336 ± 6
	(n = 7)	(n = 13)
Control vs.	408 ± 24	288 ± 15
kindled	576 ± 35†	317 ± 20
	(n = 21)	(n = 19)

Results are expressed in fmol of [^{3}H]flunitrazepam bound/mg of protein (mean ± SEM). Determinations in fascia dentata of the electroshock-treated group were obtained from the right hippocampal formation of seven pairs of electroshock and control rats; hippocampal gyri (n = 13) were obtained from both left and right hippocampal formation of the seven pairs. Fascia dentata and hippocampal gyri were obtained from both hippocampal formations of 11 pairs of kindled and control rats. Data from the left and right sides of kindled animals were pooled because no significant differences were found between the two. Analysis by Student's t test showed no significant differences between seizure-treated and control animals except where specified.
*$P < 0.005$.
†$P < 0.001$.

[^{3}H]flunitrazepam binding in the absence and presence of 2.5 μM diazepam; it accounted for 80–95% of total binding under the conditions used. The incubation mixture (vol, 1.5 ml) was 50 mM Tris citrate buffer, pH 7.1/0.027% ethanol/2.0 nM [^{3}H]-flunitrazepam containing 50–100 μg of membrane protein. Duplicate assays were conducted in the absence and presence of 2.5 μM diazepam. The reaction was continued for 80 min at 0°C and terminated by vacuum filtration through a Whatman GF/B filter. The filter was washed three times with 5-ml portions of ice-cold 50 mM Tris citrate buffer. The radioactivity on the filter was measured in an Intertechnique scintillation counter as described (6). Because all binding assays could not be performed on the same day, control and experimental animals were paired before killing unless specified otherwise. All preparative and experimental procedures were carried out with scrupulous adherence to this paired protocol.

Autoradiography. The autoradiographic procedure was adapted from Young and Kuhar (11). Hippocampal formation was removed by blunt dissection from control and experimental animals as described above. The samples were frozen by slow immersion in isopentane cooled (−70°C) in liquid nitrogen. Thin (6- to 12-μm) sections were cut in a cryostat microtome and thaw mounted onto gelatin-coated slides. Sections for study were restricted to those removed from the middle one-third of the longitudinal axis of hippocampal formation. The slides were immersed in 0.16 M Tris citrate, pH 7.1 at 4°C/2 nM [^{3}H]flunitrazepam; some solutions contained 2.5 μm diazepam to generate sections with nonspecific binding only. After incubation for 40 min at 0°C, the slide-mounted sections were washed for 2 min in buffer to reduce nonspecific binding. The slides were then rapidly dried under a stream of cold air, and flexible emulsion [Kodak NTB2 emulsion/water (1:1)]-coated coverslips were attached with glue. After exposure for 10–14 days, the coverslips were gently bent away from the tissue section, the latent autoradiograms were developed, and the tissue was fixed and stained with cresyl violet. The assemblies were reapposed and then examined with both light- and dark-field optics.

Preliminary experiments showed that binding to slide-mounted sections had properties similar to binding to membranes. Coronal sections (16 μm) of rat cerebral hemispheres were used. After incubation, the portion of the slide containing the tissue was snapped off and placed in Protosol overnight to solubilize the tissue, and the radioactivity was determined in Omnifluor. Specific binding accounted for at least 95% of total binding. The properties of specific binding to these slide-mounted sections were similar to those reported previously by Young and Kuhar (12) and to binding with membranes with respect to kinetics, binding isotherms, and displacement with nonradioactive diazepam.

RESULTS

Localization of Receptor Increases to Fascia Dentata: Microdissection Techniques. Repeated kindled seizures induced a marked increase in [^{3}H]flunitrazepam binding in membranes from fascia dentata but not hippocampal gyrus (Table 1). In kindled animals receiving a mean of 20.9 stimulations (18–23), [^{3}H]flunitrazepam binding was increased by 41% in comparison with electrode-implanted unstimulated controls. Analysis of these data showed significant differences ($P < 0.001$, Student's t-test). The slight increase (10%) of [^{3}H]flunitrazepam binding in hippocampal gyrus membranes was not significant.

Localization of benzodiazepine receptor increases after repeated electroshock seizures was similar to that after kindled seizures. In electroshock-treated animals (a total of 19 seizures), [^{3}H]flunitrazepam binding was increased by 33% in comparison with paired unstimulated controls (Table 1). Analysis of these data by Student's t test showed significant differences ($P <$ 0.005, two-tailed). By contrast, a slight nonsignificant decrease of [^{3}H]flunitrazepam binding was found in hippocampal gyrus membranes.

To determine whether the increase of [^{3}H]flunitrazepam

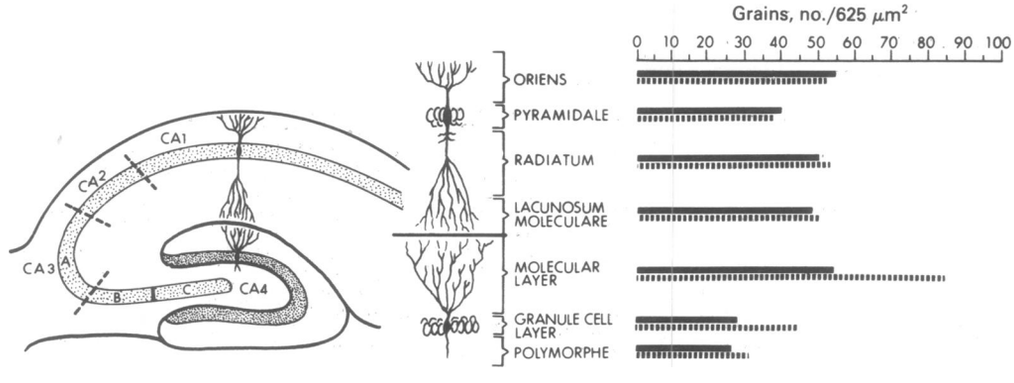

FIG. 1. (*Left*) Cross section of rat hippocampal formation with subfields denoted according to Lorente-de-No (14). (*Center*) Laminae of area CA1 and fascia dentata according to Ramon y Cajal (15) shown in relationship to a schematic pyramidal (CA1) or granule (fascia dentata) cell. (*Right*) Silver grain counts of benzodiazepine receptor autoradiographs from control and experimental animals (see Fig. 2). Grain counts in sections analyzed for nonspecific binding were <1/625 μm^2. ■■■, Experimental; ■■■, control.

binding was due to alteration in affinity or in number of binding sites, the following experiment was carried out. Membranes were prepared from fascia dentata of electroshock-treated and control animals. Binding was measured at various concentrations of [^{3}H]flunitrazepam and maximal values (B_{max}) and K_d values were determined by linear regression analyses of Scatchard plots (13). The results showed a 28% increase in the total number of binding sites in electroshock-treated compared with control animals (952 ± 43 fmol/mg of protein versus 745 ± 57). This difference was statistically significant ($P < 0.0004$, Student's t test). The K_d values for the electroshock-treated (0.82 ± 0.08 nM) and control (1.04 ± 0.16 nM) groups were not significantly different. Similar results were obtained in an experiment comparing kindled and control groups. Together with our previous observations on benzodiazepine binding isotherms in membranes from whole hippocampal formation (6), these data demonstrate that the increases in [^{3}H]flunitrazepam binding in fascia dentata in the paired protocol were due to increased numbers of binding sites.

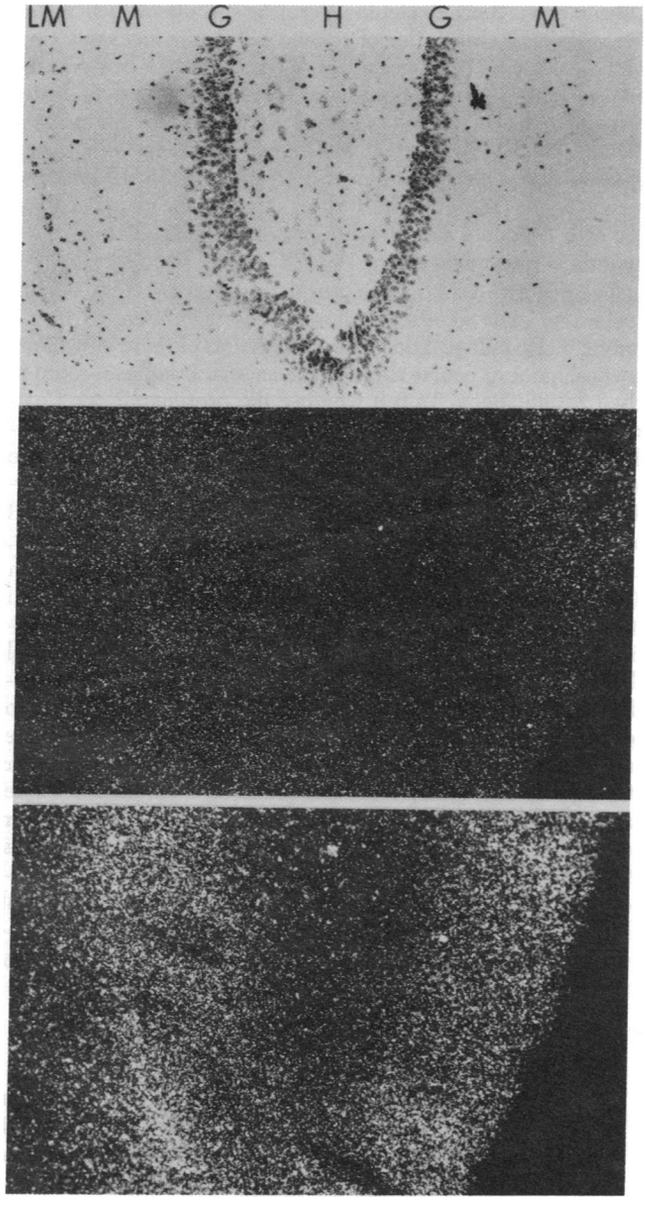

FIG. 2. (*Upper*) Light-field photomicrograph of cresyl violet stain in cross section of fascia dentata as pictured in dark field. (×43.) Dark-field photomicrographs of [^{3}H]flunitrazepam autoradiographs from control (*Middle*) and seizure-treated (*Lower*) fascia dentata. Note the increased density of silver grains over granular cell and molecular layers of experimental relative to control. Regions: LM, stratum lacunosum moleculare of CA1; M, molecular layer of fascia dentata; G, granule cell layer of fascia dentata; H, dentate hilus.

Localization of Benzodiazepine Receptor Increases in Fascia Dentata: Radiohistochemical Techniques. The relatively simplified anatomy, together with the lamination of the neuronal constituents, in fascia dentata offers important advantages for localizing a molecular alteration to a cellular element. The somata of the principal neuron, the granule cell, are arranged in a tightly packed layer termed the granule cell layer. The dendrites extend perpendicularly from the plane of the cell body layer and ramify throughout the entire thickness of the molecular layer (Fig. 1). The major excitatory afferents arise from entorhinal cortex; afferents from lateral and medial entorhinal cortex form synapses on granule cell dendrites in the outer and middle thirds of the molecular layer, respectively. The quantitatively less significant commissural and associational afferents form synapses on granule cell dendrites in the inner third of the molecular layer. If the seizure-induced increases of benzodiazepine receptors in fascia dentata are localized to a single population of neurons, the laminar pattern on autoradiographs could provide a clue to the identity of the involved neurons.

To determine the localization of the seizure-induced increases of benzodiazepine receptors within fascia dentata, [^{3}H]flunitrazepam autoradiographs were developed from thin (12-μm) sections from hippocampal formation of electroshock-treated and control animals. These autoradiographs demonstrated increases in the number of silver grains over the granule cell body and throughout the entire molecular layer (Fig. 2). The increased numbers of silver grains were apparent on low-power magnification photomicrographs (dark field) of sections from control and seizure-treated animals. These increases were verified and quantitated by grain counts through a calibrated eyepiece under high-power (×400) magnification (Table 2; Fig. 1). No marked differences were noted in dentate hilus, stratum lacunosum moleculare of CA1, or any other subregion of hippocampal formation. Similar results were obtained with multiple sections prepared from both hippocampal formations of one pair of a control and an electroshock-treated animal and two pairs of a control and a kindled seizure-treated animal. The striking distribution of the increased grain density throughout the molecular and granule cell layers is most consistent with localization to the somata and dendrites of the granule cells.

Table 2. Localization of benzodiazepine receptor increases after repeated seizure treatments: Silver grain counts from light microscopic autoradiographs

Region	Control	Electroshock	% control
Hilus	26.4 ± 1.2	30.1 ± 1.5	114
Granule cell layer	27.0 ± 1.8	44.5 ± 0.8*	165
Molecular layer	53.7 ± 1.9	84.2 ± 2.1*	157
Stratum lacunosum moleculare	47.1 ± 2.0	49.0 ± 1.5	104
Stratum radiatum	49.7 ± 1.9	53.1 ± 2.0	106
Stratum pyramidale	40.1 ± 2.0	37.6 ± 1.8	94
Stratum oriens	54.9 ± 1.9	51.8 ± 2.1	94

Results are expressed as number of silver grains per 625 μm^2 counted through an eyepiece fitted with a reticle and are mean ± SEM for 15 determinations. The slides analyzed and the photomicrographs (Fig. 2) were obtained from the same animal. The number of silver grains in sections incubated with both [^{3}H]flunitrazepam and nonradioactive diazepam was 0.7 ± .1 and did not differ between experimental and controls.

* Significant ($P < 0.001$, Student's t test, two-tailed).

Table 3. Effects of colchicine-mediated granule cell destruction on fascia dentata benzodiazepine receptors

	Biochemical analyses (n = 6)			Silver grains, no. per 625 μm^2 (n = 15)	
	[^{3}H]Flunitrazepam binding, fmol/mg of protein	Membrane protein, μg per fascia dentata	[^{3}H]Flunitrazepam binding to fascia dentata, fmol	Granule cell layer	Molecular layer
Control	578 ± 27	783 ± 45	452	38 ± 2	57 ± 2
Colchicine	289 ± 48*	237 ± 34*	73	18 ± 1*	25 ± 1*
% control				47	44

Results are mean ± SEM. [^{3}H]Flunitrazepam binding to fascia dentata was calculated by multiplying [^{3}H]flunitrazepam binding (fmol/mg of protein) by protein content per fascia dentata.
* Significant ($P < 0.001$, Student's t test, two-tailed).

Effects of Granule Cell Destruction on Benzodiazepine Receptors in Fascia Dentata: Microdissection Techniques. Localization of benzodiazepine receptor increases to granule cells suggests that granule cells normally contain benzodiazepine receptors. To test this suggestion, granule cells were destroyed by two independent methods and benzodiazepine receptor binding was measured in membranes from microdissected fascia dentata.

Previous granule cell destruction by intrahippocampal injection of colchicine caused a marked loss of benzodiazepine receptor binding sites in fascia dentata membranes. To destroy granule cells, colchicine was injected (6 nmol per injection site) bilaterally into both septal and ventral hippocampal formation as described (16). Animals were sacrificed ≈1 week later. Control animals were anesthetized but received no injections. The resulting granule cell destruction was verified histologically (data not shown) in frozen sections cut from slices removed from each hippocampal formation. Paralleling this marked granule cell destruction was a 50% reduction of [^{3}H]flunitrazepam binding (Table 3). This decrease was significant ($P < 0.001$, Student's t test). Binding isotherms in additional membranes showed this decrease to be due to decreased numbers of binding sites without significant alterations in affinity (B_{max}, control 1320 ± 123 versus colchicine 525 ± 34 fmol/mg; K_d, control 1.0 ± 0.2 versus colchine 0.9 ± 0.1 nM). This reduced binding was not simply accounted for by the presence of colchicine; inclusion of colchicine (4 μM) in the incubation mixture caused no consistent alterations of K_d or B_{max} value of [^{3}H]flunitrazepam binding to brain membranes. The 50% reduction in number of [^{3}H]flunitrazepam binding sites in the presence of only 30% remaining protein indicates a loss of 85% of [^{3}H]flunitrazepam binding sites per fascia dentata in the colchicine-treated group.

To determine whether granule cell destruction caused by a different method was associated with reduced [^{3}H]flunitrazepam binding, suckling rats were irradiated [200 rads (1 rad = 0.01 gray) on postnatal days 2 and 3, 150 rads on days 5, 7, 9, and 11] (17). Littermates were handled but not irradiated. The animals were sacrificed ≈2 months later. The irradiation effects were histologically verified in frozen sections from hippocampal formation slices removed at the time of dissection. This showed significant loss of granule cells (data not shown) and [^{3}H]flunitrazepam binding [control 450 ± 19 (n = 12) versus irradiated 314 ± 21 (n = 9), $P < 0.005$]. The decrease in protein content in the fascia dentata was used as an index of granule cell loss. The data for both colchicine and radiation treatment were plotted as [fmol bound (treated)/fmol bound (control)] versus [mg of protein per fascia dentata (treated)/mg of protein (control)]. This generated a straight line of slope 0.9 and protein intercept 0.13 ($P < 0.001$), which supports a 1:1 relationship between granule cells and benzodiazepine receptors. The positive intercept suggests that some elements of fascia dentata do not contain benzodiazepine receptors.

Effects of Granule Cell Destruction by Intrahippocampal Colchicine on Benzodiazepine Receptors: Radiohistochemical Techniques. If elimination of granule cells is the mechanism by which colchicine produces a loss of fascia dentata benzodiazepine receptors, the distribution of silver grain loss should correspond to the cell bodies and dendrites of granule cells in fascia dentata. Such a distribution was found. Significant reductions of silver grains were found over both the granule cell and molecular layers (Table 3). This further supports the idea that granule cells contain a considerable portion of benzodiazepine receptors in fascia dentata.

The majority (86%) of synapses in the outer two-thirds of the molecular layer represent entorhinal cortical afferents terminating on granule cell dendrites (18). Axons of these entorhinal cortical neurons course through the angular bundle en route to their terminations. To test whether the presynaptic terminals of these afferents contained benzodiazepine receptors, the following experiment was performed. The right angular bundle was cut with a knife with stereotaxic guidance under pentobarbital anesthesia. In control animals, the skull was trephined but no knife cut was made. The animals were sacrificed 2 days later, and the brains were serially sectioned. Evidence of denervation (19) in the dorsal right hippocampal formation was confined to the middle third of the molecular layer in the experimental animal, which corresponded to the cutting of afferents from medial but not lateral entorhinal cortex (20). Examination of [^{3}H]-flunitrazepam autoradiographs of adjacent sections disclosed no decrease in silver grain density over the middle third of the molecular layer in comparison with either the inner or the outer third (not denervated) of the molecular layer on the same side as the lesion nor in comparison with the side without lesions or control animals. Moreover, no "piling up" of silver grains was found in the area of the knife cut, as might be expected if receptors were undergoing axoplasmic transport from the cell bodies (21). These data argue against the presence of benzodiazepine receptors in presynaptic terminals and thereby strengthen the localization to granule cells.

DISCUSSION

The principal findings of this study pertain to the localization of the increased numbers of hippocampal formation benzodiazepine receptors induced by repeated seizures. Biochemical analyses of microdissected membranes localize the increase mainly to fascia dentata. Assuming the increased receptors reside in a single cell population within fascia dentata, the remarkable anatomic specificity found radiohistochemically argues for localization to the somata and dendritic tree of the granule cells. This idea is supported by lesion experiments indicating that granule cells normally contain benzodiazepine receptors. Moreover, the presynaptic terminals of the entorhinal afferents appear to be devoid of benzodiazepine receptors. Lo-

calization to other cellular constituents is unlikely, either because of the relative paucity of cells with overlapping distribution (e.g., basket cells) or because the normal distribution does not conform to this discrete pattern (e.g., glia, blood vessels).

The cellular and molecular mechanisms underlying the kindling phenomenon are obscure (7). A critical limiting factor is our ignorance of the spatial distribution of the altered neural circuitry responsible for kindling. Evidence suggests that kindling is subserved by alteration of a network of neural circuits that has a highly specific distribution, both in the area of the stimulating electrode and in "downstream" synaptic sites (9). A proposed explanation for the facilitation and amplification of information flow through these circuits is the development of long-term potentiation of synaptic communication (22, 23).

The presumptive localization of benzodiazepine receptor increases to the granule cells is particularly interesting within this framework for two reasons. First, the responsiveness of granule cells to their principal excitatory inputs readily undergoes long-term potentiation (23). Second, the granule cell is a critical relay in information flow through hippocampal formation. Cortical and many subcortical regions, including amygdala, gain access to hippocampal formation circuitry through connections with entorhinal cortex, which in turn provides an excitatory synaptic input to the granule cells (24, 25). The granule cell represents the first link of a feed-forward excitatory chain in hippocampal formation sequentially activating CA3 and CA1 pyramids (25). *In vivo* electrophysiologic studies indicate that granule cells are a key site in regulating information flow through this trisynaptic chain under physiologic conditions (26).

The granule cells may also regulate information flow through these circuits under pathologic conditions such as seizures. Exposure of hippocampal slices to convulsant agents *in vitro* results in burst firing of CA3 and CA1 pyramids, a firing pattern observed in neurons in epileptic foci (27). By contrast, granule cells themselves do not burst under these conditions. When slices are exposed to normal media, bursting of CA3 pyramids can be induced by excitatory synaptic input from granule cells (27). It seems likely that enhanced synaptic drive of CA3 pyramids, induced by long-term potentiation of excitatory synaptic activation of granule cells, could play a critical role in triggering limbic seizures. Likewise, a molecular alteration designed to reduce granule cell excitability could protect against limbic seizures.

Whether increased benzodiazepine receptors result in increased or decreased granule cell excitability is unclear. The increased numbers of benzodiazepine receptors could represent any of three major processes in kindled rats: (*i*) the molecular basis of kindling, (*ii*) the molecular basis of a process resisting development of kindled epilepsy, or (*iii*) a molecular consequence of repeated seizures with no meaningful functional significance regarding the likelihood of subsequent seizures. The anatomic specificity of the receptor localization argues against the third possibility. Arguing in favor of one of the first two possibilities is our observation that previous granule cell destruction by intrahippocampal colchicine retards the subsequent development of amygdala kindling (28). The occurrence of the receptor increases after repeated electroshock seizures does not aid in differentiating the first two possibilities, because repeated seizures induced by other agents result in partial kindling (i.e., fewer stimulations required to induce subsequent kindling compared with non-seizure-treated controls) (29, 30). Understanding the precise role of the benzodiazepine receptor increases in kindled epileptogenesis must await elucidation of the endogenous ligand for this receptor and its physiologic actions.

The present findings correlate biochemical and morphologic results pinpointing a molecular alteration to a specific neuronal population in an animal model of epilepsy. This is an important step in determining how this molecular alteration translates into altered neuronal function. The findings point to the dentate granule cells as a site for biochemical, electrophysiologic, and morphologic investigations into the basic mechanisms of amygdala kindling.

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