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Repeated seizures induce long-term increase in hippocampal benzodiazepine receptors

(drug receptors/anticonvulsants/epilepsy/kindling)

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ABSTRACT **Repeated seizures, whether induced by kindling or electroshock, caused a long-lasting (at least 24 hr) increase of [^{3}H]diazepam binding in hippocampal membranes of Sprague–Dawley rats. Scatchard analyses demonstrated that increased numbers of binding sites accounted for the increase. Neither repeated hypoxia nor repeated administration of electrical current without inducing seizures caused an increase of [^{3}H]diazepam binding. Regardless of the method used for seizure induction, the response was graded in that large numbers of seizures were required to induce significant increases, whereas fewer seizures induced only slight increases. We suggest that the receptor increases imply a heightened response to benzodiazepines and more powerful hippocampal recurrent inhibition.**

The benzodiazepines are effective anticonvulsants in various generalized and partial seizure disorders of man (1). We currently have no meaningful biochemical insights into their mechanism of action. Recent investigations have demonstrated the presence of saturable, high-affinity, stereospecific binding sites of [^{3}H]diazepam in brain membranes (2). The observation that the clinical potency of a series of benzodiazepines parallels the potency of these agents in displacing [^{3}H]diazepam binding suggests that these binding sites may represent receptors mediating the pharmacologic action of these drugs (2).

The discovery of benzodiazepine receptors may provide insight into the biochemical mechanisms of action of these drugs. It may also aid in elucidating the mechanisms underlying the pathologic conditions in which these drugs are effective.

Our laboratory has been investigating the role of receptor mechanisms in an animal model of epilepsy—namely, kindling* (3, 4). Because exogenously administered benzodiazepines are potent blockers of kindled seizures, we hypothesized that brain benzodiazepine receptors may be altered after these seizures (7). The present report demonstrates that large numbers of seizures, whether induced by kindling or electroshock, cause a long-term increase in the number of hippocampal benzodiazepine receptors.

MATERIALS AND METHODS

Kindling. Adult male Sprague–Dawley rats (Charles River) weighing at least 200 g were used in all experiments. A bipolar electrode (twisted nichrome wire, 0.254 mm diameter) was stereotaxically implanted in the basolateral nucleus of the right amygdala (coordinates: anterior–posterior, 0.50; medial–lateral, 0.48; dorsal–ventral, 0.23) under pentobarbital anesthesia (8). After a postoperative recovery period of at least 7 days, stimulations from a Grass S-88 stimulator (400–600 μA biphasic square-wave pulses, 1 msec duration, 60 Hz for 1 sec) were administered at daily intervals. The amount of current delivered was monitored in all depth electrode stimulations by measuring the voltage drop (with a Tektronix 502A oscilloscope) across a 10,000-ohm resistor in series with the stimulating circuit. The electroencephalogram (EEG) was recorded prior to and after each stimulation with a Grass model 78 polygraph. The initial stimulations evoked electrical seizures (i.e., afterdischarge recorded on the EEG) but little or no clinical seizures. The response to succeeding stimulations consisted of progressively more intense clinical seizures evolving as described by Racine (9): class 1, mouth and facial movements; class 2, head nodding; class 3, forelimb clonus; class 4, rearing; class 5, rearing and falling. The animals were defined as fully kindled after a single class 5 seizure occurred. The number of stimulations required to induce a class 5 seizure ranged from 8 to 19. Control animals underwent electrode implantation but received no stimulations. Electrode placements were not histologically verified because the stimulated amygdala was utilized for biochemical study.

Electroshock Seizures. These seizures were induced by administering 110 V (60 Hz) for 1 sec through ear clips. These seizures usually consisted of tonic extension of all four limbs followed by clonic contractions and subsequent profound postictal unresponsiveness. Cyanosis occurred in many of these seizures. Control animals were connected to the ear clips but received no stimulation.

Dissection and Membrane Preparation. The animals were killed by decapitation 24 hr after the last experimental manipulation, unless specified otherwise. The cerebral hemispheres were sectioned in the sagittal plane and the hippocampi removed from the medial aspect of the cerebral hemispheres by blunt dissection. The remaining aspects of the hemispheres were reassembled and placed into a cavity in a Lucite block that accommodated the brain at an angle of 30° above the horizontal plane. The right amygdaloid region and samples of neocortex were then dissected as described (4).

The samples were homogenized in 1 ml of cold 0.32 M sucrose/1 mM glycylglycine, pH 7.4, with a Teflon/glass homogenizer; 2 ml of 50 mM Tris citrate buffer at pH 7.1 was added and additional homogenization performed with a Polytron (setting 6 for 30 sec). After removal of the supernatant by centrifugation at 43,500 × g for 20 min, the pellet was resuspended in 2 ml of cold water by homogenization with a

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Abbreviation: EEG, electroencephalogram.

* "Kindling" is an animal model of epilepsy induced by focal electrical stimulation of the brain (5, 6). Repeated periodic administration of an initially subconvulsive electrical stimulus induces the 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 stimulations are required to induce kindling.

Table 1. [^{3}H]Diazepam binding in multiple brain regions after repeated kindled seizures (group 1)

	Cortex		Amygdala	Hippocampus	
	Left	Right	Right	Left	Right
Control	39 ± 4 (8)	64 ± 4 (8)	48 ± 3 (14)	60 ± 4 (14)	53 ± 4 (14)
Kindled	40 ± 4 (8)	62 ± 5 (8)	54 ± 8 (14)	81 ± 6 (14)*	71 ± 7 (14)†

The numbers in this table refer to the mean (±SEM) fmol of [^{3}H]diazepam bound per mg of protein in control and kindled animals; number of determinations is shown in parentheses. The animals in group 1 received a mean of 16.3 stimulations (range, 14–19). Paired *t* tests (two-tailed) comparing kindled with their paired control groups disclosed no significant ($P < 0.05$) differences except where specified.

* $P < 0.005$.

† $P < 0.001$.

Polytron and stored at −80°C. The interval between death and freezing of the membranes varied as a function of the number of animals sacrificed on a given day but did not exceed 6 hr.

The samples were thawed on the day of assay and centrifuged at 43,500 × *g* for 20 min. The pellets were resuspended in 2 ml of water. The membranes were incubated at 0°C for 30 min, centrifuged at 43,500 × *g* for 20 min, and resuspended in 2 ml of 1 mM Tris citrate buffer at pH 7.1 by homogenization with a Polytron. Aliquots 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]diazepam under equilibrium conditions. The specific activity of the [^{3}H]diazepam (New England Nuclear) was 64.06 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels). Specific binding was defined as the difference in total [^{3}H]-diazepam binding in the absence and presence of μM diazepam. Specific binding accounted for 60–80% of total binding under the standard conditions used. Henceforth, the term "binding" will refer to specific binding. The incubation mixture contained 150 μg of membrane protein, 50 mM Tris citrate buffer at pH 7.1, 0.027% ethanol, and 1.1 nM [^{3}H]diazepam in a volume of 1.5 ml. Duplicate assays were conducted in the absence and presence of μM diazepam. The reaction was continued for 40 min at 0°C and terminated by vacuum filtration through a Whatman GF/C filter. The filter was washed three times with 5 ml of ice-cold 50 mM Tris citrate buffer. The radioactivity of the filter was measured in an Intertechnique scintillation counter at an efficiency of 45% as described (3).

Preliminary studies demonstrated variation in [^{3}H]diazepam binding from day to day. This observation was verified in the present study by the variation in diazepam binding among the various control groups. Because all binding assays could not be performed on the same day, control and experimental animals were paired prior to killing. All preparative and experimental procedures were carried out with scrupulous adherence to this paired protocol.

RESULTS

[^{3}H]Diazepam Binding in Kindled Rats. Repeated kindled seizures induced a marked increase of hippocampal diazepam binding. In kindled animals receiving a mean of 16.3 stimulations (range, 14–19), [^{3}H]diazepam binding was increased by approximately 35% in both the left and right hippocampi in comparison to paired unstimulated controls (Table 1). Analysis of these data with paired *t* tests disclosed highly significant differences ($P < 0.005$ and <0.001 in left and right hippocampi, respectively). Among animals receiving a mean of 16.3 stimulations, increased hippocampal diazepam binding was present regardless of whether a single (seven animals) or multiple (seven animals) class 5 seizures occurred; the increases were greater in the former group (data not presented separately). In contrast to the results in the hippocampus, in the neocortical regions only minimal differences were present between experimental and control animals. A slight increase in binding was observed in the stimulated amygdala but was not statistically significant.

To control for the possibility that repeated administration of electrical current (in the absence of kindled seizures) alone would be sufficient to induce an increase in [^{3}H]diazepam binding, the following experiments were performed. A bipolar electrode was stereotaxically implanted in the right amygdala and 17 stimulations were administered at daily intervals. Although the total number of stimulation trains delivered to these animals was comparable to the regimen in group 1 of the kindled animals, the frequency of pulses within each stimulation train differed. In contrast to the kindled group, which received these pulses at a frequency of 60 Hz for 1 sec, this group received the pulses (600 μA biphasic square wave, 1 msec duration) at a frequency of 1 Hz for 60 sec. This pattern induced no afterdischarges or kindling effect (3). Paired controls for these animals underwent electrode implantation without subsequent stimulation. There were no significant differences in [^{3}H]diazepam binding (fmol/mg, mean ± SEM) in membranes prepared from the hippocampi 1 day after completion of stimulation: left, control (n = 10 animals) 52 ± 4, experimental (n = 10) 48 ± 3; right, control (n = 10) 43 ± 3, experimental (n = 10) 44 ± 3. Thus, electrical current *per se* does not cause an increase of hippocampal [^{3}H]diazepam binding.

The increased diazepam binding occurred in kindled animals experiencing repeated stimulation-induced seizures. Because either kindling or repeated seizures could be the critical variable, we attempted to determine which was responsible. The fact that different animals required different numbers of stimulation-induced partial seizures prior to establishment of kindling (i.e., elicitation of a single generalized class 5 seizure consisting of rearing and falling) provided the opportunity to assess these variables separately. We measured [^{3}H]diazepam binding in kindled animals requiring fewer stimulation-induced partial seizures prior to completion of kindling (mean, 10.3 stimulations; range, 8–12). Only slight increases of hippocampal [^{3}H]diazepam binding (fmol/mg, mean ± SEM) were observed: left, control (n = 13 animals) 43 ± 4, kindled 48 ± 5; right, control (n = 13) 50 ± 6, kindled 53 ± 7. These differences were not statistically significant. Because these animals were kindled to the same extent as the animals presented in Table 1 (i.e., all of these animals had a permanent enhanced sensitivity to electrical stimulation), these data suggested that the increase of [^{3}H]diazepam binding was related to the total number of seizures rather than to kindling *per se*.

Electroshock Seizures. To assess further the role of seizures *per se* in mediating these receptor alterations, series of electroshocks were administered to additional animals at 24-hr intervals. These treatments differed from kindled seizures in two important ways: (*i*) a kindling effect did not occur in that there was no development of a progressive enhanced sensitivity to the stimulus (11); (*ii*) these seizures were more intense than the kindled seizures with respect to the commonly associated

Table 2. [^{3}H]Diazepam binding in hippocampus after repeated electroshock seizures

	Group 1	Group 2
Control	54 ± 3 (14)	53 ± 3 (15)
Experimental	64 ± 4 (14)	57 ± 3 (15)

The numbers refer to the mean (±SEM) fmol of [^{3}H]diazepam specifically bound per mg of protein. The numbers in parentheses refer to the number of hippocampi analyzed; there were seven experimental animals in group 1 and eight in group 2. The animals in group 1 underwent a mean of 17 seizures (range, 15–18), whereas each animal in Group 2 underwent 7 seizures. All animals were sacrificed 1 day after the last seizure. Analysis of each experimental group and its paired control with a paired t test (two-tailed) disclosed significant differences ($P < 0.01$) in group 1 but not ($P < 0.10$) in group 2.

cyanosis, tonic motor movements, and more extensive postictal lethargy.

Group 1 of the electroshock group received a mean total of 17 seizures (range, 15–18) at daily intervals; sacrifice was 1 day after the last seizure. Hippocampal [^{3}H]diazepam binding was increased by 19% with respect to controls (Table 2, group 1). This difference was significant (paired t test; $P < 0.01$). These data indicate that repeated seizures in the absence of kindling can induce an increase of [^{3}H]diazepam binding. The factors responsible for the smaller increase of [^{3}H]diazepam binding following electroshock as compared to kindled seizures (Table 1) are not clear.

A second group of animals underwent seven electroshock seizures. In contrast to the results in the first group, the increase of [^{3}H]diazepam binding was only 8% and was not statistically significant (Table 2, group 2). Although the animals in groups 1 and 2 differed with regard to the total number of seizures and the time elapsed between the seventh seizure and death, it seemed likely that the total number of seizures was the factor inducing the receptor increases. To differentiate between these two possibilities, an additional group of animals underwent seven electroshock seizures and were sacrificed 10 days later. Hippocampal [^{3}H]diazepam binding (fmol/mg, mean ± SEM) was not significantly increased in these animals in comparison to their controls: control (n = 12 hippocampi from six animals), 57 ± 2; electroshock (n = 12 hippocampi from six animals), 58 ± 2. These observations further indicate that the total number of seizures is the variable responsible for the increase of hippocampal [^{3}H]diazepam binding.

Hypoxia commonly occurred during electroshock seizures. To determine whether repeated hypoxia itself caused an increase in hippocampal [^{3}H]diazepam binding, the following experiments were performed. Experimental animals were rendered hypoxic once each day for 17 days. Hypoxia was induced by placing the animals on a continuously shaking platform under an air-tight plastic cover and gradually replacing the air with nitrogen. The animals were removed from these hypoxic conditions after profound ataxia developed. Paired control animals underwent identical treatments except that compressed air was used instead of nitrogen. Animals were sacrificed 24 hr after the 17th daily treatment. Hippocampal [^{3}H]diazepam binding (fmol/mg, mean ± SEM) was not significantly increased in these animals in comparison to their controls: control (18 hippocampi from nine animals), 59 ± 3; experimental (18 hippocampi from nine animals), 61 ± 2. Thus, repeated hypoxia alone is not sufficient to cause a significant increase of hippocampal diazepam binding.

Binding Isotherms. To determine whether the increase of hippocampal [^{3}H]diazepam binding was due to an alteration in affinity or number of binding sites, the following experiments were performed. Membranes prepared from both hippocampi

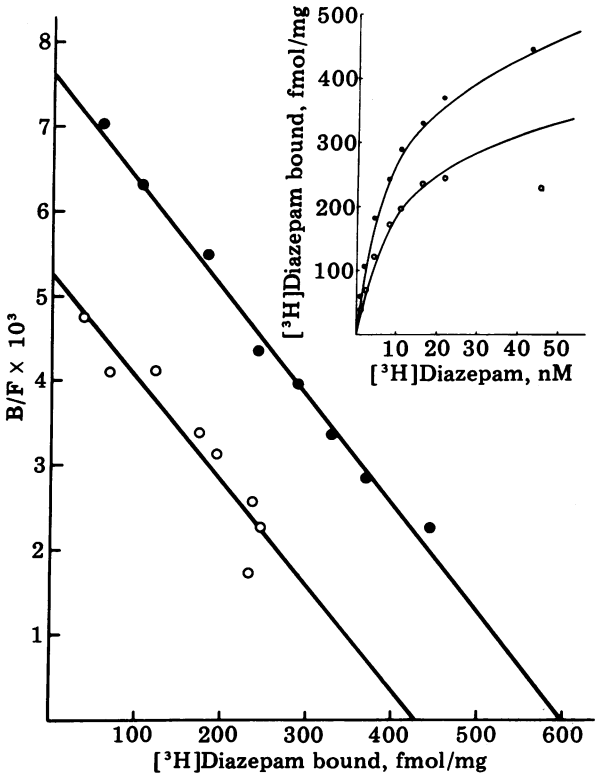

FIG. 1. Scatchard analysis of [^{3}H]diazepam binding in hippocampal membranes from control (○) and kindled (●) animals experiencing numerous seizures. The lines in the plot were generated by linear regression. (*Inset*) Same data as saturation curves of [^{3}H]diazepam binding.

of at least two kindled (at least 14 stimulations) and two control animals were pooled separately. Binding was measured with multiple concentrations of [^{3}H]diazepam and the results were plotted according to the method of Scatchard (12). The results of a representative experiment (Fig. 1; Table 3, experiment 1) disclosed a 39% increase in the total number of binding sites (B_{max}) in kindled compared to the control membranes (598 ± 14 fmol/mg of protein vs. 430 ± 37). This difference was statistically significant ($P < 0.005$, Student's t test). The dissociation constants of the kindled (7.9 ± 0.3 nM) and control (8.2 ± 1.2 nM) groups were not significantly different. The results of additional experiments with membranes prepared from kindled

Table 3. Results of linear regression analyses of Scatchard plots of [^{3}H]diazepam binding isotherms

Experiment	B_{max}	K_d
1. Control	430 ± 37	8.2 ± 1.2
Kindled	598 ± 14	7.9 ± .3
2. Control	297 ± 17	6.8 ± .6
Kindled	381 ± 30	8.3 ± 1.0
3. Control	450 ± 64	10.8 ± 2.0
Electroshock	656 ± 76	11.2 ± 2.0
4. Control	565 ± 90	11.1 ± 2.5
Electroshock	704 ± 50	12.4 ± 1.2
5. Control	384 ± 24	5.9 ± .6
Electroshock	515 ± 60	7.9 ± 1.4

B_{max}, total number of receptor binding sites in fmol/mg of membrane protein (mean ± SEM). The dissociation constants (K_d) represent the [^{3}H]diazepam concentration expressed as nM [^{3}H]diazepam (mean ± SEM). Differences present in B_{max} between experimental and control were significant ($P < 0.05$, Student's t test) in all but one experiment (experiment 4). Differences between the dissociation constants were not statistically significant.

(Table 3, experiment 2) or electroshock-treated animals (Table 3, experiments 3–5) confirmed these findings. Although the magnitude of the difference in the number of binding sites between experimental and controls varied in these experiments, increased numbers of binding sites were repeatedly observed in the experimental groups. Together, these data indicate that the increases of [^{3}H]diazepam binding observed in the paired protocols are due mainly to increased numbers of binding sites.

DISCUSSION

The principal finding of the present study is that the repeated occurrence of seizures causes an increase in the numbers of hippocampal benzodiazepine receptors 24 hr following the last seizure. Increases occurred in two different types of electrically induced seizures. Repeated administration of electrical current without inducing seizures did not cause an increase of benzodiazepine receptors. Likewise, repeated hypoxia did not cause an increase of receptors. Regardless of the method used for seizure induction, the response was graded in that large numbers of seizures were required to induce significant increases, whereas fewer seizures induced only slight increases.

Short-term increases of cerebral cortical benzodiazepine receptors have been found after electroshock- or pentylenetetrazol-induced seizures (13). The onset of the increases was within 15 min after a single seizure, and the peak increase (20%) occurred 30 min after the seizure. Binding had returned to normal by 60 min. By contrast, the increases of hippocampal benzodiazepine receptors observed here required more time to develop (significant increases were not observed earlier than 14 days) and persisted longer (at least 24 hr after the last seizure). The relatively long durations of the increases observed here are akin to the durations of previously described alterations of brain β-adrenergic and muscarinic cholinergic receptors (3, 4).

The hippocampal benzodiazepine receptor increases reported here represent an endogenous response to seizures which may have important implications for regulation of neuronal excitability and seizure control. Electrophysiologic study of cultured spinal neurons has demonstrated that the benzodiazepines selectively potentiate γ-aminobutyric acid-mediated inhibition (14, 15). Considerable evidence indicates that, in the hippocampus, γ-aminobutyric acid is the neurotransmitter responsible for recurrent inhibition (16, 17). In accord with these findings, intravenous administration of diazepam potentiates hippocampal recurrent inhibition (18). Because increased numbers of receptors are commonly associated with an enhanced biologic response (19, 20), these receptor increases may translate into a heightened response to exogenously administered benzodiazepines and more powerful recurrent inhibition. Detailed localization of the receptor increases with autoradiographic techniques will permit direct testing of this hypothesis with the hippocampal slice preparation.

The long-term effectiveness of the benzodiazepine anticonvulsants is often limited by development of tolerance (21). Tolerance refers to the loss of drug effectiveness despite administration of previously adequate or even increased amounts of the drug. The mechanisms underlying benzodiazepine tolerance are unknown. If the seizure-induced receptor increases are important in the anticonvulsant effectiveness of the benzodiazepines, chronic exposure to benzodiazepines could cause a receptor down-regulation, thereby resulting in tolerance.

In addition to these potential implications for understanding mechanisms of action of the benzodiazepines, the receptor increases may be important in endogenous control of neuronal excitability. By analogy with the opiate receptor, the existence of the benzodiazepine receptor implies the existence of an endogenous ligand for this receptor. This possibility has been strengthened by identification of several putative endogenous ligands (22, 23). Characterization of the endogenous ligand and elucidation of its electrophysiologic actions (i.e., an agonist or antagonist of benzodiazepines) will be essential to understanding whether the receptor increases mediate increased or decreased neuronal excitability.

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1. Mattson, R. H. (1972) in *Antiepileptic Drugs*, eds. Woodbury, D. M., Penry, J. K. & Schmidt, R. P. (Raven, New York), pp. 497–516.
2. Squires, R. F. & Braestrup, C. (1977) *Nature (London)* **266**, 732–734.
3. McNamara, J. O. (1978) *Brain Res.* **154**, 415–420.
4. McNamara, J. O. (1978) *Exp. Neurol.* **61**, 581–591.
5. Goddard, G. V., McIntyre, D. C. & Leech, C. K. (1969) *Exp. Neurol.* **25**, 295–330.
6. Wada, J. A. (1976) *Kindling* (Raven, New York).
7. Babington, R. G. & Wedeking, P. W. (1973) *Pharmacol. Biochem. Behav.* **1**, 461–467.
8. Pellegrino, L. J. & Cushman, A. J. (1967) *A Stereotaxic Atlas of the Rat Brain* (Appleton-Century-Crofts, New York).
9. Racine, R. J. (1972) *Electroenceph. Clin. Neurophysiol.* **32**, 281–294.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
11. Ramer, D. & Pinel, J. P. J. (1967) *Exper. Neurol.* **51**, 421–433.
12. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672.
13. Paul, S. M. & Skolnick, P. (1978) *Science* **202**, 892–894.
14. Choi, D. W., Farb, D. H. & Fischbach, G. D. (1977) *Nature (London)* **269**, 342–344.
15. Macdonald, R. & Barker, J. L. (1978) *Nature (London)* **271**, 563–564.
16. Andersen, P. (1975) in *The Hippocampus*, eds. Isaacson, R. L. & Pribram, K. H. (Plenum, New York), Vol. 2, p. 155.
17. Curtis, D. R., Duggan, A. W., Felix, D. & Johnston, G. A. R. (1970) *Nature (London)* **229**, 1222–1224.
18. Tsuchiya, T. & Fukushina, K. (1978) *Eur. J. Pharmacol.* **48**, 421–424.
19. Kebabian, J. W., Zatz, M., Romero, J. A. & Axelrod, J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3735–3739.
20. Spron, J. R., Harden, T. K., Wolfe, B. B. & Molinoff, P. B. (1976) *Science* **194**, 624–625.
21. Browne, T. R. (1978) *N. Engl. J. Med.* **299**, 812–816.
22. Asano, T. & Spector, S. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 977–981.
23. Toffano, G., Guidotti, A. & Costa, E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4024–4028.