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Diazepam receptor: Specific nuclear binding of [^{3}H]flunitrazepam

(nuclear receptor/benzodiazepines/psychotropic drugs/central nervous system pharmacology)

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ABSTRACT **Autoradiographic localization of [^{3}H]flunitrazepam in nuclei of the rat cerebral cortex was further confirmed by biochemical analysis of specific nuclear binding. Highly purified rat cerebral cortex nuclei were shown to bind [^{3}H]flunitrazepam specifically. The $K_{d(app)}$ for nuclear binding was 28 nM for the nuclei compared with a $K_{d(app)}$ of 1.1 nM for binding of [^{3}H]flunitrazepam to synaptosomal membrane fractions of the same tissue. Inhibition of the nuclear binding with inosine and hypoxanthine was greater than inhibition of the synaptic membrane fractions. These results lead us to conclude that specific binding may occur at both the synaptic membrane and the nuclear levels and that different endogenous ligands may compete at each site for binding. Furthermore, the possibility exists for translocation and alteration of the bound ligand complex from membrane site to nuclear site.**

It is now quite clear that specific sites exist in the brain for binding diazepam and certain structurally related ligands, all belonging to the class of compounds, the benzodiazepines. Diazepam is an extremely important drug because of its antianxiety effects with little apparent toxicity; it is also active as an anticonvulsant and a muscle relaxant.

Binding of the benzodiazepines has been demonstrated in a large number of laboratories (1–8), and workers in these laboratories have shown that the binding exhibits many of the properties of a true pharmacologic receptor. However, because of the difficulty of providing good animal models for human anxiety, it has not been possible to prove that the binding site is a true neurohormone receptor. Studies have shown that although the binding site exhibits many of the requirements for being called a receptor site, the necessary requirement that the binding elicits a pharmacologic response has yet to be demonstrated.

Even though the true receptor nature of the binding has not been elucidated, several attempts have been made to isolate an endogenous ligand that inhibits the specific binding. Hypoxanthine and inosine in very high concentrations (mM) have been shown to partially inhibit the binding of [^{3}H]benzodiazepine (9, 10), and a highly purified inhibitor isolated from urine has been described (11). Other experiments (12) have been done to examine porcine brain fractions that contain entities between 500 and 100,000 M_r for competitive inhibition of specific benzodiazepine binding in rat brain subfractions, and inhibition by both high and low M_r fractions has been found. The high M_r (40,000–70,000) fraction, termed benzodiazepine-competitive factor I (BCF-I), was 5 times as potent as the low M_r (1000–3000) fraction (BCF-II). The designations BCF-I and BCF-II were chosen because the entities compete with benzodiazepines at their binding site (12).

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In previous work we had found that [^{3}H]flunitrazepam was bound more tightly to the benzodiazepine site than was [^{3}H]-diazepam (13) and that the highest binding of [^{3}H]flunitrazepam occurred in the cortex "synaptosomal membrane" fraction. However, in totally unrelated studies on the potential toxicity of diazepam (unpublished results) we found, much to our surprise, that when an *in vivo* intubation of [^{3}H]flunitrazepam was given, the predominant autoradiographic labeling was in the brain nuclei and not in the membranes (Fig. 1).

The present paper reports evidence that purified nuclei from rat cerebral cortex specifically bind [^{3}H]flunitrazepam. The nuclear binding has a higher $K_{d(app)}$ (i.e., lower affinity) than that of the synaptosomal membranes, and the nuclear binding is inhibited to a greater extent by inosine and hypoxanthine than is the binding at the membrane level. Hypothetical models for the binding are presented.

MATERIALS AND METHODS

Histological and Ultrastructural Preparation of Nuclear Fractions of Forebrain. Nuclear fractions, prepared as described below, were received in pellet form, fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.4), rinsed, and postfixed in 1% OsO_4 in phosphate buffer. The pellets were minced, dehydrated in ethanol, and embedded in Epoxy resin (14). Sixty-nanometer sections were cut on an LKB Ultratome III, stained with uranyl acetate and lead citrate, and photographed on a Zeiss EM-10A. For orientation and survey screening, 1-μm-thick sections were stained with toluidine blue.

Light Microscopic Autoradiography of Rat Whole Brain. Normal rats were given a bolus injection of 20 μCi of [^{3}H]flunitrazepam in water via oral intubation (1 Ci = 3.7×10^{10} becquerels). Thirty minutes later the animals were killed and the whole brains were fixed in Bouin's solution and embedded in paraffin. Six-micrometer sections of brain were coated with Kodak NTB_2 emulsion, dipped in scintillation fluid, exposed in light-tight boxes, and developed in Kodak D-19 developer after 21 days.

Binding Assay. [*methyl*-^{3}H]Flunitrazepam was obtained from New England Nuclear (40 Ci/mmol). Pure, unlabeled flunitrazepam was a gift of Roche Laboratories. The forebrain specifically bound the most [^{3}H]flunitrazepam of all areas of the brain (2). Forebrain sections of 150- to 200-g Sprague–Dawley or Holtzman rats were homogenized in 10 vol of 0.32 M sucrose, and the homogenate was centrifuged at $1000 \times g$ for 10 min. Fractionation procedures for the brain sections have been described (2, 13); fraction B was used as the synaptosomal fraction. Purified nuclei were prepared as described below.

To determine the specific binding, we exposed 500-μl sam-

Abbreviation: BCF, benzodiazepine-competitive factor.

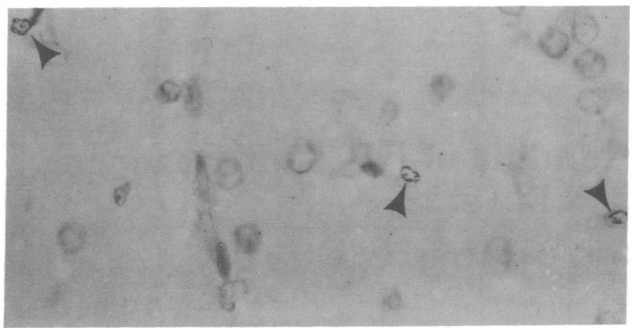

FIG. 1. Autoradiograph of rat cerebral cortex after a bolus oral intubation of [^{3}H]flunitrazepam. Note the three labeled nuclei (arrows). Labeling tends to be associated with the outer portion of the nucleus. (×174.)

ples of the fractions, resuspended in 50 mM Tris buffer/0.32 M sucrose, to either 5 nM [^{3}H]flunitrazepam or 5 nM [^{3}H]flunitrazepam plus 3 μM unlabeled flunitrazepam. The assay tubes were then incubated at 37°C for 15 min with shaking strictly at a pH of 7.3, followed by equilibration for 30 min in an ice bath. To collect the fraction binding the [^{3}H]flunitrazepam, we added 10 ml of ice-cold 50 mM Tris buffer (pH 7.5) to each incubation tube and poured the contents over Whatman GF/A glass-fiber filters with suction. The filters were washed free of unbound flunitrazepam with an additional 10 ml of Tris buffer and placed in 5 ml of Bray's solution. [^{3}H]Flunitrazepam was measured in a Nuclear Chicago scintillation counter. Specifically bound [^{3}H]flunitrazepam, as given herein, was the activity in the assay tube incubated with only the [^{3}H]flunitrazepam minus the activity in the appropriate assay tube with the [^{3}H]flunitrazepam and the large excess of unlabeled flunitrazepam, as is the convention in determining specific binding. All assays contained 5 μM [^{3}H]flunitrazepam since 10% of the available flunitrazepam was bound at this concentration (13). This allowed for greater or lesser binding to be measured in the various experiments while a 600 M excess was still maintained in the nonspecific assay tubes. Additions of xanthine, hypoxanthine, uracil, and inosine to the assay were made as described (10), maintaining an assay pH of 7.3.

Protein Determination. Protein in the nuclear fraction or in the synaptosomal membrane fraction being assayed was determined by the method of Lowry *et al.* (15).

Preparation of Nuclei. Nuclei were prepared by isolating cerebral cortical neuronal cells from rats (200–250 g) by the method of Sellinger *et al.* (16) and preparing nuclei from these neuronal cells by the method of Knüsel *et al.* (17) as previously described (18). The nuclei were extremely pure and homogeneous. Purity of the preparation during the isolation procedure was monitored by light microscopy of preparations in 0.06% Azure C in 0.25 M sucrose. All nuclear preparations were assayed for the plasma membrane marker enzyme 5′-nucleotidase (19) and UDPase, a smooth endoplasmic reticulum marker (20); these markers were not detected in any of the preparations.

RESULTS

Purity and Morphology of Rat Cerebral Cortex Nuclei. In Fig. 2 is shown an electron micrograph of the nuclei used in the biochemical studies reported herein. The nuclei show a high degree of structural integrity, and all morphologic analyses showed an absence of cytoplasmic membrane material in the purified preparations.

Kinetic Analysis of [^{3}H]Flunitrazepam Binding to Rat Cerebral Cortex Nuclei. The data in Figs. 3 and 4 demonstrate the following. Binding of [^{3}H]flunitrazepam to the nuclei was

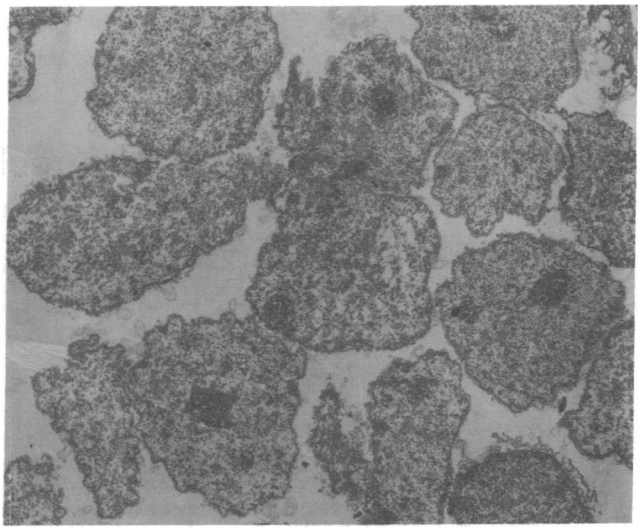

FIG. 2. Electron micrograph of the nuclear fraction of rat forebrain. The fraction is relatively free of nonnuclear fragments, and the nucleolemmae remain intact. (×2740.)

saturable (Fig. 3), and the $K_{d(app)}$ for the binding was 28 nM. In exactly similar experiments in this laboratory (13), binding of [^{3}H]flunitrazepam to synaptosomal membranes had a $K_{d(app)}$ of 1.1 nM. That the $K_{d(app)}$ is more than an order of magnitude different clearly shows that one is dealing with completely different interactions at the membrane and nuclear levels; indeed, it is of interest that the nuclear $K_{d(app)}$ is higher than that of the synaptosomal membrane-binding $K_{d(app)}$.

The Scatchard analysis of the data (Fig. 4) shows that all of the sites at the nuclear level have the same intrinsic binding constants, consistent with a single macromolecule–ligand interaction. It is not known whether the binding represents a translocation of the membrane–ligand form of the binding to a nuclear site or whether the nuclear binding is coincidental to the membrane binding. The pharmacologic significance of the binding shown in Figs. 3 and 4 seems of undeniable importance because nuclear binding or localization is seen to a great extent *in vivo* (Fig. 1).

Inhibition of Specific [^{3}H]Flunitrazepam Binding to Either Synaptic Membranes or Purified Nuclei. The data presented in Table 1 show that at very high concentrations [^{3}H]-flunitrazepam binding to synaptic membranes (fraction B) is inhibited significantly by inosine and hypoxanthine but not by

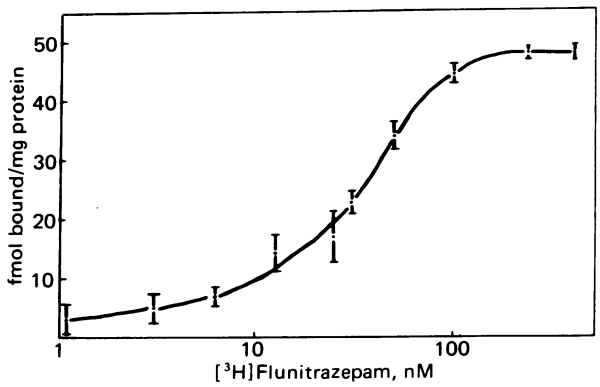

FIG. 3. Binding of [^{3}H]flunitrazepam to purified forebrain nuclear macromolecules as a function of concentration of [^{3}H]flunitrazepam. Each assay tube contained 2 mg of purified nuclear protein and specific binding was determined as given in the text. Each point is the mean ± 1 SD.

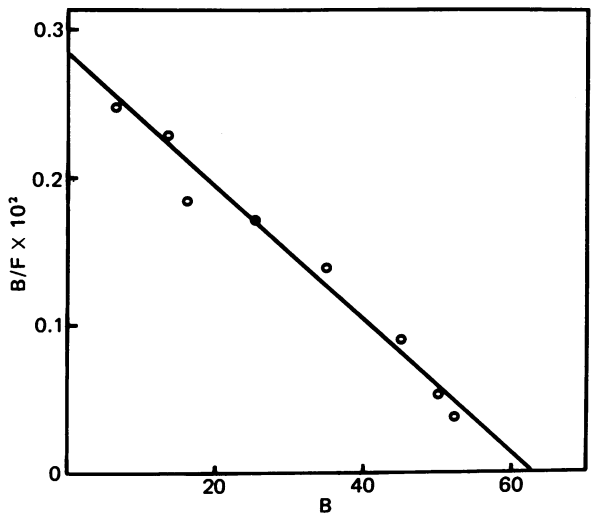

FIG. 4. Scatchard analysis of the data presented in Fig. 3. The fact that a straight line was obtained indicates that all of the binding sites have the same intrinsic binding constant (commonly referred to as one binding site) with affinity, $K_{d(app)}$, 28 nM. B, bound flunitrazepam; F, free flunitrazepam.

uracil or xanthine. These data are consistent with those of the workers who first reported this inhibition (9, 10). Interestingly, binding of the [^{3}H]flunitrazepam to the nuclear fraction is also inhibited by inosine and hypoxanthine, but not by uracil and xanthine. Of even greater interest is that the inhibition by the purine and its nucleoside (hypoxanthine and inosine) is much greater in the nuclear fraction than in the synaptic membrane fraction. Referring to Table 1, for example, 4 mM inosine inhibits the binding of [^{3}H]flunitrazepam 24% in the synaptic membrane fraction and 49% in the nuclear fraction; 4 mM hypoxanthine inhibits specific [^{3}H]flunitrazepam binding to synaptic membranes 18% and to nuclei 40%. Thus, these inhibitors have a much more profound effect against the nuclei–ligand binding than against the synaptic membrane–ligand binding.

DISCUSSION

The results presented clearly indicate an interaction between [^{3}H]flunitrazepam and cerebral cortex nuclei *in vivo* and, more important, a specific binding of flunitrazepam *in vitro* to nuclei. Indeed, "physiologic neurotransmitters" or pharmacologic psychotropic agents are implicated to be present at the neuronal nuclear site; usually these agents are postulated to interact at the synaptic membrane, cytoplasmic enzyme, or synaptic vesicle level. The finding of a direct binding *in vivo* and *in vitro* of a benzodiazepine to nuclei may necessitate a rethinking of its (as well as other psychotropic agents') molecular mode of action or toxicity, dependency, and tolerance (21), whether these results pertain to "receptor mechanisms" or not.

The fact that flunitrazepam specifically binds more tightly to the synaptosomal membrane binding site (K_d = 1.1 nM) than to the nuclei (K_d = 28 nM) leads to the postulation of a model in which the ligand first interacts at the membrane level and then, either as free ligand or ligand–binding macromolecule complex, is translocated to the nucleus. Another model might be dual interactions, with some molecules interacting at the membrane level (efficacy?) while others interact at the nuclear level (toxicity?).

Of great interest is the fact that the specific nuclear binding of [^{3}H]flunitrazepam is more highly inhibited by the purine and nucleoside than the specific membrane binding. Because the nucleus has high concentrations of purines and nucleosides and because nucleosides and purines bind to nuclear constituents such as DNA and histones, it is possible that the control mechanisms or endogenous ligand for benzodiazepines resides in molecular hypoxathine or inosine.

Table 1. Inhibition of specific [^{3}H]flunitrazepam binding to both "synaptic" membranes and purified nuclei from rat cerebral cortex

Potential inhibitor	Final conc., mM	% of control* Synaptic membrane fraction	% of control* Purified nuclei fraction
Inosine	4.0	76 ± 2	51 ± 2
	0.4	80 ± 4	74 ± 4
	0.04	96 ± 2	96 ± 4
Hypoxanthine	4.0	82 ± 6	60 ± 3
	0.4	90 ± 2	78 ± 3
	0.04	101 ± 3	88 ± 6
Uracil	4.0	94 ± 2	92 ± 5
	0.4	92 ± 4	96 ± 2
	0.04	96 ± 2	90 ± 7
Xanthine	4.0	97 ± 3	101 ± 2
	0.4	99 ± 7	86 ± 9
	0.04	96 ± 4	92 ± 3

Synaptic membrane refers to fraction B as described in previous publications (2, 13) and in *Materials and Methods*. Assays were carried out in the presence or absence of the potential inhibitor. Results are expressed as % bound [^{3}H]flunitrazepam found in the presence of the inhibitor compared with that in an assay in which buffer replaced the potential inhibitor. All assays were carried out at pH 7.3.

* Mean ± 1 SD.

Because hypoxanthine and inosine do not inhibit [^{3}H]flunitrazepam as much at the synaptosomal membrane, it is possible that control mechanisms or endogenous ligand activity may reside in the polypeptide BCF-I and BCF-II inhibitors which have been described (12). In any event, it is obvious that the two sites of specific binding allow for more than one endogenous inhibitor to be active.

In conclusion, although a true receptor mechanism has not been established for the benzodiazepine binding to nuclei, the specific binding has been described. Aside from the pharmacologic importance, the elucidation of just what role the flunitrazepam nuclear binding plays in the molecular action of benzodiazepines should prove exciting.

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1. Squires, R. F. & Braestrup, C. (1977) *Nature (London)* **266**, 732–734.
2. Bosmann, H. B., Case, K. R. & DiStefano, P. (1977) *FEBS Lett.* **82**, 368–372.
3. Mohler, H. & Okada, T. (1977) *Life Sci.* **20**, 2101–2110.
4. Braestrup, C. & Squires, R. F. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3805–3809.
5. Chang, R. S. & Snyder, S. H. (1978) *Eur. J. Pharmacol.* **48**, 213–218.
6. Knochman, R. L. & Mackerer, C. R. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 773 (abstr.).
7. Mohler, H. & Okada, T. (1977) *Science* **198**, 849–851.
8. Speth, R. C., Wastek, G. J., Hruska, R. E., Reisine, T. D., Kobayashi, T. M. & Yamamura, H. I. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 773 (abstr.).
9. Marangos, P. J., Paul, S. M., Greenlaw, P., Goodwin, F. K. & Skolnick, P. (1978) *Life Sci.* **22**, 1893–1900.

10. Skolnick, P., Marangos, P. J., Goodwin, F. K., Edwards, M. & Paul, S. (1978) *Life Sci.* **23,** 1473–1480.
11. Nielsen, M., Gredal, O. & Braestrup, C. (1979) *Life Sci.* **25,** 678–686.
12. Colello, G. D., Hochenbery, D. M., Bosmann, H. B., Fuchs, S. & Folkers, K. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 6319–6323.
13. Bosmann, H. B., Penney, D. P., Case, K. R., DiStefano, P. & Averill, K. (1978) *FEBS Lett.* **87,** 199–202.
14. Spurr, A. R. (1969) *J. Ultrastructure Res.* **25,** 31–39.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
16. Sellinger, O. Z., Azcurea, J. M., Johnson, D. E., Ohlsson, W. G. & Loden, Z. (1971). *Nature (London) New Biol.* **230,** 253–256.
17. Knüsel, A., Lehner, B., Kuenzle, C. C. & Kistler, G. S. (1973) *J. Cell Biol.* **59,** 762–765.
18. Bosmann, H. B. & Case, K. R. (1975) *Neurobiology* **5,** 35–41.
19. Bosmann, H. B. & Pike, G. Z. (1971) *Biochim. Biophys. Acta* **227,** 402–412.
20. Bosmann, H. B. (1970) *Biochim. Biophys. Acta* **220,** 560–568.
21. DiStefano, P., Case, K. R., Colello, G. D. & Bosmann, H. B. (1979) *Cell Biol. Int. Rep.* **3,** 163–167.