Neurol. **169**, 133 (1976); M. Constantine-Paton and M. I. Law, *Science* **202**, 639 (1978).
12. R. L. Meyer, *Diss. Abstr. Int. B* **35**, 1510 (1975). Fibers deflected into inappropriate lateral positions of the opposite tectum would innervate these regions when the normal optic fibers to these regions were cut by a tectal incision.
13. R. L. Meyer, *Exp. Neurol.* **56**, 23 (1977).
14. In three of the unsuccessful cases, no ipsilateral units or only a few weak ones were recordable. In the fourth fish, the deflected fibers were inadvertently inserted at the medial edge of tectum thereby cutting fibers of passage to the posterior tectum (*7*). The map was comparable to that of previous experiments (*6*) in which these fibers were intentionally cut.
15. Paraffin embedding, 30-μm sections, and 1 week's exposure were specified after the method of W. M. Cowan, D. I. Gottlieb, A. E. Hendrickson, J. L. Price, and T. A. Woolsey [*Brain Res.* **37**, 21 (1972)]; for details see (*5*, *6*, *11*).
16. Pairs of field positions were classified as clearly reversed or nonreversed (normal polarity or confused). Each pair was unique so that fields from the most anterior tectal positions were included only as the object of a comparison. Occasionally a comparative unit could not be obtained at exactly 250 μm anterior along the row of recording points. In this case the closest anterior units > 250 μm were compared. Rarely, one tectal penetration produced two separated receptive fields; a position midway between was used for such comparisons.
17. S. C. Sharma, *Exp. Neurol.* **41**, 661 (1973); R. L. Levine and M. Jacobson, *Brain Res.* **98**, 172 (1975).
18. In a number of previous studies in which a part of retina or tectum has been surgically rotated or otherwise transplanted, the resulting projection has sometimes been contrary to the original polarity of the transplanted tissue. The problem has been that the surgery itself can lead to an alteration or regulation of the original polarity so that the projection may actually be in accordance with the existing polarity of the transplanted tissue. Through the work of Hunt, Jacobson, and others, such regulation of polarity has, in fact, been shown to be the case at least when the surgery is performed at embryonic stages. For juvenile and adult stages, the question has remained unanswered. In the present experiment, this kind of regulative change could not occur because the integrity of both retina and tectum were preserved. This demonstration that selective interfiber interactions can predominate over intrinsic polarity suggests that regulation need not be the explanation for previous transplantation experiments in juvenile and adult animals. A recent preliminary report by J. Rho [*Biophys. J.* **21**, 137A (1978)] indicates, in fact, that regulation is not occurring. In an exhaustive and ingenious series of tectal transplantations, Rho observed elements of both appropriate and inappropriate polarity in one graft. This means that either regulation is exceedingly complex and varied or that it is not the explanation. Taken together, Rho's results and the present findings suggest that polarity of a projection onto a transplanted piece of tectum can be appropriate or inappropriate depending on the degree to which fibers invading the transplant can communicate with fibers in surrounding tectum. Variability in healing of the transplanted tissue or in the rapidity with which fibers invade it would then account for the variability of previous results. For detailed discussion of this literature, see R. K. Hunt and M. Jacobson [*Curr. Top. Dev. Biol.* **8**, 203 (1974)]; R. L. Meyer and R. W. Sperry [in *Plasticity and Recovery of Function in the Central Nervous System*, D. G. Stein, J. J. Rosen, N. Butters, Eds. (Academic Press, New York, 1974), p. 45]; and R. Levine and M. Jacobson [*Exp. Neurol.* **43**, 527 (1974)].
19. T. J. Horder, *J. Physiol. (London)* **216**, 53P (1971); M. Yoon, *Am. Zool.* **12**, 106 (1972); *Exp. Neurol.* **37**, 451 (1972).
20. I thank R. W. Sperry, C. R. Hamilton, K. Gaston, and L. Wolcott for their comments on the manuscript; J. Macenka for histology; and L. Wolcott for illustrations. This work was supported by PHS grant MH-03372 to R. W. Sperry.

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GABA Receptors in Clonal Cell Lines: A Model for Study of Benzodiazepine Action at Molecular Level

Abstract. *A "receptor unit" for γ-aminobutyric acid (GABA), which includes brainlike receptor binding sites for tritium-labeled GABA and benzodiazepines (diazepam, clonazepam, and flunitrazepam) and a thermostable endogenous protein (GABA modulin) that inhibits both GABA and benzodiazepine binding, has been demonstrated in membranes prepared from NB_{2a} neuroblastoma and C6 glioma clonal cell lines. In these cells, as in brain, diazepam (1 micromolar) prevents the effect of GABA modulin, and in turn GABA (0.1 millimolar) increases the binding of [^{3}H]diazepam. The neuroblastoma and, to a lesser extent, the glioma cells represent a suitable model to study the interactions and the sequence of membrane and intracellular events triggered by the stimulation of benzodiazepine and GABA receptors.*

In crude synaptic membranes prepared from brain, benzodiazepines increase the affinity of γ-aminobutyric acid (GABA) receptors by competing with an endogenous thermostable protein. This protein has been termed "GABA modulin" because it allosterically modulates the high-affinity binding of GABA to its postjunctional receptor sites (*1*, *2*). The potency of several benzodiazepines in competing with GABA modulin correlates with their binding affinity for specific sites in crude synaptic membrane preparations and with their in vivo ability to relieve anxiety (*3*). This relationship has suggested that the action of benzodiazepines on GABA modulin represents a possible molecular mechanism for their well-documented facilitatory action on GABA transmission in vivo (*4*). Since GABA modulin could be released from its storage sites in brain and bind to synaptic membranes during homogenization (*1*), it could not be concluded from experiments with crude brain synaptic membranes whether interaction with GABA modulin accounts for benzodiazepine modification of GABA receptors in vivo.

We now present evidence that mouse neuroblastoma NB_{2a} (NB) cells and rat C6 glioma (C6) cells are an adequate model for studying the supramolecular organization of GABA receptors and for monitoring the action of benzodiazepines at the molecular level. For such studies, an ideal cell line should contain on its membrane: (i) the benzodiazepine receptor, (ii) the GABA receptor regulating a Cl^- channel, and (iii) GABA modulin. Moreover, to satisfy the postulates that derive from experiments with brain tissue, it should be shown that these three processes interact with each other in the following ways: (i) GABA modulin should inhibit the high-affinity binding of GABA and benzodiazepines (*1*, *2*); (ii) the activation of GABA receptors should change the flux of Cl^- across the membrane (*5*); (iii) the K_d values for binding of GABA (*6*) and benzodiazepines (*7*) should be similar to those in brain membranes (*8*); and (iv) the addition of benzodiazepines should change the membrane binding sites for GABA from a homogeneous class with a K_d of 200 nM to two classes (K_{d1} = 200 nM, K_{d2} = 20 nM) (*1*, *2*), and vice versa: the addition of GABA should lower the K_d for benzodiazepine binding (*8*).

The high-affinity receptors for the benzodiazepines and GABA located on the membranes of NB and C6 cells have properties similar to those of receptors located on brain membranes (Table 1). The K_d's of diazepam, clonazepam, and flunitrazepam are approximately equal in C6 (5 to 6 nM), NB (3 to 9 nM), and rat brain cortex (3 to 7 nM) membranes (*9*). In the membranes of NB cells, as in brain membranes, the affinity of [^{3}H]-clonazepam and [^{3}H]flunitrazepam for the receptor is higher than that for [^{3}H]diazepam (Table 1).

The density of binding sites is three- to fourfold higher in brain synaptic membranes than in the membranes of NB cells. The B_{max} values for the three [^{3}H]benzodiazepines are similar in membranes of NB cells; however, in C6 cells, [^{3}H]clonazepam labeled only half as many binding sites as did [^{3}H]diazepam (Table 1).

The membranes prepared from brain and those prepared from clonal cell lines have similar kinetics for the high-affinity binding of GABA to receptors. Scatchard analysis of the saturation curve obtained with freshly prepared membranes from NB revealed only one receptor component. When these membranes were frozen, thawed, and treated with Triton X-100 (*1*), Scatchard analysis revealed two populations of GABA receptors. The binding of GABA to the high-affinity site of both NB and C6 cells was saturable; GABA was displaced by

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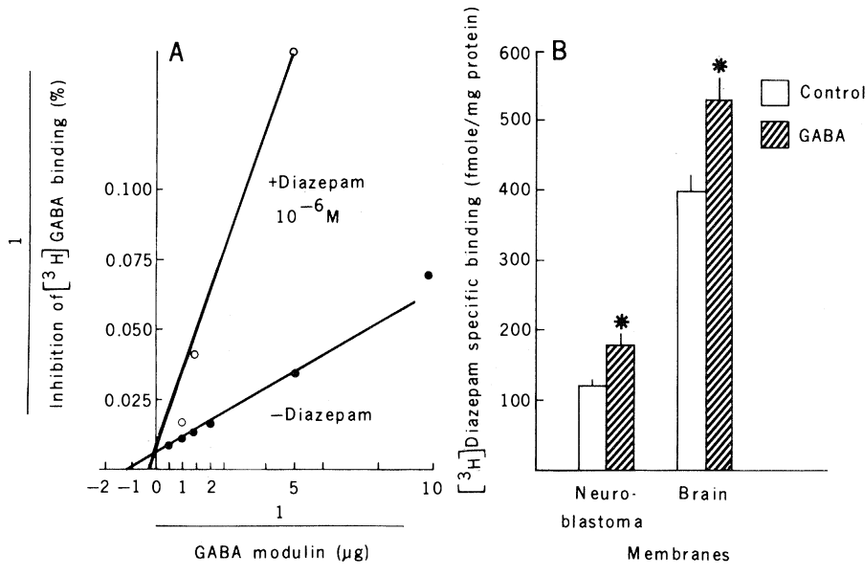

Fig. 1. (A) Interaction between diazepam and GABA modulin on binding of [^{3}H]GABA to mouse NB_{2a} neuroblastoma membranes. Crude membranes from NB cells were prepared by successive freezing, thawing, and washing with tris-citrate buffer plus treatment with Triton X-100 (*1*). This membrane preparation was frozen at −20°C for another 16 to 18 hours. After thawing and washing twice with tris-citrate buffer, the membranes (approximately 250 μg of protein per milliliter) were incubated at 0°C for 15 minutes with $10^{-6}M$ diazepam or solvent before addition of GABA modulin. The data are graphed by the Lineweaver-Burke method. This plot shows an apparent competitive interaction between diazepam and GABA modulin. GABA modulin was extracted from NB cell membranes as described for Table 1. Partial purification was achieved by filtering the boiled and dialyzed extract on a Sephadex G-100 column (*1*). (B) Effect of GABA on [^{3}H]diazepam binding. Fresh crude membranes from mouse NB cells and rat brain cortex were obtained as described (*1*). The fresh membranes were frozen at −20°C for 16 to 18 hours. After thawing and washing with 50 mM tris-citrate buffer (pH 7.1), the membranes (approximately 300 to 350 μg of protein per milliliter) were used for assay. A typical reaction mixture contained, in a total volume of 1 ml, approximately 300 μg of membrane proteins, 6 nM [^{3}H]diazepam (39 Ci/mmole, New England Nuclear), and $10^{-4}M$ GABA. The mixture was incubated at 0°C for 15 minutes. Specific [^{3}H]diazepam binding is defined as the difference between total [^{3}H]diazepam binding and that obtained in the presence of $5 \times 10^{-5}M$ flunitrazepam. $^*P < .05$ when compared with control group.

muscimol [concentration producing 50 percent inhibition (IC_{50}) = $5 \times 10^{-9}M$] and bicuculline methiodide (IC_{50} = $10^{-5}M$). The pH optimum for GABA binding was ~ 7 for membranes of both NB and C6 cells. In membranes prepared from brain cortex, the high-affinity site for GABA binding is unmasked after GABA modulin has been removed by freezing, thawing, and repeated washing with Triton X-100 and tris-citrate buffer. This procedure increases the affinity of GABA receptors for GABA in membranes of C6 and NB cells and also removes a significant amount of the GABA modulin (Table 1).

Partially purified preparations of GABA modulin obtained from membranes of NB cells inhibit in a dose-related fashion the high-affinity Na^+-independent [^{3}H]GABA binding to membranes of NB cells which had been previously frozen, thawed, treated with Triton X-100, and repeatedly washed with tris buffer (Fig. 1A). Furthermore, diazepam ($10^{-6}M$) prevents the action of GABA modulin on the high-affinity binding of [^{3}H]GABA. The kinetics of this interaction appear to be competitive (Fig. 1A). Similar results were obtained with membranes from C6 cells. In addition, in membranes of NB or C6 cells, the activation of the GABA receptor increases the binding for benzodiazepines. When membranes prepared from NB or C6 cells were treated with GABA ($10^{-4}M$)

Table 1. Comparison of kinetic constants for benzodiazepine and GABA binding in membranes prepared from clonal cell lines and rat brain. Mouse NB and rat C6 cells were grown to confluency in Dulbecco's modification of Eagle's medium containing 10 percent fetal calf serum (*14*). For benzodiazepine binding studies, the membranes (crude mitochondrial fractions) (*1*) were frozen for 12 hours and then washed with 200 volumes of 50 mM tris-citrate buffer (pH 7.1) just before assay. The nonspecific binding was determined by using $5 \times 10^{-5}M$ cold flunitrazepam with [^{3}H]diazepam, $5 \times 10^{-6}M$ diazepam with [^{3}H]clonazepam, and $5 \times 10^{-6}M$ diazepam with [^{3}H]flunitrazepam. [^{3}H]GABA binding was determined in fresh or frozen membranes treated with Triton X-100 (*1*). In freshly prepared membranes the total binding per milligram of protein for 20 nM [^{3}H]GABA was 3500 count/min, and the nonspecific binding was 2500 count/min. In frozen membranes treated with Triton X-100 the total binding per milligram of protein was 4500 count/min and the nonspecific binding was 2000 count/min. GABA modulin content was assayed with 20 nM [^{3}H]GABA and 200 μg of brain synaptic membranes, frozen and washed several times with Triton X-100 to remove endogenous GABA modulin. One unit (U) of GABA modulin is the amount that produces 20 percent inhibition of [^{3}H]GABA binding. The soluble GABA modulin represents the inhibitory material extracted by disrupting the cell membranes with homogenization (polytron setting 6 for 30 seconds) in H_2O and then washing with 50 mM tris-citrate buffer. The aqueous and tris buffer supernatants (after centrifugation at 40,000g for 20 minutes) were pooled and heated at 95°C for 10 minutes. The sample was then dialyzed for 72 hours against the same tris buffer and centrifuged at 40,000g for 10 minutes. The clear supernatant appropriately diluted was for measuring inhibitory activity. Each value is the mean of three separate experiments; GABA modulin is given as units per milligram of membrane protein. Variation from the mean was never greater than 15 percent. [methyl-^{3}H]Diazepam (39 Ci/mmole) and [2,3-^{3}H(N)]GABA (40 Ci/mmole) were from New England Nuclear. Generally labeled [^{3}H]clonazepam (20 Ci/mmole) and [^{3}H]flunitrazepam (20 Ci/mmole) were gifts from Hoffmann-La Roche.

Membrane	Specifically bound [^{3}H]benzodiazepines						Specifically bound [^{3}H]GABA						GABA-modulin (U/mg)
	Diazepam		Clonazepam		Flunitrazepam		Fresh		Frozen + Triton X-100				
									Low affinity		High affinity		
	K_d (nM)	B_{max} (pmole/mg)	K_d (nM)	B_{max} (pmole/mg)	K_d (nM)	B_{max} (pmole/mg)	K_d (nM)	B_{max} (pmole/mg)	K_d (nM)	B_{max} (pmole/mg)	K_d (nM)	B_{max} (pmole/mg)	
NB_{2a} neuroblastoma	9.2	0.24	3.7	0.23	3.4	0.23	200	0.39	300	0.9	19	0.22	25
C6 glioma	5.0	0.15	5.6	0.8			210	0.41	260	0.2	26	0.21	20
Brain cortex	6.9	0.84	2.2	1.2	3.6	0.98	220	2.5	130	4.9	15	0.75	10

before addition of 6 n*M* [^{3}H]diazepam, the amount of specifically bound [^{3}H]-diazepam was increased by approximately 30 percent. This increase was similar to that observed in brain membrane preparations (Fig. 1B).

Clonal lines of neuroblastoma and glioma cells have proved to be a suitable substrate for studying molecular mechanisms in the organization of catecholamine (*10*) and opiate receptors (*11*). The data reported here support the view that NB and, to a lesser degree, C6 cell lines are also adequate models for studying regulation of the GABA "receptor unit." This unit is composed of GABA and benzodiazepine receptors and GABA modulin. The physiological interaction of GABA modulin with GABA and benzodiazepine receptors in the membranes of NB and C6 glioma cells is indirectly suggested by studies with intact cells: (i) in intact NB cells (*12*), [^{3}H]clonazepam and [^{3}H]diazepam label a similar number of benzodiazepine receptors, with a K_d essentially identical to that shown in isolated neuroblastoma membranes; (ii) diazepam (10^{-6}*M*) added to NB or C6 cell cultures produces 50 percent release of the inhibitor into the medium and a concomitant 50 percent decrease of the inhibitor in the cell membranes.

In addition, the regulation of the GABA receptor units located on NB or C6 cell membranes is in many aspects similar to the regulation of those located on brain membranes. In NB and C6 clonal cell lines, as in brain, GABA binding is regulated by GABA modulin and its kinetics are modified by the activation of specific benzodiazepine receptors. Moreover, the GABA receptor can also modify the benzodiazepine receptor and as a result it can increase the affinity of the specific binding sites for benzodiazepines. Whereas the benzodiazepines act competitively with GABA modulin (*2*), it is not known whether GABA also regulates diazepam binding by acting on GABA modulin. One could speculate that the physiological agonist of the benzodiazepine receptor is GABA modulin. Conversely, one could also propose that an endogenous benzodiazepine agonist modulates the function of the GABA modulin, but such an agonist has not yet been found.

It is pertinent to discuss the physiological role linked to the interactions between GABA and GABA modulin and the pharmacological implications of the consequences of the activation of the benzodiazepine receptors. One possibility is that GABA modulates a Cl^- channel in NB and C6 cell lines. Initial results with NB cells indicate that the activation of GABA receptors causes an increase in the inward Cl^- flux (*13*); whether the cooperative interaction of the benzodiazepine and GABA receptors extends also to the GABA regulation of Cl^- channels remains to be investigated.

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References and Notes

1. G. Toffano, A. Guidotti, E. Costa, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4024 (1978).
2. A. Guidotti, G. Toffano, E. Costa, *Nature (London)* **275**, 553 (1978).
3. A. Guidotti, G. Toffano, M. Baraldi, J. P. Schwartz, E. Costa, in *GABA-Neurotransmitters* (Munksgaard, Copenhagen, 1979), pp. 406–415; A. Guidotti, M. Baraldi, J. P. Schwartz, E. Costa, in *Advances in Pharmacology and Therapeutics*, P. Simon, Ed. (Pergamon, Oxford, 1978), pp. 33–41.
4. W. Haefely, A. Kulcsar, H. Möhler, L. Pieri, P. Polc, R. Schaffner, in *Mechanisms of Action of Benzodiazepines*, E. Costa and P. Greengard, Eds. (Raven, New York, 1975), pp. 131–151; E. Costa, A. Guidotti, C. C. Mao, A. Suria, *Life Sci.* **17**, 167 (1975); D. W. Choi, D. H. Farb, G. D. Fischbach, *Nature (London)* **296**, 342 (1977); R. MacDonald and J. I. Barker, *ibid.* **271**, 563 (1978); D. W. Gallager, *Eur. J. Pharmacol.* **49**, 133 (1978); H. M. Geller, D. A. Taylor, B. J. Hoffer, *Naunyn-Schmiedebergs Arch. Pharmacol.* **304**, 81 (1978); S. N. Kozhechkin and R. V. Ostrovskaya, *Nature (London)* **269**, 72 (1977).
5. K. Krnjevic, *Physiol. Rev.* **54**, 418 (1974); E. Roberts, *Biochem. Pharmacol.* **23**, 2637 (1974).
6. S. R. Zukin, A. B. Young, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4802 (1974); R. W. Olsen, in *GABA in Nervous System Function*, E. Roberts *et al.*, Eds. (Raven, New York, 1974), pp. 287–304; S. J. Enna and S. H. Synder, *Mol. Pharmacol.* **13**, 442 (1977).
7. R. F. Squires and A. Braestrup, *Nature (London)* **266**, 732 (1977); H. Möhler and T. Okada, *Science* **198**, 849 (1977); R. C. Speth, G. J. Wastek, P. C. Johnson, H. I. Yamamura, *Life Sci.* **22**, 859 (1978); C. R. Mackerer, R. L. Kochman, B. A. Bierschenk, S. S. Bremner, *J. Pharmacol. Exp. Ther.* **206**, 405 (1978).
8. J. F. Tallman, J. W. Thomas, D. Gallager, *Nature (London)* **274**, 383 (1978); G. J. Wastek, B. Speth, T. D. Reisine, H. I. Yamamura, *Eur. J. Pharmacol.* **50**, 445 (1978).
9. Preliminary results have been reported (*3*).
10. X. O. Breakefield, *Life Sci.* **18**, 267 (1976); P. Schubert, S. Heinemann, W. Carlisle, H. Tarikas, B. Kimes, J. Patrick, J. H. Steinbach, W. Culp, B. L. Brandt, *Nature (London)* **249**, 224 (1974).
11. W. A. Klee and M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3474 (1974); W. A. Klee, A. Lampert, M. Nirenberg, in *Opiates and Endogenous Opioid Peptides*, H. W. Kosterlitz, Ed. (North-Holland, Amsterdam, 1976), pp. 153–159.
12. The cells were suspended in 150 m*M* tris-citrate buffer (*p*H 7.1). [^{3}H]Diazepam and [^{3}H]clonazepam binding was assayed at 0°C by the same procedure as described for cell membranes (Table 1).
13. M. Baraldi, A. Guidotti, E. Costa, *Neurosci. Abstr.*, in press.
14. J. P. Schwartz, D.-M. Chuang, E. Costa, *Brain Res.* **137**, 369 (1977).

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Menopausal Flushes: A Neuroendocrine Link with Pulsatile Luteinizing Hormone Secretion

Abstract. *Menopausal flush episodes were found to be invariably associated with the initiation of pulsatile pituitary release of luteinizing hormone. This was not accompanied by a significant change in circulating catecholamine or prolactin concentrations. Since pulsatile luteinizing hormone release results from episodic secretion of luteinizing hormone releasing factor by the hypothalamus, these findings suggest a link between the neuroendocrine mechanisms that initiate such episodic secretion and those responsible for the onset of flush episodes.*

Although estrogen withdrawal unquestionably plays a major role in the development of menopausal flushes, the physiological mechanism for the initiation of flushes and of transient physical changes during flush episodes (*1, 2*) remains elusive. It has been suggested that menopausal flushes are manifestations of vasomotor instability due to a transient increase in adrenergic activity (*3, 4*), but evidence to support this is lacking. The study described here was therefore designed to search for neuroendocrine correlates of spontaneous flushes in hypogonadal women.

A total of 55 flush episodes were studied in six postmenopausal women. All studies were carried out with the subjects at bed rest in a quiet room with a stable, ambient temperature. A normal diet with the exclusion of caffeine and nicotine was provided and all subjects were fully awake during the 8- to 10-hour studies. Onset of flush episodes was reported by the subjects and retrospectively confirmed by objective observation. Finger temperature was measured by using thermistors, and electrocardiogram and pulse rate were monitored. These parameters were continuously graphed by an eight-channel physiological recorder. Blood samples were obtained from an indwelling venous cannula at 2- to 15-minute intervals between flush episodes for determination of serum luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin (PRL) concentrations by radioimmunoassay (*5*) and plasma dopamine (DA), norepinephrine (NE), and epinephrine (E) concentrations by radioenzymatic assay (*6*).

The onset of each of the 55 flushes was characterized by a sudden intense sensa-

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