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A Neuroblastoma × Glioma Hybrid Cell Line with Morphine Receptors

(cell membranes/narcotics/neurobiology/tissue culture)

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ABSTRACT **A neuroblastoma × glioma hybrid cell line with well-developed neural properties was found that has high-affinity morphine receptors. The average cell contains approximately 3 × 10^5 receptors. In contrast, parent cells and other neuroblastoma or hybrid cell lines tested had few or no morphine receptors.**

Membrane preparations that bind narcotic analgesics such as morphine and its congeners have been described by a number of laboratories (1–9). Such binding satisfies the criteria first enunciated by Goldstein *et al.* (10) for a narcotic receptor in that it is specific for the pharmacologically active D (−) enantiomers of the narcotics. Furthermore, there is a generally satisfactory correlation between binding affinities measured for various opiates and their potencies as analgesics *in vivo* (5) as well as a somewhat more satisfactory correlation between binding strength and narcotic agonist potency measured with isolated intestinal preparations (11). The membrane preparations studied to date were isolated from brain or intestine and, therefore, are derived from mixtures of cell types. We hoped to obtain a relatively homogenous cell population for our studies of opiate action by examining clonal lines of neuroblastoma cells (12) for morphine receptors. Somatic cell hybrids also were studied, since previous results showed that genes for neural properties can be activated (13) or deactivated (14) in hybrids formed by the fusion of neuroblastoma cells with other cell types. New phenotypes thus generated can be inherited and thereby perpetuated in a fairly stable fashion.

In this report, we wish to describe cell lines with and without morphine receptors.

MATERIALS AND METHODS

Chemicals were obtained from the following sources: [^{3}H]dihydromorphine (51.6 Ci/mmole) from New England Nuclear Corp.; morphine sulfate from Merck and Co.; naloxone hydrochloride was donated by Endo Laboratories, Inc.; the other narcotics used were kindly supplied by Dr. Everette May; fetal bovine serum was from Colorado Serum Co.; DMEM (Dulbecco-Vogt modification of Eagle's minimal essential medium) was from GIBCO (Catalogue no. H21). Other chemicals were of reagent grade purity.

Cell Lines. The neuroblastoma cell lines used were derived from the mouse C-1300 tumor; their properties have been described previously (12). Neuroblastoma × glioma hybrids, NG108-5 and NG108-15, were obtained* by Sendai virus-induced fusion of neuroblastoma clone N18TG-2, resistant to 6-thioguanine (14), and C6Bu-1, a 5-bromodeoxyuridine-resistant clone of the rat glioma C6 (15). Clone NL1F is a hybrid formed by fusion of neuroblastoma clone N4TG1 and mouse L cell clone B82 (14).

Neuroblastoma and C6Bu-1 cells were grown in petri dishes or flasks in 90% DMEM, 10% fetal bovine serum, in a humidified atmosphere of 90% air, 10% CO_2, and 37° as described (12). Hybrid cells were grown in the same medium supplemented with 0.1 mM hypoxanthine, 1 μM aminopterin, and 12 μM thymidine. For drug binding experiments, the cells usually were grown to confluency and harvested by scraping (from petri dishes) or shaking (from flasks) in their growth medium. The cells were centrifuged at approximately 500 × *g* for 5 min at room temperature and the pellets (generally less than 1 ml) were washed two times with 50 ml of D1 [137 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose (16)] and suspended in a solution of 0.32 M sucrose, 0.01 M Tris·HCl (pH 8) at 3° so that the concentration of protein was close to 0.5 mg/ml. Uniform cell suspensions were achieved by aspirating the suspension in a 10-ml plastic pipet three times. Cell suspensions were kept at 3° and usually used immediately for binding studies. Cell suspensions that were frozen and stored at −60° retained the specific binding of [^{3}H]dihydromorphine.

Routine Assay Method. A cell suspension prepared as just described (900 μl) was added to a plastic test tube containing either 10 μl of water or 10 μl of 0.1 mM morphine sulfate. A solution, 100 μl, containing [^{3}H]dihydromorphine (10 nM, 51.2 Ci/mmole) dissolved in 0.32 M sucrose was added to each tube in a darkened room and the samples were mixed with a vortex mixer. After incubation at 37° for 10 min, tubes were centrifuged at 19,000 rpm (45,000 × *g*) for 10 min in a Sorvall RC2B centrifuge at 0° and supernatant fluids were removed with a 5-ml syringe equipped with a blunt 19-gauge needle and then discarded. Each tube and surface of the pellet was washed with 1 ml of 0.32 M sucrose and the washed pellet was suspended in 1 ml of 1% Triton X-100 and assayed for radioactivity after mixing with 7.5 ml of Triton–toluene scintillation fluid (17). All operations involving dihydromorphine were performed in very dim, indirect light. Each sample was assayed in triplicate or quadruplicate.

Neuroblastoma cell lines used were subcultured 5 to 30 times; hybrid cells were subcultured 10 to 20 times. In most cases a number of different subcultures were tested. Specific binding is defined as fmoles of [^{3}H]dihydromorphine bound per mg of protein (upon incubation of cells with nM [^{3}H]di-

* B. Hamprecht, T. Amano, and M. Nirenberg, in preparation.

hydromorphine) that are displaced by a 1000-fold, or higher, excess of unlabeled morphine.

Binding affinities of morphine and of other narcotics were estimated from experiments in which a range of concentrations of unlabeled opiates was tested for its ability to displace bound [^{3}H]dihydromorphine as described previously (9).

RESULTS

The specific binding of [^{3}H]dihydromorphine was tested with various cell lines derived from the nervous system as shown in Table 1. Little or no specific binding of dihydromorphine was detected with neuroblastoma clone N18TG-2 or rat glioma clone C6BU-1. However, specific binding sites for [^{3}H]dihydromorphine were found with a hybrid cell line, NG108-15, originating from the fusion of the neuroblastoma N18TG-2 and glioma C6Bu-1*. Relatively few specific narcotic binding sites were detected, under the conditions used, with a sister neuroblastoma × glioma hybrid cell line, NG108-5, neuroblastoma clones S20 and S20Y, and a neuroblastoma × L cell hybrid, NL1F. The low values may indicate the presence of a reduced number of receptors or receptors with a reduced affinity for morphine. Narcotic receptors were not detected with neuroblastoma lines N1E, N1E-115, N4, N18, or N18-TG-2, or with C6BU-1. Thus, two classes of cells were found: cells with morphine receptors, and cells that do not have these receptors in quantities sufficient to detect with the assay used.

Characterization of the Narcotic Receptors of NG108-15 Hybrid Cells: A portion, only, of the binding of [^{3}H]dihydromorphine is saturable in that it is displaced by nonradioactive morphine (Fig. 1), and indeed, this phenomenon is the basis of our assay procedure. Nonsaturable (that is nondisplaceable) binding of [^{3}H]dihydromorphine has been found in all tissues tested and is thought to be nonspecific binding. Only the saturable component of the binding shown in Fig. 1 is considered to be specific binding.

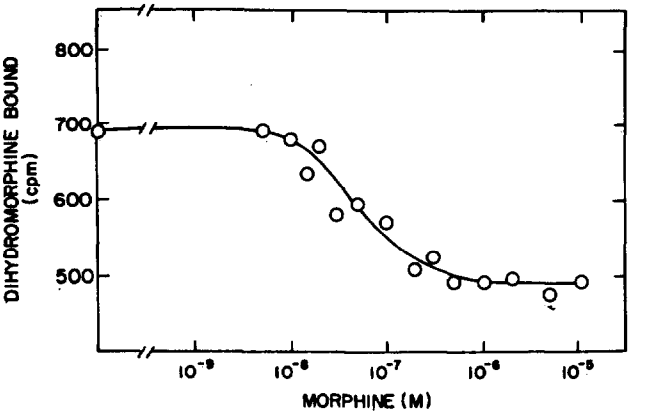

FIG. 1. The displacement of bound [^{3}H]dihydromorphine by different concentrations of unlabeled morphine from a suspension of NG108-15 cells (subculture 10, 468 μg of protein per tube). Each tube contained 1 nM [^{3}H]dihydromorphine and the indicated concentration of morphine.

The specific binding sites discriminate between (+) and (−) enantiomers of the narcotics as shown for the pair dextrorphan and levorphanol in Fig. 2. The latter compound is seen to displace specifically bound dihydromorphine at concentrations more than two orders of magnitude lower than does its analgesically inactive enantiomer, dextrorphan (18). A similar result has been obtained with the (−) and (+) enantiomers of metazocine, the latter of which is inactive as an analgesic (19) and as a displacer of dihydromorphine, at least at concentrations of μM and below.

The number of binding sites present in suspensions of NG108-15 can be estimated by measuring the amount of specific binding as a function of [^{3}H]dihydromorphine concentration. We find that, at saturation, approximately 0.6 pmoles of dihydromorphine are bound specifically per mg of cell protein (Fig. 3). The *insert* to the Figure shows the data

TABLE 1. *Specific binding of [^{3}H]dihydromorphine to neuroblastoma and hybrid cell lines*

Cell lines		fmoles of [^{3}H]-dihydromorphine bound/mg of protein[a]	No. of experiments	Neurotransmitter synthesis	Ref.
A. *Parents*					
Mouse neuroblastoma	N18TG-2	0 ± 1	2	None	[b]
Rat glioma	C6BU-1	1 ± 1	5	None	(15)
B. *N18TG2 × C6BU-1 hybrids*					
	NG108-15	17 ± 6	13	Acetylcholine	[b]
	NG108-5	4 ± 2	11	Acetylcholine	[b]
C. *Neuroblastoma*					
	NS20	3 ± 2	3	Acetylcholine	(12)
	NS20Y	4 ± 2	7	Acetylcholine	(12)
	N1E	0	1	Catechols	(12)
	N1E-115	2 ± 1	9	Catechols	(12)
	N4	0	1	None	(12)
	N18	2 ± 1	6	None	(12)
D. *Neuroblastoma × L cell hybrid*					
(N4TG-1) × (B82)	NL1F	6 ± 3	3	None	(14)

[a] Specific binding of [^{3}H]dihydromorphine is that amount of the bound radioactivity which is displaced by 1 μM unlabeled morphine (see the *Materials and Methods* section for details).

[b] B. Hamprecht, T. Amano, and M. Nirenberg, in preparation.

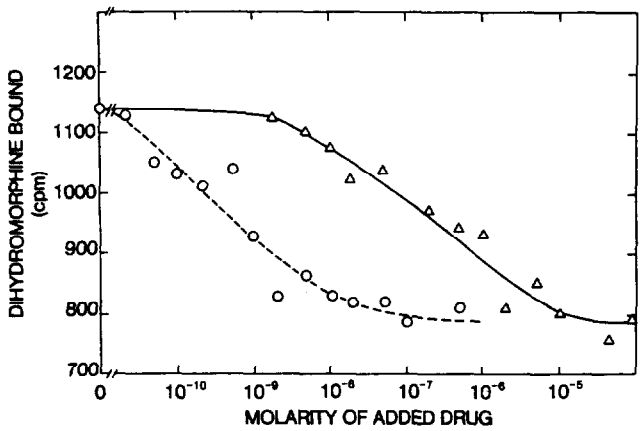

FIG. 2. The displacement of bound [^{3}H]dihydromorphine by levorphanol (○), [(−)3-hydroxy-*N*-methylmorphinan] and dextrorphan(△)[(+)3-hydroxy-*N*-methylmorphinan] at the concentrations shown from a suspension of NG108-15 cells (subculture 15).

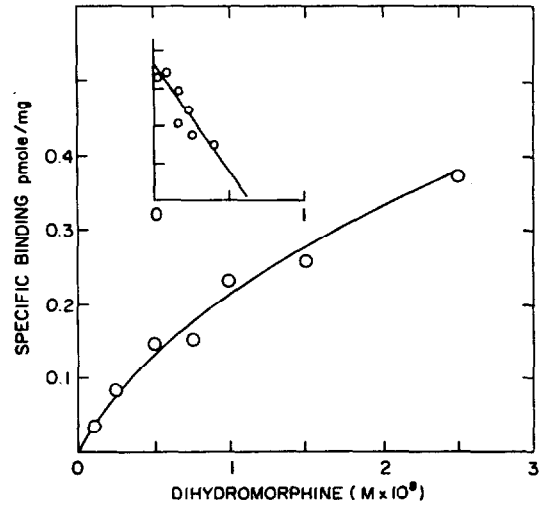

FIG. 3. The specific binding of [^{3}H]dihydromorphine to a suspension of NG108-15, measured as the difference between the amount of binding found in the absence and presence of 10 μM unlabeled morphine, as a function of [^{3}H]dihydromorphine concentration. The cells used were subculture 12 and were grown in the presence of dibutyryl cyclic AMP.

plotted according to Scatchard (20) and is consistent with there being only one class of dihydromorphine binding site with a dissociation constant of 20–30 nM.

We have examined the ability of a number of other narcotic analgesics as well as of the specific antagonist, naloxone, to bind to the receptors found in NG108-15 suspensions, as measured by their ability to displace specifically bound [^{3}H]dihydromorphine. In Table 2 the binding affinities measured in this way with NG108-15 suspensions and also with a crude mitochondrial fraction of rat brain as the source of receptors are shown. Note that the binding affinities of the NG108-15 preparations are generally somewhat lower than those of the brain preparation, but that the relative binding strengths are similar in the two preparations. The order of binding affinities is seen to be well correlated with the affinities found by Kosterlitz and Watt (11) for the pharmacological action of these agents on segments of guinea pig ileum.

An important question concerns the location of the narcotic receptor sites. Trypsin is often used as an aid in detaching cells from surfaces and is believed not to penetrate the cell membrane. Treatment of a cell suspension of NG108-15 with 0.05% trypsin for 10 min at 37° resulted in a decrease of the specific binding of [^{3}H]dihydromorphine from 26 to 8 fmoles/mg of protein under standard conditions. Although most of our experiments were performed with cell suspensions, we found that the specific binding of [^{3}H]dihydromorphine to homogenates of NG108-15, prepared in 0.32 M sucrose, was unchanged when compared with whole cells. When a homogenate of NG108-15 was centrifuged to equilibrium in a 58–20% w/w continuous sucrose gradient, the bulk of the morphine receptors were found at a sucrose concentration (measured by refractometry) equivalent to a density of 1.16 g/ml. These results are consistent with the working hypothesis that the receptor is localized on the plasma membrane.

TABLE 2. *Binding affinities of a series of narcotics to suspensions of NG108-15 and to brain homogenates*

Compound	Apparent dissociation constant[a] (nM)		Activity in guinea pig ileum[c]
	NG108-15	Rat brain[b]	
Levorphanol	0.3	0.8	7
Morphine	20	3	88
Pentazocine	50	10	150
Methadone	80	20	—
Codeine	1000	800	8800
Naloxone	10	1	1.2
Dextrorphan	150	100	—

[a] Binding was measured as the concentration required to displace ½ of the specifically bound [^{3}H]dihydromorphine in our usual assay system. Under our conditions of assay this number should be close to a true dissociation constant.

[b] Measured as described in *Materials and Methods* and (9). (R. A. Streaty and W. A. Klee, unpublished observations.)

[c] These are apparent dissociation constants (nM) for the antagonist activity of these compounds in the isolated ileum (11).

DISCUSSION

The results show that a neuroblastoma × glioma hybrid cell line (NG108-15) has the ability to bind narcotic analgesics in a stereospecific manner and with high affinity, whereas little or no specific binding was observed with parental cells and other cell lines tested. The relative binding affinities of narcotic analogues to NG108-15 cells were approximately the same as those found with rat brain homogenates and match well the pharmacological potency of the narcotics reported for isolated intestinal segments. Thus, there is good reason to believe that the neuroblastoma × glioma hybrid cells have morphine receptors similar to those found in brain and guinea pig ileum.

In addition to morphine receptors, NG108-15 cells also have other neuronal characteristics that are not found with parental cells, such as choline acetyltransferase*, intracellular acetylcholine, and clear and dense core vesicles, 500 and 1500 Å in diameter (21). In addition, the cells have long neurites, electrically excitable membranes, and nicotinic acetylcholine receptors.†

† B. Hamprecht, T. Amano, W. Kemper, and M. Nirenberg, unpublished observations.

Many properties of neuronal cell lines in culture have been shown to be regulated (22). Thus, the specific activities of choline acetyl transferase and cholinesterase* as well as the extent of morphological differentiation of NG108-15 cells can be increased many-fold in cultures which are grown in the presence of dibutyryl cAMP or are maintained in stationary phase (21). We did not detect regulation of the number of morphine receptor sites, since logarithmically dividing NG-108-15 cells had the same number of morphine receptors as sister cultures maintained in the presence of dibutyryl cAMP that were highly differentiated with respect to cell morphology. On the other hand, we observed with one population of NG108-15 that the cells lost much of their ability to bind dihydromorphine in a specific manner after 19 to 20 subcultures. Thus, it should be possible to obtain defective cell lines with respect to the synthesis, structure, or function of morphine receptors.

At saturation of dihydromorphine we estimate that there are 0.6 pmoles of bound morphine per mg of cell protein. This is approximately twice the specific activity of rat brain homogenates measured under identical conditions. Since 1 mg of protein corresponds to about 1.2×10^6 cells, there are approximately 300,000 receptor sites per cell. This is a large number and is similar to the number of nicotinic acetylcholine receptors of cultured sympathetic ganglion neurons of the chick (23).

One mechanism that has been proposed for the action of morphine is to inhibit the release of acetylcholine (24). Since NG108-15 cells both synthesize acetylcholine and release this compound into the medium, it should be possible to test this hypothesis. In addition, the recent demonstration by Collier and Roy of the specific reversal by narcotic analgesics of prostaglandin E-stimulated cAMP formation in rat brain homogenates (25) provides a possible mechanism for the action of these drugs. In collaboration with Shail K. Sharma, we have confirmed these findings with homogenates prepared from NG108-15, in that morphine was found to inhibit adenylate cyclase activity of NG108-15.

The availability of relatively homogeneous populations of cells with and without morphine receptors and with other neuronal properties provides opportunities to explore questions relating to the mechanism of action of morphine and, possibly, phenomena such as morphine dependence and tolerance.

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1. Pert, C. B. & Snyder, S. H. (1973) *Science* **179**, 1011–1014.
2. Terenius, L. (1973) *Acta Pharmacol. Toxic.* **32**, 317–320.
3. Simon, E. J., Hiller, J. M. & Edelman, I. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1947–1949.
4. Pert, C. B. & Snyder, S. H. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2243–2247.
5. Wong, D. T. & Horng, J. S. (1973) *Life Sci.* **13**, 1543–1556.
6. Kuhar, M. J., Pert, C. B. & Snyder, S. H. (1973) *Nature* **245**, 447–450.
7. Hiller, J. M., Pearson, J. & Simon, E. J. (1973) *Res. Commun. Chem. Pathol. Pharmacol.* **6**, 1052–1062.
8. Pert, C. B., Pasternak, G. & Snyder, S. H. (1973) *Science* **182**, 1359–1361.
9. Klee, W. A. & Streaty, R. (1974) *Nature* **248**, 61–63.
10. Goldstein, A., Lowney, L. I. & Pal, B. K. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1742–1747.
11. Kosterlitz, H. W. & Watt, A. J. (1968) *Brit. J. Pharmacol. Chemother.* **33**, 266–276.
12. Amano, T., Richelson, E. & Nirenberg, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 258–263.
13. Minna, J., Nelson, P., Peacock, J., Glazer, D. & Nirenberg, M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 234–239.
14. Minna, J., Glazer, D. & Nirenberg, M. (1972) *Nature New Biol.* **235**, 225–231.
15. Amano, T., Hamprecht, B. & Kemper, W. (1974) *Exp. Cell Res.*, in press.
16. Ham, R. G. & Puck, T. T. (1962) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. 5, pp. 90–119.
17. Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.* **37**, 854–857.
18. Eddy, N. B., Halbach, H. & Braenden, O. J. (1957) *Bull. W.H.O.* **17**, 569–863.
19. May, E. L. & Eddy, N. B. (1959) *J. Org. Chem.* **24**, 1435–1437.
20. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672.
21. Daniels, M. & Hamprecht, B. (1974) *J. Cell Biol.*, in press.
22. McMorris, F. A., Nelson, P. G. & Ruddle, F. H. (1973) *Neurosci. Res. Progr. Bull.* 412–536.
23. Greene, L. A., Sytkowski, A. J., Vogel, Z. & Nirenberg, M. W. (1973) *Nature* **243**, 163–166.
24. Smith, C. B. (1972) in *Chemical and Biological Aspects of Drug Dependence*, eds. Mule, S. J. & Brill, H. (CRC Press, Cleveland, Ohio), pp. 495–504.
25. Collier, H. O. J. & Roy, A. C. (1974) *Nature* **248**, 24–27.