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7. We suspect that the enrichment of Au in these particles (by factors of 26 to 42) is due to contamination. Although these samples were coated with only Pd for SEM examination instead of Au, it appears that they have picked up 10^9 atoms of Au from the sample-coating device.
8. A 604-μg sample of a bona fide C1 chondrite (Revelstoke) analyzed in this laboratory by essentially the same techniques employed in this investigation gave no better fit to the abundance pattern over the submicrogram stratospheric particles (R. Ganapathy, unpublished data).
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Opiate (Enkephalin) Receptors of Neuroblastoma Cells: Occurrence in Clusters on the Cell Surface

Abstract. *A bioactive, fluorescent derivative of enkephalin, Tyr-D-Ala-Gly-Phe-Leu-Lys-rhodamine, was used to determine the distribution of opiate receptors in living neuroblastoma cells. The receptors appeared in clusters on the cell surface, and no internalization was detected. No specific fluorescence or clusters were observed in the presence of [D-Ala², Leu⁵]enkephalin or at 4°C, and the clusters were much reduced under ionic conditions (that is, with 100 millimolars sodium) that specifically decrease the binding of opiate agonists.*

The enkephalins are two naturally occurring pentapeptides, Tyr-Gly-Gly-Phe-Met (Met-enkephalin) and Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin), with morphine-like activities (*1*, *2*). Met-enkephalin and Leu-enkephalin interact with opiate receptors in brain membrane preparations (*3*) and with specific cell surface receptors in cultured neuroblastoma cells (*4*, *5*). These peptides have been widely implicated as having neurotransmitter or neuromodulator functions at the synaptic junction.

Cultured neuroblastoma cells contain large numbers of opiate (enkephalin) receptors and have been extremely useful in elucidating the cellular and molecular actions of opiates and opioid peptides (*4*, *5*). Neuroblastoma-glioma hybrid cells (strain NG108-15) and N4TG1 neuroblastoma cells have about 100,000 and 20,000 enkephalin-binding sites per cell, respectively (*4*, *5*). The interaction of enkephalin with receptors in neuroblastoma cells has been studied in detail by using ^{125}I-labeled derivatives of the metabolically stable enkephalin analog [D-Ala², D-Leu⁵]enkephalin (*5*).

Image-intensified fluorescence microscopy has proved useful in visualizing the patterns and the mobility of receptors for epidermal growth factor, insulin, and α_2-macroglobulin in intact cells (*6*). In the present studies, a bioactive, fluorescent analog of enkephalin was used to visualize enkephalin receptors in neuroblastoma cells. The receptors appeared to form clusters on the cell surface, and no internalization was detected.

The Tyr-D-Ala-Gly-Phe-Leu-Lys-rhodamine was synthesized by conventional procedures, derivatization being achieved by reaction of the blocked hexapeptide with tetramethylrhodamine isothiocyanate followed by removal of protecting groups; final purification was by thin-layer chromatography (*7*). The rhodamine peptide inhibits the binding of ^{125}I-labeled[D-Ala², D-Leu⁵]enkephalin to brain membrane preparations and NG108 cells with apparent IC_{50} values (*2*) of about 5.9 and 5.5 nM, respectively. Cells of strain NG108-15 were grown in Dulbecco's MEM containing HAT and 10 percent FBS; whereas cells of strain N4TG1 were grown in Dulbecco's MEM containing only 10 percent FBS. At confluence, the cells were removed from the flask by incubating them with 1 mM EDTA for 5 minutes at 37°C. The detached cells were washed three times with 50 mM tris-HCl, pH 7.7, containing 0.25M sucrose, 2 mM $MgCl_2$, and 5 mM glucose; they were then suspended in the same buffer and incubated with the rhodamine-labeled enkephalin analog before being examined by fluorescence microscopy (*8*). The cells were not fixed for these studies. The results with cells of strain NG108 are shown in Fig. 1.

Many patchy areas of bright fluorescence, spreading over the cell surface, were detected with $5 \times 10^{-8}M$ rhodamine derivative (Fig. 1A). These clusters were not seen in the presence of $5 \times 10^{-6}M$ [D-Ala², Leu⁵]enkephalin (Fig. 1B), or with other cell lines (for example, 3T3 fibroblasts, KB epidermoides) that lack opiate receptors. Whereas enkephalin suppressed completely the appearance of clusters, a few clusters could be detected in the presence of high concentrations ($10^{-4}M$) of naloxone, and a slightly greater number were visible with $10^{-4}M$ morphine than with naloxone. These findings are consistent with the findings that both naloxone and morphine have very much lower affinities than enkephalin for receptors in neuroblastoma cells, and that the affinity for morphine is lower than that for naloxone (*4*, *5*). At 4°C no discrete clusters could be discerned, even after incubation for 2 hours (Fig. 1C). In the presence of 0.1M NaCl, only a few patches were detected (Fig. 1D), and the brightness and number of patches were diminished substantially (compared with Fig. 1A). Very similar phenomena were observed with N4TG1 cells (data not shown), except that there were fewer patches compared to NG108-15 cells.

These results are consistent with the known characteristics of opiate receptors, and with our previous studies (*5*) of the interaction of ^{125}I-labeled [D-Ala², D-Leu⁵]enkephalin with receptors in neuroblastoma cells. The binding was reduced in the presence of sodium and at 4°C, even after prolonged periods of incubation. The fluorescent clusters probably represent the specific binding sites (receptors) for enkephalins. The nonspecific binding of rhodamine-enkephalin, although weak, is uniformly distributed over the cell, making it difficult to determine whether some enkephalin receptors are distributed diffusely as well as in patches. We do not know whether the receptors occur naturally in patches or whether their distribution is altered as a result of binding; the binding (*5*) of enkephalins to NG108 cells is a relatively slow process, even at 37°C, and the appearance of patches coincides closely with the increase in binding as determined with ^{125}I-labeled analogs. Thus a redistribution of occupied receptors into patches may occur more rapidly than binding, making the latter process rate-limiting.

In contrast to epidermal growth factor (EGF) and insulin receptors on fibroblasts (*6*), where fluorescence labeling shows that the receptors are initially distributed uniformly and quickly form patches that are subsequently internalized (*6*), the enkephalin-labeled receptor patches appeared only slowly and were not internalized. The visible patch-

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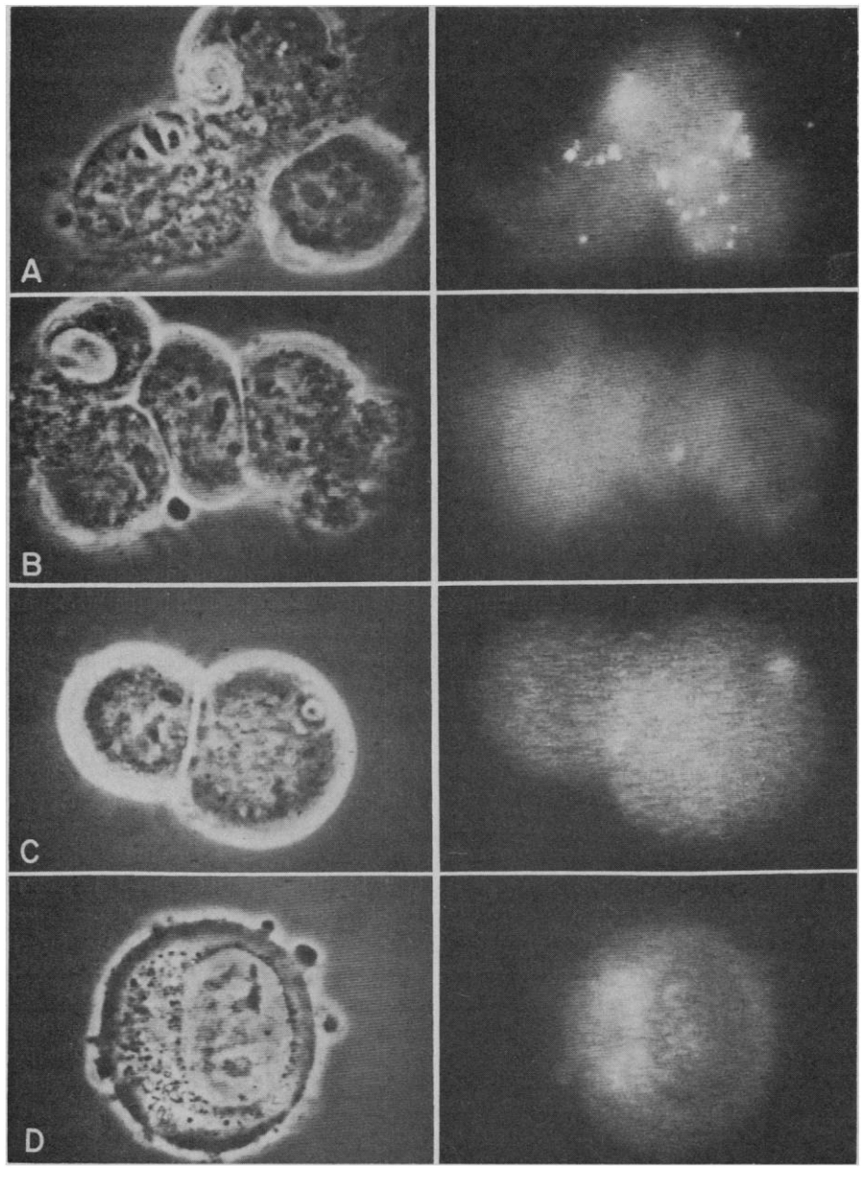

Fig. 1. Rhodamine-enkephalin binding to NG108-15 cells. On the left, phase micrographs (×1575); on the right, fluorescence micrographs of the same field. (A) Cells incubated for 40 minutes at 24°C with $5 \times 10^{-8} M$ rhodamine derivative. (B) Cells incubated in the presence of $5 \times 10^{-6} M$ [D-Ala^2, Leu^5]enkephalin. (C) Cells incubated for 2 hours at 4°C. (D) Cells incubated in the presence of 0.1M NaCl at 24°C.

es began to appear after 10 minutes and reached their maximum number after 40 minutes at 24°C; no further changes were detected with longer incubations at 24°C or 37°C. Careful examination by focusing (Fig. 2) revealed that all of the patches were on the cell surface, and the characteristic saltatory motion of endocytic vesicles, seen with EGF- and insulin-labeled receptors (*6*), was not observed in these studies. Furthermore, most of the patches disappeared if the cells were washed and incubated at 37°C three times with fresh medium to remove the ligand in the medium and allow the cell-bound enkephalin to dissociate. These results are consistent with the observation (*5*) that more than 95 percent of the cell-bound ^{125}I-labeled [D-Ala^2, D-Leu^5]enkephalin can dissociate from the cells even if the binding had taken place at 37°C during prolonged periods (*9*). Thus, it is likely that enkephalin receptors in neuroblastoma cells probably occur as surface clusters that do not internalize.

The relevance of these clusters to the biological functions of enkephalin receptors is unclear. Several recent studies suggest that receptor cross-linking or aggregation may be important for hormonal action. Bivalent antibodies to the insulin receptor block insulin binding, bind to insulin receptors, and trigger many of the same biological responses caused by insulin (*10, 11*). Monovalent Fab′ fragments are ineffective, but addition of antibodies to Fab′ fragments to cross-link the Fab′-receptor complexes restores the insulin-like activity (*10*). Furthermore, under certain conditions bivalent but not univalent antibodies against insulin or EGF can dramatically enhance the activity of very low concentrations of

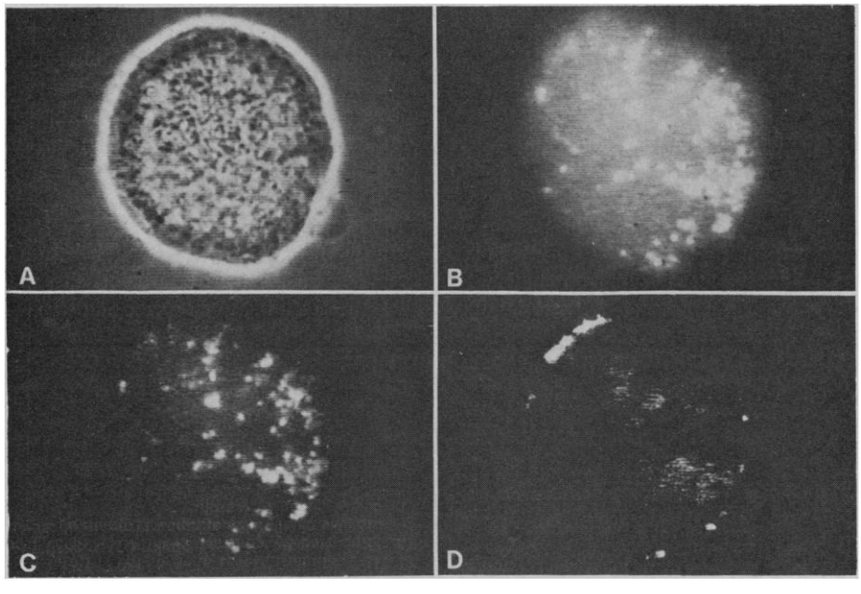

Fig. 2. Clusters of enkephalin-receptor complexes on the cell surface. The NG108-15 cells were incubated for 40 minutes at 24°C with $5 \times 10^{-8} M$ rhodamine derivative. (A) Phase micrograph (×1890) and (B to D) three fluorescence micrographs of the same field with different levels of focusing on the cell surface. (B) The cell contains nonspecific binding of rhodamine-enkephalin as in Fig. 1. (C and D) By increasing the contrast on the TV monitor, the nonspecific binding is made undetectable.

these hormones in fibroblasts (*12, 13*). A biologically inactive analog of EGF has been described which retains its ability to bind but does not induce aggregation of receptors (*13*). Bivalent antibodies to EGF restore both the bioactivity and the morphological cross-linking (patch formation) of this derivative toward that observed with the native hormone. These studies suggest that microaggregation of receptors into groups of two or more may be essential for the biological responses of at least some hormones.

Most opiate receptors probably occur in the membrane of the synapse (*14*), and the size of the synapse is about 0.5 μm (*15*). It is possible that opiate receptors are densely distributed on postsynaptic membranes and that aggregation of the occupied receptors may be necessary for generating physiological effects. The fluorescent analog described in the present study may be useful in studying the mechanisms of receptor redistribution (*16*) and in determining precisely the localization of opiate receptors in the central and peripheral nervous systems.

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8. Binding of the rhodamine enkephalin to NG108 cells was visualized with an RCA silicon intensifier target TV camera (TC1030H) attached to a Zeiss Photomicroscope III equipped with epifluorescence. Video output was recorded on a Panasonic recorder (NV-8030) and displayed on a Hitachi TV monitor (VM-905AU), from which Polaroid photographs were taken.
9. The presence of physiological concentrations of calcium ions in cells treated with or without EDTA does not alter the appearance of clusters or the absence of internalization. Because of the dramatic reduction in the binding of enkephalin in the presence of 100 m*M* Na^+, the clusters cannot be detected. However, the nearly complete dissociation of cell-bound ^{125}I-labeled [D-Ala^2, D-Leu^5]enkephalin indicates that no internalization occurs in the presence of sodium ions (*5*).
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Neuronal Chemotaxis: Chick Dorsal-Root Axons Turn Toward High Concentrations of Nerve Growth Factor

Abstract. *Micropipettes containing 2 to 50 biological units of β nerve growth factor (NGF) were placed near growing axons of chick dorsal-root ganglion neurons in tissue culture. The axons turned and grew toward the NGF source within 21 minutes. This turning response to elevated concentrations of NGF appears to represent chemotactic guidance rather than a general enhancement of growth rate.*

Chemotaxis, the attraction of living protoplasm to a chemical substance, may help guide axons to their target tissues. Several studies in vitro (*1–3*) suggest that β nerve growth factor (NGF), a protein known to enhance axonal outgrowth from dorsal-root and sympathetic ganglia (*4*), is a chemotactic agent. These studies, however, leave unresolved the question of whether NGF actually guides axonal growth or simply enhances the survival or growth rate of axons that happen to be growing near the NGF source. We sought to distinguish between these possibilities by continuously observing the growth cones of chick dorsal-root axons that were exposed to a localized source of NGF. We found that these axons turn and grow toward the NGF source within 21 minutes, even if the background concentration of NGF is sufficient to support survival and rapid axonal growth.

Table 1. Turning response of dorsal-root ganglion axons to NGF.

Concentration of NGF (BU/ml)		Number of axons	
Background	Micropipette	Positive response*	No response†
1	50	40	0
1	1	0	40
1	1 (+ BSA‡)	0	5
1	1 (+ FCS§)	0	5
0	1	0	5
1	2	5	0

*Rate of turn, 3.3 ± 0.2 deg/min. †Rate of turn, 0.01 ± 0.14 deg/min. ‡Bovine serum albumin (0.1 mg/ml) added. §Fetal calf serum (2.5 μl/ml) added.

Lumbosacral dorsal-root ganglia from White Leghorn chick embryos 7 and 12 days of age were excised and placed onto glass cover slips coated with a mixture of collagen and poly-L-lysine (25:1 by weight). The explants were incubated in air at 34°C in 35-mm plastic petri dishes with nutrient medium similar to Ham's F-12 except buffered with 1,4-piperazinediethanesulfonic acid (Pipes). The medium was supplemented with 5 to 10 biological units (BU) of NGF per milliliter (*5, 6*). After 24 hours, the cover slips were placed in an observation chamber (34°C) and were viewed with an inverted phase-contrast microscope (×750).

A micropipette (tip diameter, 2 to 4 μm) filled with NGF (1 to 50 BU per milliliter of perfusion medium) was used as a localized NGF source. The tip of the micropipette was placed about 25 μm from the tip of a growth cone at approximately 45° to the axon's longitudinal axis (Fig. 1a) and slightly above the surface of the medium. A separate perfusion system added perfusion medium to one side of the observation chamber while continuously removing medium from the opposite side with a vacuum line. This system produced a flow of medium (25 ml/hour) past the axon in a direction opposite to the initial direction of axonal growth. The NGF, flowing from the micropipette at the rate of 1 to 2 μl/hour, was carried along with this background flow, producing a higher concentration of NGF on the side of the growth cone nearest the micropipette (this was initially determined with methylene blue).

Figure 1 shows a dramatic example of

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